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# CHARACTERIZATION OF *ARTHROSPIRA* (*SPIRULINA*) STRAINS

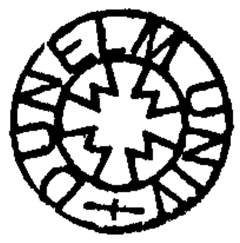
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**University of Durham**
**In accordance with the requirements for the degree of**
**Doctor of Philosophy**

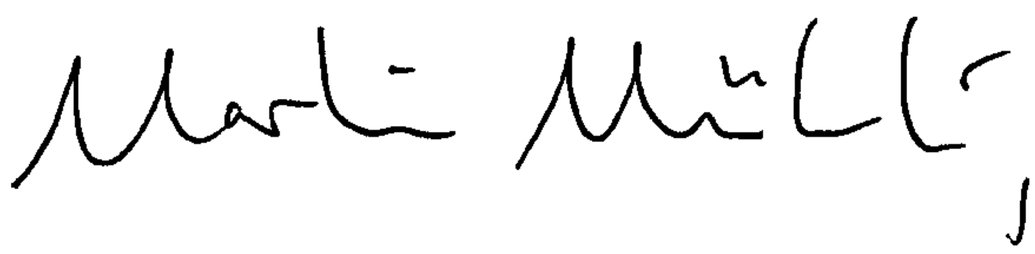
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# DECLARATION

I declare that the work contained within this thesis submitted by me for the degree of Doctor of Philosophy is my own original work, except where otherwise stated, and has not been submitted previously for a degree at this or any other University.



Martin Mühling

# PUBLICATIONS

Data presented in this thesis have been presented at the following conferences:

- Mühling M, Scott M, Harris N, Belay A, Whitton BA (1997) Characterization of *Arthrospira* and *Spirulina* strains: Phenotypic characteristics. Proc. IX$^{th}$ Int. Symp. Phototrophic Prokaryotes, Vienna, Austria.

- Mühling M, Whitton BA, Harris N, Wilmotte A, Belay A, Ota Y (1999) Variations in the fatty acid composition of *Arthrospira* ('*Spirulina*') strains. 8$^{th}$ Int. Conf. Appl. Algol. (ICAA) Montecatini, Italy.

- Whitton BA, Mühling M, Harris N, Wilmotte A, Belay A, Ota Y (1999) Heterotrophic growth of cyanobacteria with special considerations of *Arthrospira* ('*Spirulina*'). 8$^{th}$ Int. Conf. Appl. Algol. (ICAA) Montecatini, Italy.

In addition, data resulting from the collaboration with Dr Annick Wilmotte (University of Liège), who worked on the molecular taxonomy of the same set of *Arthrospira* strains, has been presented in the following publication (Appendix C):

- Scheldeman P, Baurain D, Bouhy R, Scott M, Mühling M, Whitton BA, Belay A, Wilmotte A (1999) *Arthrospira* ('*Spirulina*') strains from four continents are resolved into only two clusters, based on amplified ribosomal restriction analysis of the internally transcribed spacer. FEMS Microbiol. Lett. 172: 213-222. (Appendix C.)

# ABSTRACT

A culture collection of clonal, axenic cultures of 35 *Arthrospira* strains and five strains, which were duplicate subcultures of the original isolates, was established. In addition, eight morphological mutants were isolated from cultures of these strains. All strains are different from those belonging to the genus *Spirulina* as concluded from TEM and PyMS studies. All *Arthrospira* strains, duplicates and different morphotypes were screened for variation in morphological, ultrastructural, physiological and biochemical characters for taxonomic purposes.

Special emphasis was put on the analysis of factors which influence the morphology of the trichome helix, as this is the feature most characteristic for *Arthrospira* strains. For example, the orientation of coiling was found to be influenced by genetic drift or the growth temperature, but can also be reversed by mechanical impact.

Studies were also made on the ability to utilize sugars for growth. None of the strains grew on sucrose, but many grew on glucose and/or fructose. Growth in the presence of sucrose under photoheterotrophic conditions required an adaptation process, though sucrose is not being utilized for growth.

Analysis of fatty acid composition of all strains revealed quantitative differences between strains, most markedly in the unsaturated fatty acid fraction. Repeat experiments showed the same results each time, indicating the value of this approach for identification purposes. Similarly, lectin-binding to cell surface structures proved to be a useful approach for differentiation between strains.

Analysis of the whole cell composition by pyrolysis mass spectrometry (PyMS) did not confirm the clusters based on other phenotypic characters, but showed that there is a high similarity between duplicate strains or different morphotypes derived from the same stock.

Numerical analysis of the data for 28 characters resolved the helical strains in two phenotypic clusters which show a high correlation to the two molecular clusters based on ARDRA of the ITS of the same set of strains.

The helical trichome morphology of the strains showed the highest correlation to either of the clusters. Comparison of species descriptions and morphological characters of the strains, as determined in the presented work, indicate, that phenotypic cluster I is composed of *Arthrospira maxima*, *A. fusiformis* and *A. indica*, while *A. platensis* forms phenotypic cluster II.

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This project was carried out parallel to the molecular analysis of the same set of strains. I am very grateful to Dr Annick Wilmotte (University of Liège, Belgium) for her collaboration and discussions.

I am further indebted to my colleagues in Laboratory 9, each of them contributed to this work. A special thanks goes to Mark Scott, who carried out a major part of the phosphatase enzyme assays and culturing of the strains during the first 18 months. I am also grateful to John Gemmell for discussions and technical support. I thank Drs Meenakshi Banerjee and Roberto García-Ruiz for useful discussions and advice.

I am also indebted to colleagues at the University of Warwick. I thank Dr Stefan Radjiewski for his advice on numerical analysis and Dr Ferdinand Wagner for reading parts of the manuscript.

The project presented in this thesis covers numerous areas of research. To acquire the necessary experimental skills in each of the fields is, however, often only possible with the support of colleagues. I thank Dr Alan C. Ward (University of Newcastle) for his help with pyrolysis mass spectrometry and his advice on numerical analysis. I am indebted to Prof. Chris Hawes (Oxford Brookes University, Oxford) and Drs Trevor A. Booth and Gordon W. Beakes (University of Newcastle) for their help with the confocal microscopy. Several colleagues in the department also contributed to this project. I am thank Mrs Christine Richardson for teaching me transmission electron microscopy as well as for her help with the microanalysis-system and Dr Charles Brennan for his help with scanning electron microscopy. Drs Adrian Brown and Paul O'Hara are thanked for their help with the gas chromatograph for fatty acid identification and Prof. Anthony R. Slabas for use of equipment.

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## ABBREVIATIONS

| | |
|---|---|
| ACP | acyl-carrier protein |
| AFLP | amplified fragment length polymorphism |
| RAPDs | randomly amplified polymorphic DNA (PCR) |
| AFM | Atomic force microscopy |
| ARDRA | amplified ribosomal DNA restriction analysis |
| ATCC | American Type Culture Collection, Rockville, Maryland, USA |
| BHT | butylated hydroxy toluene |
| CCAP | Culture Collection of Algae and Protozoa, Ambleside, Cumbria, England, UK |
| CCALA | Culture Collection of Algal Laboratory, Institute of Botany, Trebon, Czech Republic |
| CCMEE | Culture Collection of Microorganisms from Extreme Environments, Florida State University/Florida A&M University, Tallahassee, Florida, USA |
| DCMU | 3-(3,4-dichlorphenyl)-1,1-dimethylurea |
| DIC | Dainippon Ink & Chemicals Inc., Biochemical Division, Tokyo, Japan |
| DMSO | Dimethyl sulfoxide |
| EDTA | ethylenediaminetetra-acetic acid |
| FAMES | fatty acid methyl esters |
| EZ | enriched (solidified (1% w/v)) Zarrouk's medium |
| FITC | fluorescein isothiocyanate |
| h | hour(s) |
| IAM | Institute of Applied Microbiology, University of Tokyo, Japan |
| ITS | internally transcribed spacer |
| M | molar |
| min | minute(s) |
| mM | milimolar |
| N | nitrogen |
| NIES | National Institute for Environmental Studies Collection, Tsukuba, Ibaraki, Japan |

| | |
|---|---|
| NIVA | Norwegian Institute for Water Research, Oslo, Norway |
| OTU | operational taxonomic unit |
| P | phosphorus |
| PCA | principal component analysis |
| PCD | principal coordinates analysis |
| PCC | Pasteur Culture Collection of Cyanobacterial Strains, Paris, France |
| PDEase | phosphodiesterase activity |
| PMEase | phosphomonoesterase activity |
| pNP | p-nitrophenol |
| pNPP | p-nitrophenyl phosphate |
| PyMS | Pyrolysis Mass Spectrometry |
| $r_{cs}$ | cophenetic correlation coefficient |
| SAG | Sammlung von Algenkulturen der Universität Göttingen, Germany |
| s | second(s) |
| $S_J$ | Jaccard coefficient |
| $S_{SM}$ | simple matching coefficient |
| SEM | Scanning electron microscopy |
| TEM | Transmission Electron Microscopy |
| UPGMA | unweighted pair group method with arithmetic averages |
| UTEX | Culture Collection of Algae at the University of Texas at Austin, Austin, Texas, USA |
| WPGMA | weighted pair group method with arithmetic averages |

# CHAPTER 1 INTRODUCTION

## 1.1 Cyanobacteria

### 1.1.1 What are cyanobacteria?

Cyanobacteria or blue-green algae are photosynthetic prokaryotes possessing the ability to synthesize chlorophyll *a* (Whitton & Potts, 2000). Although cyanobacteria are able to anoxygenic photosynthesis using hydrogen sulphide instead of water, generally water is being used as electron donor for photosynthesis, thus leading to the release of oxygen (Ho & Krogman, 1982).

### 1.1.2 Taxonomy of cyanobacteria

Cyanobacteria have originally been classified by their ability to synthesize the blue phycobilin pigment phycocyanin. The high content of this pigment under certain environmental conditions is responsible for the bluish colour of the organisms and hence the original nomination as blue-green algae. Cyanobacteria have been grouped for long together with all other (eukaryotic) algae. Their close taxonomic relationship to bacteria, however, had already been recognized by Cohn (1853) and De Bary (1884). Finally, Stanier and Van Niel (1962) elucidated the fundamental differences in the cellular organisation between prokaryotes and eukaryotes and Stanier (1974) provided the convincing argument that cyanobacteria have the same prokaryotic cellular organisation as bacteria. This knowledge led also to the now generally accepted nomination as cyanobacteria (1979).

The taxonomic classification of cyanobacteria has been traditionally based purely on morphological characters applying the International Code of Botanical Nomenclature. Almost entirely on the basis of observations on field material Geitler (1932) produced a comprehensive treatise which recognized approximately 1300 species in 145 genera. However, applying this key no consideration is given to the question as to whether the phenotype described more than sixty years ago is genetically similar to the present sample of similar morphology.

An attempt to overcome this limitation and to use more characters of different nature than morphology was undertaken by Stanier and coworkers (eg Stanier et al., 1971; Rippka et al., 1979). The reclassification by this "Stanier" system was not merely based on morphological criteria, but included physiological, biochemical and genetic information (Rippka et al., 1979). This grouping, which concerned mainly the generic level, is however still far from "perfect", as it does not provide a phylogenetic and therefore natural grouping of organisms. Using rDNA sequence data, Giovanni et al. (1988) showed that strains grouped closely by the Stanier system, but may still be only distantly related in terms of phylogeny.

## 1.2 *Arthrospira* and *Spirulina*

### 1.2.1 Morphology of the genera *Arthrospira* and *Spirulina*

The genera *Arthrospira* and *Spirulina* belong to the order Oscillatoriales which includes all non-heterocystous, filamentous cyanobacteria that undergo binary fission in a single plane and do not produce akinetes (Castenholz, 1989). *Arthrospira* and *Spirulina* are distinguished from other oscillatoriacean genera by their helical trichome morphology (Castenholz, 1989; Fig. 1.1). The life cycle of *Arthrospira*, however, represents the typical, rather simple, life cycle of the Oscillatoriales (Fig. 1.2A). The apical part of a trichome breaks away by the formation of specialized cells, the necridia, which undergo cell lysis (Cifferi, 1983). The necridial cells are distinguished by their biconcave shape and lack of pigments. However, independent of the strain, necridia are not always observed and the fragmentation of trichomes may, thus, occur without the sacrifice of a cell (usual observations). The resulting single cell or short chains of cells (ca. 2-8; Fig. 1.2B), the hormogonia, are highly motile and give rise to a new trichome by binary fission vertically to the longitudinal axis of the trichome.

Fig. 1.1 *Arthrospira* and *Spirulina*.

1.1A shows *Arthrospira* strain D0893, and 1.1B represents *Spirulina subsalsa* strain D0871.

Fig. 1.2 Life cycle of *Arthrospira*.

1.2A Life cycle of *Arthrospira* sp. For details see text. (Taken from Ciferri, 1983.) 1.2B Hormogonium formation of *Arthrospira* strain D0885/H1. A hormogonium is released from the 'mother' trichome leaving behind a clearly visible sheath (arrow). The size bar represents 10 μm.

Fig. 1.1A




Fig. 1.1B




Fig. 1.2A

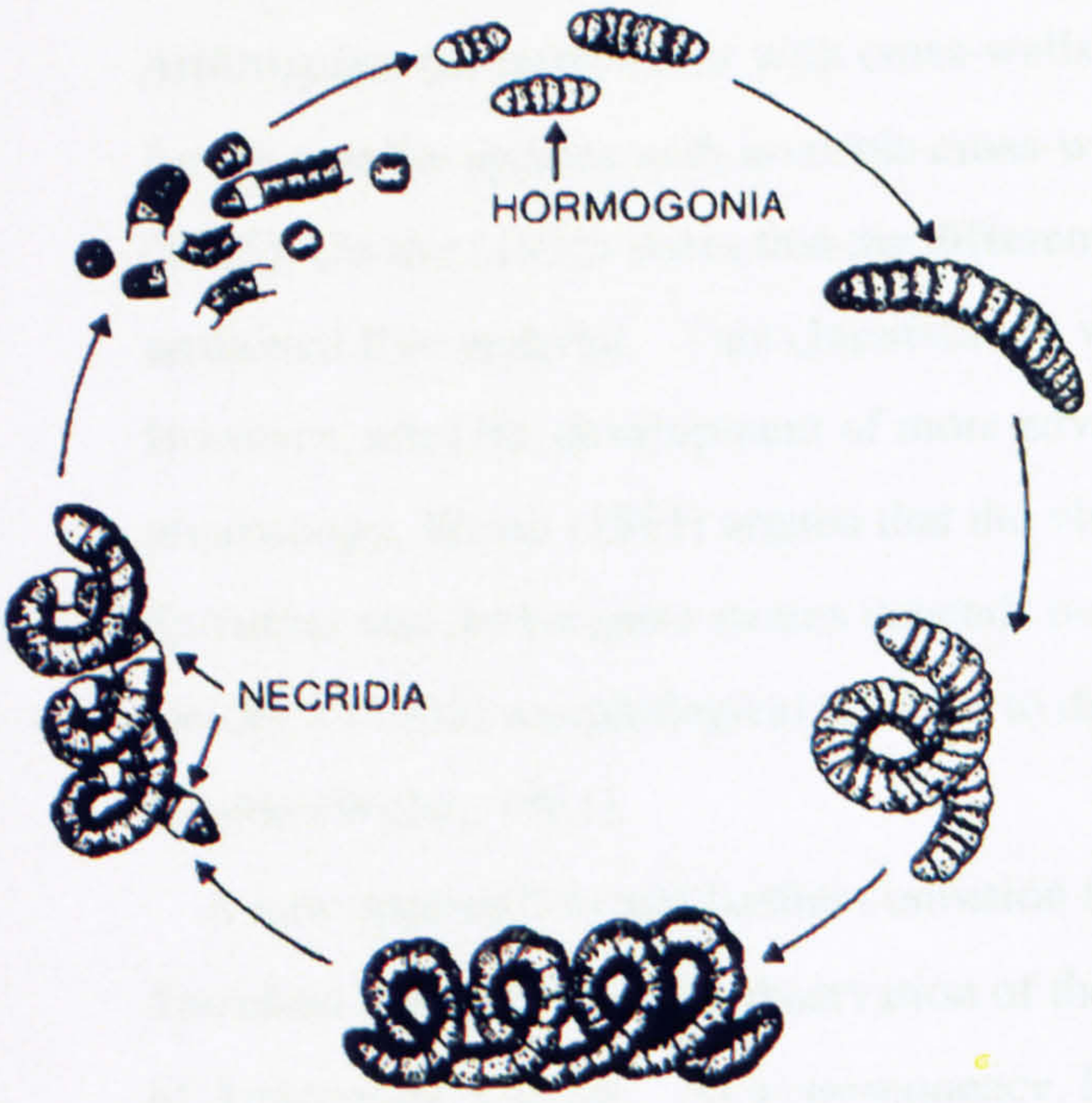
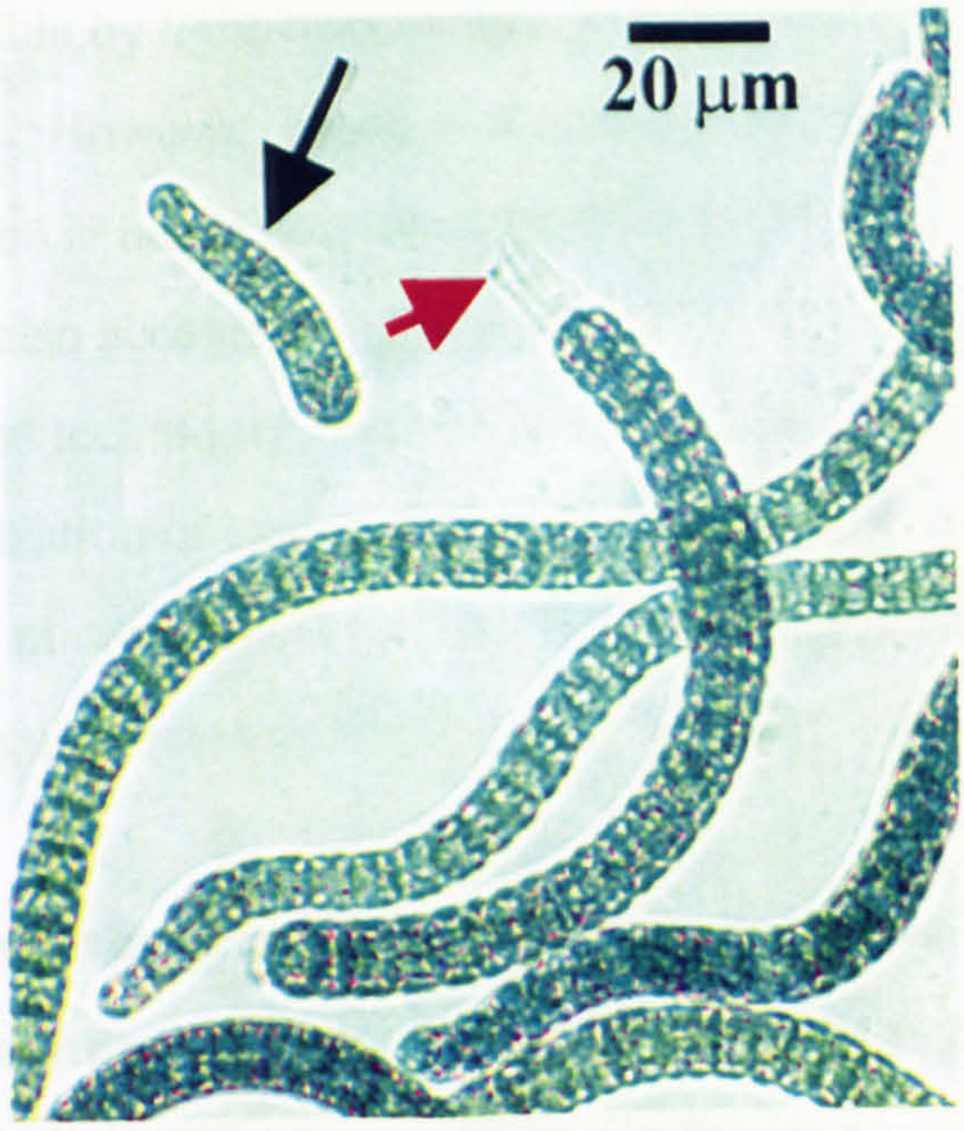



Fig. 1.2B



### 1.2.2 History of the taxonomy of the genera *Arthrospira* and *Spirulina*

Although the introduction of two separate genera is now generally accepted, there has been much dispute in the past and the resulting taxonomic confusion is tremendous. A short historic survey of the taxonomic understanding of the two genera may illustrate this.

*'Classical' concepts*

The genus *Spirulina* was established by Turpin in 1827 for *Spirulina oscillarioides* (Turpin, 1827). In 1852, Stizenberger introduced the genus *Arthrospira* (Stizenberger, 1852) for helical cyanobacteria from highly alkaline (pH 9-10) environments, that are distinguished from *Spirulina oscillarioides* Turpin by cross-walls and bigger trichomes. Later on, taxonomists (eg Gomont, 1892) followed these descriptions, grouping species of bigger dimensions with cross-walls visible under the light microscope into the genus *Arthrospira* and the smaller form with, observed under the light microscope, typical "unicellular trichomes" (Gomont, 1892) into the genus *Spirulina* Turpin. Gardner (1917) was the first who questioned the taxonomic determination of new isolates as *Spirulina* Turpin or *Arthrospira* Stizenberger based on the presence of cross-walls visible under the light microscope. In 1925 Figini confirmed Gardner's (1917) doubts demonstrating that, using appropriate stains, septa were visible in 13 different species of *Spirulina*, including the smallest strains known at this time (Figini, 1925). In his great treatise of cyanobacteria, Geitler (1932) grouped both genera into a single genus, *Spirulina*, constituting two sections *Arthrospira,* the large forms with cross-walls visible by light-microscopy, and *Euspirulina*, for the smaller species with invisible cross-walls. However, based on the results of Figini (1925), Geitler (1932) states that the differentiation is based only on observations of unstained live material. This classification was also accepted by Desikachary (1959). However, after the development of more advanced techniques, such as phase contrast microscopy, Welsh (1961) argued that the visualization of cross-walls in live material of *Spirulina* and *Arthrospira* strains depends on the microscopical technique used. Therefore, Geitler's (1932) morphological markers to distinguish between the two subgenera are not tenable (Welsh, 1961).

A new approach to add further confusion to the taxonomy of the genera *Arthrospira* and *Spirulina* was based on the observation of the occurrence of straight trichomes in cultures of *Arthrospira* species. As a consequence, Bourrelly (1970) to consider *Spirulina* as a

subgenus of *Oscillatoria*, as the straight morphotype of *Spirulina* (*Arthrospira*) is indistinguishable to the straight filaments of *Oscillatoria*.

*'Modern' approach*

Rippka et al. (1979) did not regard *Arthrospira* as a genus distinct to *Spirulina*. However, based on substantial difference in the mean DNA base composition between a *Spirulina* and an *Arthrospira* strain (Herdman *et al.*, 1979a), Rippka et al. (1979) suggest that the *Arthrospira/Spirulina* taxonomy has to be revised using more advanced techniques.

Investigations of the ultrastructure of *Arthrospira* strains, provided substantial evidence for a valid distinction of the two genera. While *Arthrospira* has a single row of pores around the trichome at each site of the cross-walls, *Spirulina* has several rows of pores at the concave site of the coil at each site of cross-walls (Guglielmi & Cohen-Bazire, 1982). Furthermore, cylindrical bodies were found in *Spirulina* (*Arthrospira*) *platensis* (van Eykelenburg, 1979; Tomaselli et al., 1996), but not in *Spirulina subsalsa* (Tomaselli et al., 1996).

γ-linolenic acid was found to represent a major part of the unsaturated fatty acids in *Spirulina* (*Arthrospira*) *platensis* (Nichols and Wood, 1968). Cohen et al. (1987) investigated the fatty acid composition of 18 *Arthrospira* strains and one *Spirulina* strain. All *Arthrospira* strains tested contained γ-linolenic acid, while *Spirulina subsalsa* did not (Cohen et al. (1987).

The first molecular approach to resolve the taxonomy of the two genera involved the sequence analysis of the 16S rDNA used as molecular marker (Nelissen et al., 1994). The sequence similarity of the 16S rRNA sequence was found to be higher between the two *Arthrospira* strains tested, than between the two *Arthrospira* strains and a *Spirulina* strain (99.7% and 91.2%, respectively) (Nelissen et al., 1994). Comparison with known 16S rRNA sequences of other cyanobacteria revealed that *Arthrospira* is not as closely related to *Spirulina* as often thought in the past, but belong to a group constituting *Oscillatoria*, *Lyngbya* and *Microcoleus*, while *Spirulina* showed similarity to unicellular cyanobacteria (Nelissen et al., 1994). Furthermore, the ITS region of *Arthrospira* strains contained the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes, while the ITS region of *Spirulina* contained only the $tRNA^{Ile}$ gene (Nelissen et al., 1994).

*How to handle this confusion?*

In conclusion, the scientific evidence justifies the separation of the two genera and provides also taxonomic markers for the correct identification of either of the genera. Nevertheless, the confusion over the nomination is manifested in the literature and is today complicated by the marketing of the industrial product *Arthrospira* under the taxonomically wrong name Spirulina (Section 1.4). To solve the confusion, Tomaselli et al. (1996) suggested to use "in scientific journals and books only the proper generic designation", while "in all other cases (eg commercial products or formulations) the common name "*Spirulina*" may be used, providing that the correct scientific denomination of the organism cultivated (ie *A. platensis, A. maxima or A. fusiformis*), and if possible its origin (eg Lake Chad, Lake Texcoco), is given" (Tomaselli *et al.*, 1996).

In the following the taxonomic genus *Spirulina* is referred to as *Spirulina* and the food product as Spirulina. Furthermore, where the literature is quoted concerning work on *Arthrospira* species but using the taxonomically wrong name *Spirulina*, the taxonomically correct nomination *Arthrospira* is added in parentheses after the genus name (ie *Spirulina* (*Arthrospira*) *platensis*).

### 1.2.3 Taxonomy of the genus *Arthrospira*

Under the International Code of Botanical Nomenclature the starting point for the valid names of non-heterocystous, filamentous cyanobacteria (family *Oscillatoriaceae*) is with Gomont (1892) (Castenholz and Waterbury, 1989).

*'Classical' concepts*

Gomont (1892) described three species of *Arthrospira, A. jenneri* (Fig. 1.3A), *A. platensis* (Fig. 1.3B) *and A. miniata*. The main characters distinguishing *A. jenneri* and *A. platensis* were differences in the length between two turns (= helix pitch; 21-31 µm and 43-57 µm, respectively) and the helix diameter (9-15 µm and 26-36 µm, respectively), while *A. miniata* showed a much smaller trichome diameter and cylindrical cells (longer than wide) (Gomont, 1892). Subsequently, several new species and varieties have been described in individual publications (Appendix B).

As a consequence, Geitler (1932) described already 13 botanical species of *Arthrospira* which are distinguished by trichome diameter, cell dimensions (ie cells square or longer

than wide), helix dimensions (ie helix pitch, diameter of helix) and length of the trichomes. However, based on the studies of Rich (1931) on the variability of the trichome helix of *Spirulina* (*Arthrospira*) *platensis* (Fig. 1.4), Geitler (1932) warns that several of his "species" may well belong to the same species.

Despite the introduction of many species, only a few of those have attracted more attention. As concluded from the description and original drawings, *Spirulina fusiformis* Woron. 1934 showed variation in the trichome morphology, which was either fusiform to regular or barrel-shaped, but always with a strong attenuation of the helix towards the trichome apices (Fig. 1.5). A further character of *Spirulina* (*Arthrospira*) *fusiformis* was its length of only 10 to 320 μm (Woronichin, 1934). Rich (1931) reported similar observations of variation of the trichome morphology of an African isolate of *Arthrospira platensis* (Fig. 1.4). This may indicate, that the species regarded by Rich (1931) as *Arthrospira platensis* might be identical with *S. fusiformis* Woron., a view also expressed by Elenkin (1949). More recently, Hindák (1985) studied the variation in trichome morphology of an *Arthrospira fusiformis* isolate from Lake Bogoria (Kenya). His observations confirm that the different trichome morphologies represented by Rich (1931) are indeed different morphotypes of the same species, *Spirulina fusiformis* Woron. (Hindák, 1985). Therefore, it seems likely that *Spirulina fusiformis* Woron. 1934 should be regarded as synonym of *Arthrospira platensis* Gomont 1892, as the latter was described first.

One of the most recent species description concerned an isolate from India, which was assigned to a new species, *A. indica* Desikachary et Jeeji Bai 1992 (Desikachary & Jeeji Bai, 1992). This isolate showed variation of the trichome morphology similar to *Spirulina fusiformis* Woron. (Woronichin, 1934), but possessed generally a calyptrate endcell (thickened cell wall of apical cell), while, based on the original drawings of Woronichin (1934), *Spirulina fusiformis* Woron. is thought to lack calyptrate end cells. However, Desikachary and Jeeji Bai (1996) argued, that *Arthrospira indica* should be considered as a synonym of *A. fusiformis* should a calyptra be demonstrated for *A. fusiformis*.

Fig. 1.3 Gomont's (1892) species descriptions.

The drawings were taken from Gomont (1892).

1.3A *A. platensis* Gomont 1892

1.3B *A. maxima* Gomont 1892

1.3C *A. miniata* Gomont 1892

Fig. 1.4 Morphological variability of *Arthrospira platensis* after Rich (1931).

The fusiform state of the trichome (I) is represented at a lower magnification. The drawings were taken from Rich, 1931.

Fig. 1.5 *Arthrospira fusiformis* Woron.

Note the different helix orientation of the trichomes. (Taken from Woronichin, 1931.)

Fig. 1.3

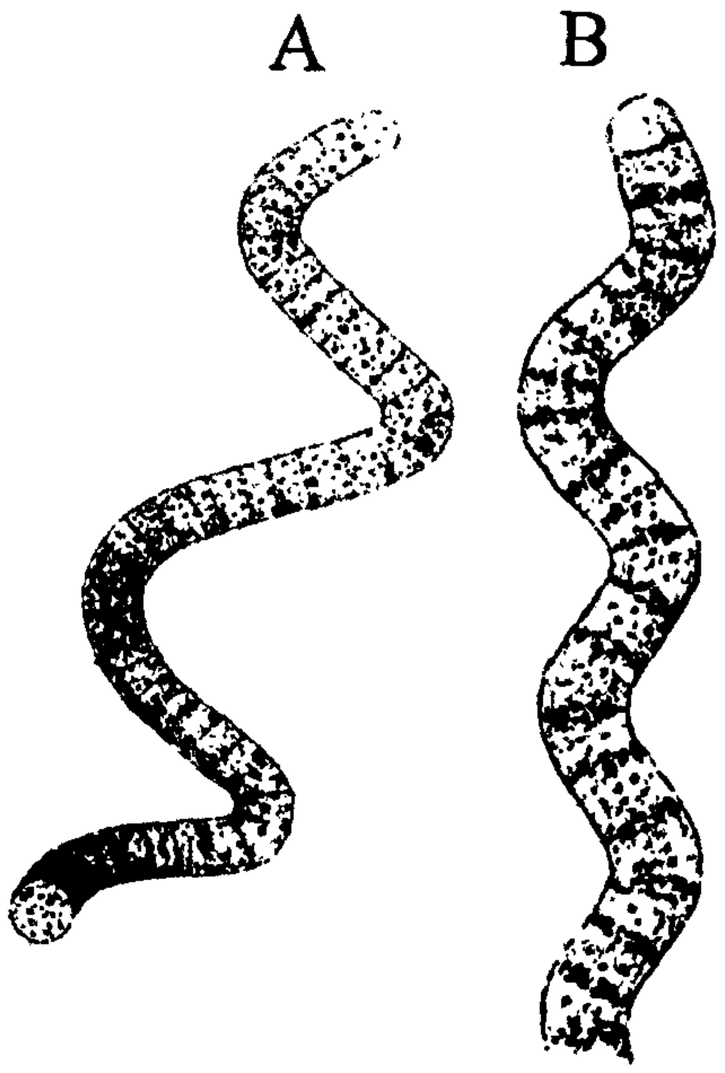



Fig. 1.4

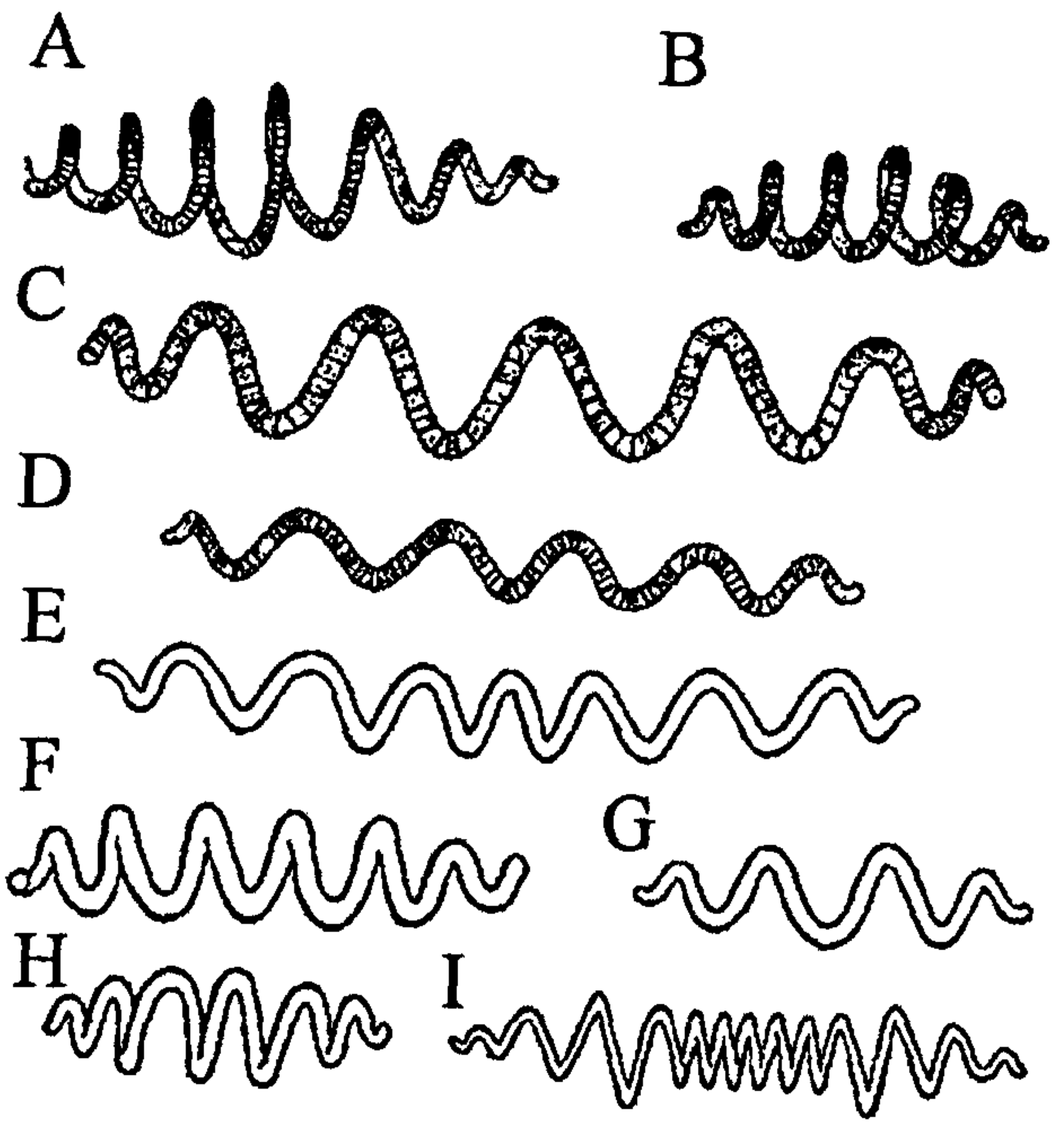
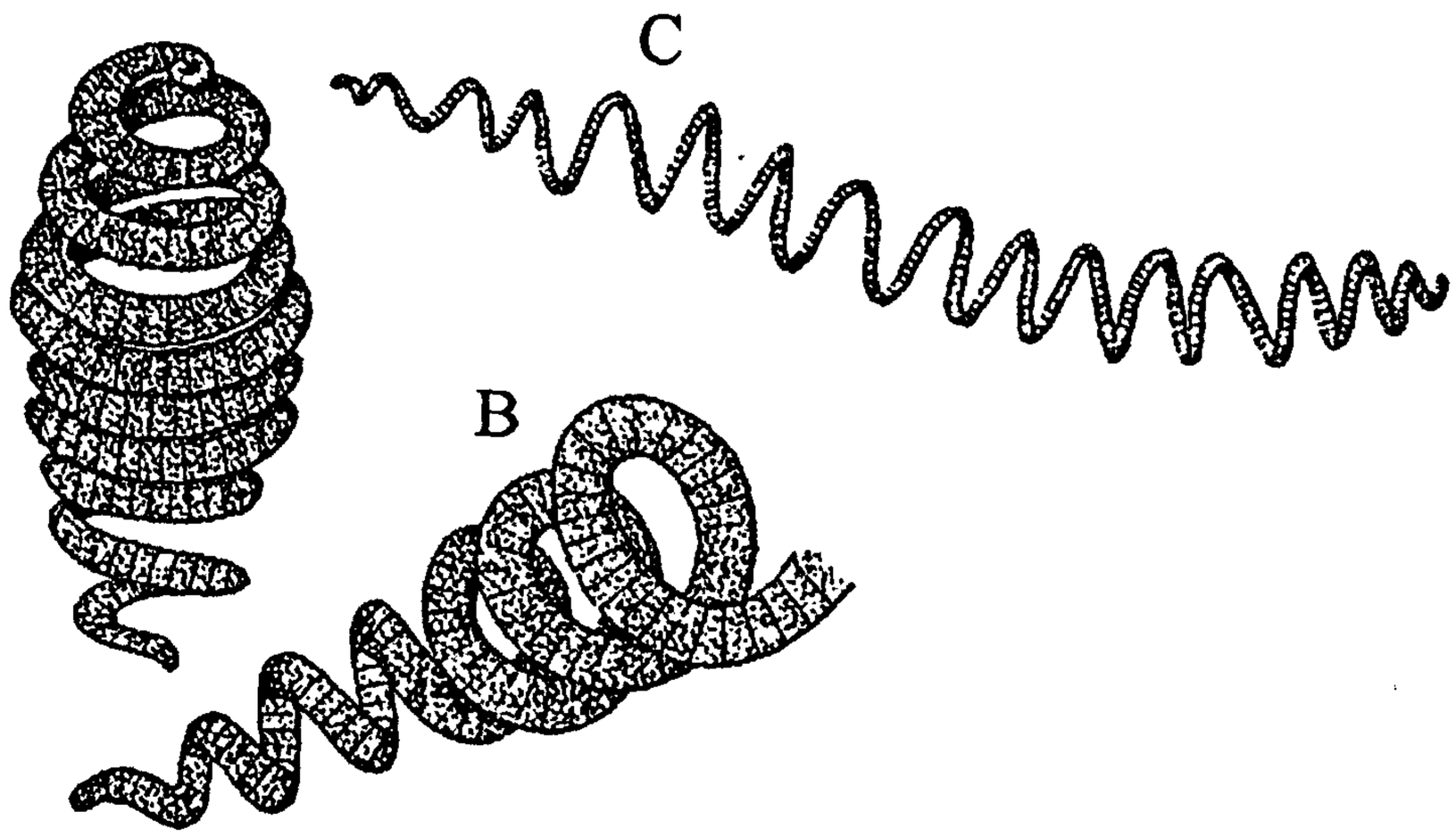



Fig. 1.5



None of the recent isolates or strains at culture collections have been assigned to most of these new species by main stream taxonomists. The species, new isolates were generally assigned to, were in most cases *A. platensis* Gomont 1892, *A. maxima* Setch. et N.L.Gardner 1917, *S. fusiformis* Woron. 1934 and *A. indica* Desikachary et Jeeji Bai 1992. This may be mainly due to the fact that the variability of the trichome morphology has been recognized and prevents now the recognition of a variety of species which are possibly only different morphotypes of known species. This however, leaves the taxonomists still with the aftermath of the previous establishments of a large number of species.

Komárek and Lund (1990) have attempted to solve some of the confusion, namely which species are synonyms of the following *Arthrospira* species: *Arthrospira platensis, A. maxima, A. jenneri, A. fusiformis*. According to Komárek and Lund (1990) *Arthrospira platensis* and *A. jenneri* are benthic species and, therefore, do not possess gas vacuoles, a view first described by Fott and Karim (1973) and supported by Hindák (1985). Their distribution is probably pantropical, isolates being known mainly from tropical and subtropical America, Africa and Europe (Komárek & Lund, 1990). In contrast, *Arthrospira maxima* and *A. fusiformis* are planktonic and, thus, gasvacuolated species, occurring in Africa and tropical and central Asia (Komárek & Lund, 1990). Accepting these four species, Komárek and Lund (1990) created a list of synonyms in the literature.

*Molecular taxonomy of Arthrospira strains*

As recent as 1997, Viti et al. (1997) carried out the first molecular screening to solve the dispute on the taxonomy of the genus *Arthrospira* at the intrageneric level. The authors studied the genotypic diversity of nine *Arthrospira* strains and one morphologically different subclone using total DNA restriction profile analysis (Viti et al., 1997). The results showed that the molecular clustering of the strains was in agreement with the nomination of the strains (*Arthrospira platensis*, *A. maxima*) based on morphological criteria. Although the molecular data seemed to confirm the morphological criteria, the screening program was based on a very limited diversity of strains. All four strains designated to *Arthrospira maxima* were isolates from the same lake (Lake Texcoco, Mexico), and the five strains belonging to *Arthrospira platensis* were isolates from three lakes in Chad and one in Algeria.

Nelissen et al. (1994) showed already that the 16S rRNA sequence does not provide a useful marker as it proved to be too conserved to distinguish at the intrageneric level (Section 1.2.2.2). In contrast, the ITS region showed only 83.3% similarity between the two strains tested (PCC 7345 (= D0911 of the presented work; Section 2.2), PCC 8005 (= D0914 of the presented work)). This knowledge formed the basis for a second molecular screening program of *Arthrospira* strains and has been carried out parallel to the presented work (Scheldeman et al., 1999; Appendix C). The authors employed amplified ribosomal DNA restriction analysis of the ITS (ARDRA) a method used previously for the taxonomic analysis of cyanobacteria (Lu et al., 1997). It was based on the same set of *Arthrospira* strains the presented work is based on (Section 2.2), except two strains, which are not included in this study, and three strains, which are duplicates of strains included in the presented work. As concluded from the ARDRA of the ITS the 37 strains from four different continents are resolved in only two molecular clusters (Scheldeman et al., 1999). Strain D0914 and a straight morphotype of an Indian isolate designated as *Arthrospira indica* Desikachary et Jeeji Bai 1992 formed a small subcluster within cluster I.

### 1.2.4 *Arthrospira* as food source

*Discovery of use of* Arthrospira *as a food*

The history of *Arthrospira* as (health) food covers only the last 30 years. *Arthrospira* was originally discovered as a food source in 1940 when Dangeard described the results of the analysis of a sample of green dried material from the market place of a village near Lake Chad (Africa) (Fig. 1.6A), where it was sold by the natives as food under the name Dihé. Dangeard (1940) found that the sample consisted of "a true puree of filamentous, spiral shaped blue-green alga". Up to today the natives harvest mats of the buoyant cyanobacterium from Lake Chad or other alkaline lakes around this lake and dry it in paddles on the sandy shores (Fig. 1.6B). However, due to the war and the report being published in a minor journal (and in French language), no notice was taken of Dangeard's (1940) findings.

Approximately 25 years later, the Belgian botanist Léonard rediscovered Dihé during the Belgian Trans-Saharan expedition which he reported about in a Nature publication (Léonard, 1966). Furthermore, in the same year Farrar (1966) presented in an other Nature publication historical evidence that the Aztecs were producing a type of green dried bread

called tecuitlatl from a cyanobacterium of unknown origin. Based on the historical descriptions and the high alkaline environment of the soda lake (Lake Texcoco) near Mexico City, where the Spanish conquerors described the use of tecuitlatl, it can be assumed that *Arthrospira* was also consumed by the Aztecs. This time much notice was taken by these reports. The fact, that this organism has been exploited for at least several hundreds of years by native populations of the natural environment of *Arthrospira*, indicated the potential for the food market and triggered the interest of private industry.

*Nutritional and pharmaceutical value of Spirulina*

The nutritional value of *Arthrospira* was confirmed for the first time by the Institut Français du Pétrole (1967). The protein content of 62 to 68% (of dry weight) was made up of 18 amino acids and included all amino acids essential for the human body. Furthermore, a series of vitamins (provitamin A, vitamin $B_1$, $B_2$, $B_6$, $B_{12}$, C) was also found being present. Due to the prokaryotic cell wall the rate of absorbance of the nutrients is much higher compared to other, eukaryotic (health) food algae, such as *Chlorella* and *Scenedesmus* (Henrikson, 1997). The discovery of γ-linolenic acid in *Spirulina* (*Arthrospira*) *platensis* added an other cell compound of nutritional and pharmaceutical value (Nichols and Wood, 1968). The high content of unsaturated fatty acid increases also the nutritional quality of *Arthrospira*. More recently, a sulfated polysaccharide, designated calcium-spirulan, has been shown to inhibit the Herpes simplex virus type 1 (Hayashi et al., 1993). Further studies showed that there is great potential of calcium-spirulan in the successful application against other viral infections (Hayashi et al., 1996) including HIV-1 (Ayehunie, 1998).

*Spirulina production*

The morphology and ecology of *Arthrospira* provides several advantages for the production of biomass at an industrial scale. The filamentous nature of *Arthrospira* allows relatively easy and inexpensive harvesting using a screen (Switzer, 1980). The high alkaline environment is inhospitable to most other algae and permits maintenance of monoalgal cultures in large outdoor ponds (Fig. 1.6C). This allows continuous culture at a rate equal to daily growth (Switzer, 1980).

Fig. 1.6 *Arthrospira* as source of food.

1.6A. Sale of Dihé at the market place of N'garangou (Chad). 1.6B. Drying of *Arthrospira* at the sandy shores of Lake Chad (Chad). Photo courtesy of Dr Michel Brouers (University of Liège, Belgium). The photographs (A, B) were taken on 19. March 1998.

1.6C. Aerial photograph of the 22 hectares production area of Earthrise Farms (Calipatria, California). Earthrise Farms is one of the largest producers of Spirulina. Photo courtesy of Yoshimichi Ota, President of Earthrise Farms.

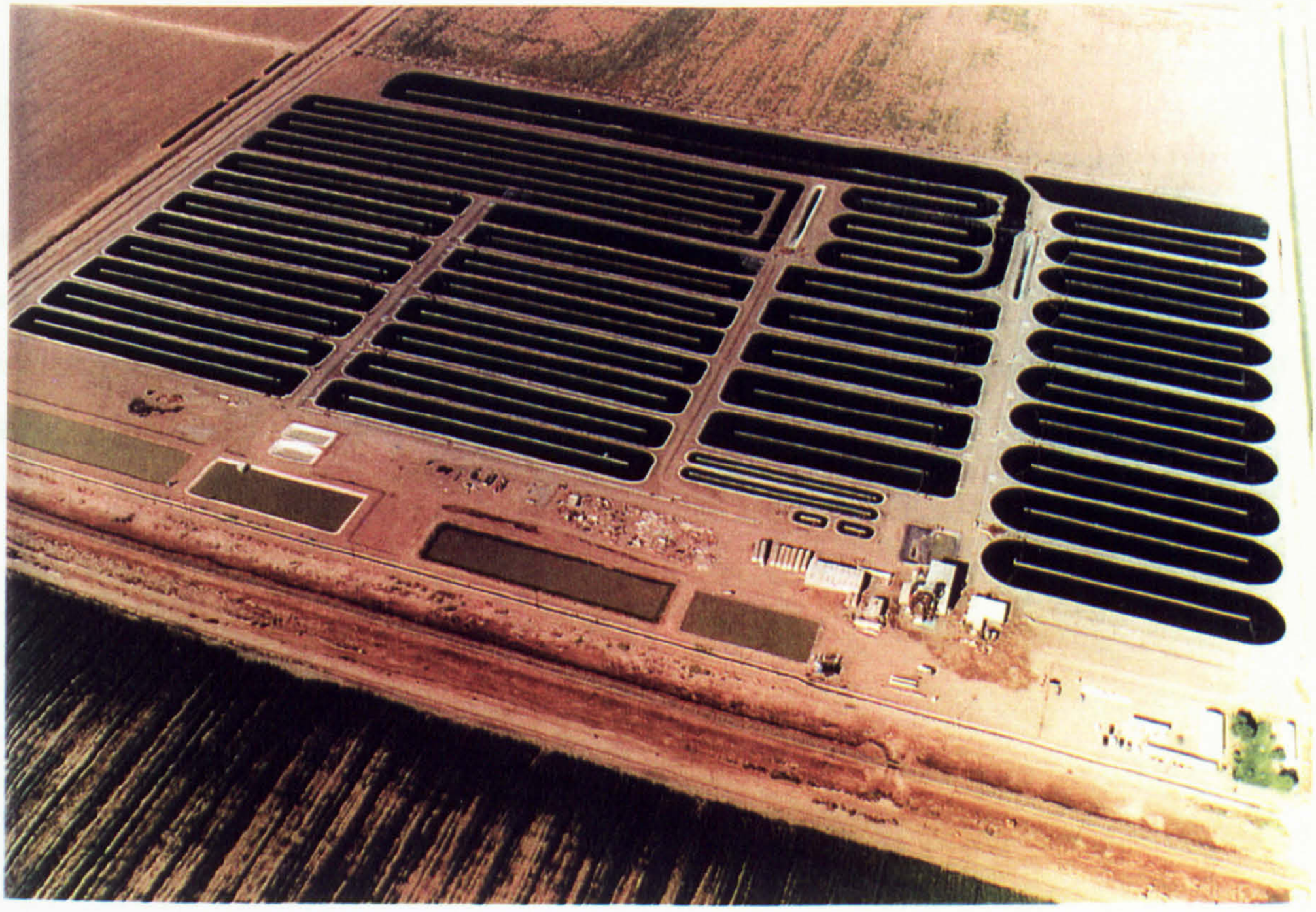
A)



B)



C)



### 1.2.5 Culture collection of *Arthrospira* and *Spirulina* strains

#### 1.2.5.1 Strains

*Arthrospira*

Comparative screening programmes of a large set of *Arthrospira* strains were limited so far to 24 (Jeeji Bai and Seshradi, 1996; screening of morphological characters) or 18 (Cohen et al., 1987; screening of fatty acid composition) strains. Other research groups tested fewer strains (eg Viti et al., 1997). The main reason for the limited number of strains tested is the laborious maintenance of a large number of strains in the absence of a generally applicable method for long-term storage of *Arthrospira* strains which represents a problem also for major culture collections. PCC contains currently the largest set (six strains) of *Arthrospira* strains of all public culture collections (Rippka & Herdman, 1992). A further limitation of accumulation of a large set of strains is the reluctance of researchers to hand over strains. Therefore, it is not surprising that the largest number of strains screened in the past is held at locations with a natural occurrence of *Arthrospira* strains, and hence local isolates (eg Shri AMM Murugappa Chettiar Research Centre, Madras, India; Desikachary & Jeeji Bai 1996), or at industrial or long-term funded research institutes (eg Jacob Blaustein Institute for Desert Research, Israel; Cohen et al., 1987).

*Spirulina*

Although *Spirulina* strains are of economic importance due to their relatively high content of phycoerythrin (Tomaselli et al., 1995), no screening program has been carried out of a large a set of strains as has been done for *Arthropsira*. The most extensive study carried out concerns a screening programme of the fatty acid composition of six *Spirulina* strains (Cohen and Vonshak, 1991).

### 1.2.5.2 Media

*Arthrospira*

Most researchers and culture collections have used a medium based on that of Zarrouk (1966) to culture *Arthrospira* strains. CCAP, SAG and NIES maintain *Arthrospira* strains in a medium which is either identical or very similar to Zarrouk's medium, but PCC maintains *Arthrospira* strains in a medium with lower N and P concentrations. BG-11 contains only 60% and ASN-III only 30% of the nitrogen of Zarrouk's medium and only 6% (BG-11) or 4% (ASN-III) of the phosphorus of Zarrouk's medium (Rippka & Herdman, 1992). UTEX uses a medium very similar to Zarrouk's medium for two of its four *Arthrospira* strains. The other two (D0875, D0876) are marine isolates and are maintained in seawater enriched with N (= 14% of Zarrouk's medium) and P (1.1% of Zarrouk's medium). Furthermore, modifications to the trace metal content are frequently used. Omission of vanadium, chromium, tungsten and titanium makes the trace element content similar to that of BG-11 medium (Waterbury & Stanier, 1981). CCAP, SAG and NIES culture *Arthrospira* strains in media with typically much lower concentrations of some of these elements.

*Spirulina* strains

*Spirulina* strains are generally cultured in a medium similar to ASW:BG, which is used by CCAP (CCAP, 1998). This medium is made up in two parts, which are autoclaved separately and mixed aseptically, (1:1), when cool. Part 1 is identical with BG-11; part 2 contains seawater, tricine (2.8 mM), additional nitrate and phosphate (1.32 mM $NO_3^-$, 53.2 mM $PO_4^{3-}$) and an aqueous extract of soil.

### 1.2.5.3 Production of axenic cultures of cyanobacteria

Most areas of investigation of any bacterial group depends largely on the production of axenic (bacteria-free) cultures. The difficulty to obtain axenic cultures of cyanobacteria is well resembled by the great variety of purification methods. Besides techniques which make use of gliding movement and phototaxis of motile cyanobacteria (Allen, 1952; Allen, 1973; Bowyer & Skerman, 1968; Bunt, 1961 Vaara et al., 1979), a series of streaking procedures have been reported, eg numerous restreakings on mineral medium (Castenholz, 1970; Stanier et al., 1971; Van Baalen, 1961; Waterbury & Stanier, 1978). However, agar which is mainly

used as solidifying agent usually contains impurities (Allen & Gorham, 1981; Bretcher & Kaiser, 1978; Krieg & Gerhardt, 1981), which may lead to inhibition of cyanobacterial growth (Allen & Gorham, 1981).

Other types of purification techniques made use of mechanical separations such as micromanipulation (Bowyer & Skerman, 1968), filtration (Heaney & Jarworski, 1977) and equilibrium centrifugation (Sitz & Schmitt, 1973). Treatment of cyanobacterial cultures with toxic chemicals, such as phenol (Carmichael & Gorham, 1974; McDaniel et al., 1962), sodium hypochlorite (Fogg, 1942), detergents (McDaniel et al., 1962), sodium sulfide (Parker, 1982), or treatment with antibiotics (Pinter & Provasoli, 1958; Ferris & Hirsch, 1991), UV or gamma irradiation (Koch, 1965; Kraus, 1966) or elevated temperature (Allen & Stanier, 1968; Wieringa, 1968) have also been employed in order to purify cyanobacteria from any contaminating bacteria.

Only one procedure for obtaining an axenic culture of *Spirulina* (*Arthrospira*) *platensis* has been reported (Torzillo et al., 1985). The authors used a combination of differential filtration (on nylon net and 8 μm membranes), filament fragmentation, centrifugation and dilution of the obtained 2-4 celled trichome fragments.

### 1.2.5.4 Long-term storage of cyanobacteria

There are two main problems that are encountered in the absence of a successful and reliable method for long-term storage. Firstly, serial subculturing of the strains is not only time consuming but also increases the risk of contamination. Secondly, serial subculturing can also result in genetic drift to a genotype that is best adapted to the laboratory culture conditions. This also leads to the loss of a pool of great genetic variation.

It has been shown for cyanobacteria that genetic drift can affect morphological and physiological properties during serial subculturing, ie loss of the helical trichome morphology of *Arthrospira* strains (Section 1.3.2) and loss of the ability to photoheterotrophic growth of *Synechocystis* PCC6803 (Hihara & Ikeuchi, 1997), emphasizing the need for a successful method for long-term storage. The most common method used is cryo-preservation in liquid nitrogen, but drying techniques have proven useful for cyanobacteria which are desiccation tolerant (Whitton, pers. comm.).

## 1.3 Morphology and motility of cyanobacteria

Despite the relatively small size, cyanobacteria have traditionally been classified on the basis of their morphological criteria (Section 1.1.2). However, attempts to identify cyanobacteria in culture using this field-based system for classification may often lead to many difficulties and ambiguities (Doers & Parker, 1988). Therefore, care must be taken to avoid selection of markers which are under the influence of the environment or genetic selection pressure. It will not always be possible to do so, but consideration of a wide range of markers will minimize the effect of an "ambiguous" one, especially if the latter ones are known as problematic to the user of the taxonomic key.

Analysis of the 17 original species descriptions (Appendix A) of *Arthrospira* spp. collected in the presented work, and several keys dealing with the identification of isolates belonging to the genus *Arthrospira* (eg Geitler, 1932; Desikachary & Jeeji Bai, 1996), shows that the main morphological characters used for taxonomic purposes were characters describing the trichome helix (length of pitch, diameter, attenuation of helix towards the apices), the trichome (attenuation towards the apices), the cell size (width (ie trichome width); length) and shape of end cell. Other taxonomists use additional characters, such as granulation, sheath and motility of *Arthrospira* strains (Tomaselli, 1997). Komárek and Lund (1990) used the presence of gas vacuoles for the grouping of *Arthrospira* spp. into benthic and planktonic species. There is only one report (Buell, 1938) that considers helix orientation as a taxonomic marker.

As helix parameters have been so far the characters generally used for taxonomic grouping of *Arthrospira* strains, the literature on helical growth forms is reviewed in some detail.

### 1.3.1 Helical growth of prokaryotes

A helical morphology is a widespread character of life, with examples among eukaryotic algae (*Microspora lauterbornii*: Printz, 1964), several taxonomically diverse genera of gram-negative bacteria (*Saprospira*, *Spirillum*, *Oceanospirillum*: Krieg, 1984) and cyanobacteria (*Arthrospira*, *Spirulina*, *Anabaenopsis*).

### 1.3.2 Loss of helical morphology of *Arthrospira* spp.

Rippka et al. (1979) described the helical shape of *Arthrospira* and *Spirulina* has been described by as "a stable and constant property" of the genus that distinguishes it from *Oscillatoria, Pseudanabaena* and the LPP-group (*Lyngbia, Phormidium* and *Plectonema*). However, as early as 1931, Rich (1931) described for the first time the occurrence of straight trichomes in laboratory cultures of *Arthrospira* strains. This observation was confirmed by several taxonomists (eg Thomasson, 1960; Bousson, 1971; Lindblom, 1972; Marty & Busson, 1970). Lewin (1980) also confirmed these observations and argued, that the separation of *Oscillatoria* and *Arthrospira* is therefore "untenable".

### 1.3.3 Influence of environment on helix dimensions

Due to the importance for taxonomic purposes and the obvious changes in dimensions, much research has focussed on factors influencing the helical trichome morphology of *Arthrospira* spp., such as growth medium (Bousson, 1971; Watanabe & Ichimura, 1977), light and temperature (Van Eykelenburg, 1979) or a combination of both (Jeeji Bai & Seshardi, 1980). In general, increasing growth temperature was found to result in a tightening of the trichome helix of *Arthrospira* strains (Bousson, 1971; Van Eykelenburg, 1979), and potassium, phosphorus and sulphur deficiencies increased their diameter (Bousson, 1971). Phosphorus and sulphur deficiencies also led to a lengthening of the filaments (Bousson, 1971). A more complex picture was obtained when the influence of the combination of light and nutrient availability on the helix morphology of *Arthrospira* was tested. Jeeji Bai and Seshardi (1980) described three different morphological variants of *Spirulina (Arthrospira) fusiformis*: the S-type with trichomes having more or less a regular and long pitch, the C-type with trichomes having a distinct barrel shape with short pitch, and the H-type with trichomes having a dumbbell shape with tight closed helix. The S-type variant predominated in cultures with low light and nutrients, whereas the C-type variant was the most abundant type under high light and nutrient conditions. When a combination of high light and low nutrients were provided the helix morphology changed from C- to H-type variant. Another factor that influences the helical morphology of a trichome is salinity, which leads to decreasing helix diameter (Watanabe & Ichimura, 1977).

Changes in helix morphology were thought to result from a rearrangement of the polysaccharide chains within the cell wall (van Eykelenburg, 1979). Such a rearrangement was also suggested to be responsible for the slow spiralling of a filament when placed on a agar plate (Van Eykelenburg & Fuchs, 1980). After rewetting of the filaments by addition of a drop of water the spiral conformation snapped immediately back to a helical one. The authors explained this phenomenon by dehydration/hydration of the oligopeptides in the peptidoglycan layer of the cell wall, thought to be responsible for maintaining the helical trichome shape (Van Eykelenburg & Fuchs, 1980).

### 1.3.4 Helix reversal

A particular character which has caught some attention is the reversal of the orientation of a helical morphology. Already in 1751Carl von Linné (Linnaeus) seems to have been aware of helix reversal in plants, as shown by his drawings in Philosophia Botannica (Tabula V). Darwin (1888) described experiments to elucidate the reason for the reversal of helix orientation of the tendrils of climbing plants. More recently, Goriely and Tabor (1997a,b,c) investigated helix reversal from a mathematical viewpoint. Based on these studies, Goriely and Tabor (1998) developed a theoretical model describing the reversal of helix orientation as observed in climbing plants and often also in telephone cords: both tendrils and telephone cords show a snag at the point of reversal. The reversal of helix orientation of tendrils occurs when the growing tendril touches a support. As neither the stem nor the support can rotate, the tendril curls on itself, thus causing the reversal of the helix. In telephone cords the reversal occurs when the cord is stretched and twisted so that it unwinds slightly and some of the tension is released (Goriely & Tabor, 1998).

Among prokaryotes most attention has been given to helix-producing mutants of the rod-shaped gram-positive *Bacillus subtilis* (Mendelson, 1976), which were unable to divide after cell replication. Several factors influence helix orientation in these mutants, including temperature and $Mg^{2+}$ concentration (Mendelson & Karamata, 1982), D-alanine (Surana et al., 1988) and D-cycloserine (Mendelson, 1988). In the case of cyanobacteria, Buell (1938) described two new species of *Spirulina* (*Arthrospira*), which were distinguished from one another by their helix orientation (*Spirulina amethystina* clockwise and *S. aeruginea* anti-clockwise ). In contrast to Buell's (1938) findings, the drawings of *Spirulina fusiformis*

Woron. (Woronichin, 1934; Fig. 1.5) show trichomes with either clockwise or anti-clockwise helix orientation, while Hindák's (1985) drawings of *Arthrospira fusiformis* show both helix orientations within the same trichome.

### 1.3.5 Motility of cyanobacteria

A great deal of literature has been published on prokaryotic motility, but most of it deals with the flagellated, swimming forms. However, cyanobacteria do not possess flagella and, therefore, are not able to swim using such a mechanism. There is only one cyanobacterium known capable of swimming. A few marine forms of *Synechococcus*, isolated from the Sargasso Sea and South Atlantic Ocean can swim with a speed of 25 $\mu m\ s^{-1}$ without flagella or any other surface structures (Waterbury et al., 1985). The mechanism used by these isolates to swim is still unclear. Pitta and Berg (1995) demonstrated that self-electrophoresis, a mechanism used by some bacteria, is not responsible for the swimming motility. An alternative explanation was introduced by Ehlers et al. (1996) who suggested that travelling waves of surface fibrils along the cell surface may allow the *Synechococcus* isolates to swim. All other cyanobacteria, when motile, glide. Gliding can be defined as "self-propulsion across a solid or semi-solid material without the aid of any visible organ (ie flagellum) or apparent change in the shape of the organism" (Castenholz, 1982). Excretion of slime has long been thought to be responsible for the gliding motility of cyanobacteria (Burkholder, 1934). The discovery of junctional pores in the cell wall of several filamentous cyanobacteria (Ris & Singh, 1960) indicated that slime excretion represents the propulsive mechanism for gliding. To test the hypothesis of gliding by slime excretion Hoiczyk and Baumeister (1998) investigated the junctional pore system of *Phormidium uncinatum* and *Anabaena variabilis*. Structural analysis of the isolated pore complexes in combination with light microscopic observations of the slime secretion process strongly suggested that slime directly generates the necessary thrust for locomotion for the gliding of filamentous cyanobacteria (Hoiczyk & Baumeister, 1998). Halfen and Castenholz (1971b) also calculated the necessary energy demand for the gliding of the filamentous cyanobacterium *Oscillatoria princeps* if slime excretion provides the mechanical propulsion. The researchers concluded that the energy expense would be approximately 5% of the total

ATP produced by oxidative phosphorylation (Halfen & Castenholz, 1971b). Similar energy expenditures have been suggested for other bacteria (Morowitz, 1954).

However, the gliding motility of filamentous cyanobacteria is not based alone on the secretion of slime. During gliding the filaments of cyanobacteria rotate around their longitudinal axis. Fibrils, which are helically wound along the trichomes and are located between the peptidoglycan layer and the outer membrane were found to be responsible for the rotation of the filaments of *Oscillatoria princeps* during gliding (Halfen & Castenholz, 1970). Later research, however, showed that these fibrils may well be located outside the outer membrane of *Oscillatoria princeps* (Hoiczyk & Baumeister, 1995). This led to the hypothesis that slime secretion works in combination with the fibrillar surface structures during gliding of cyanobacteria. But also fibrils located underneath the outer membrane are now thought to be involved in motility of filamentous cyanobacteriae (Adams et al., 1999). Further analysis of the helical fibrils, located at the surface of the cyanobacterial cell wall, revealed that they consist of a $Ca^{2+}$-binding glycoprotein and are necessary for gliding motility of cyanobacteria as well as $Ca^{2+}$ in the medium (Hoiczyk & Baumeister, 1997). $Ca^{2+}$ has already been found to influence motility of *Spirulina subsalsa* (Abeliovich and Gan, 1982) and is also required for swimming of the *Synechococcus* isolates (Pitta et al., 1997).

## 1.4 Ultrastructure of cyanobacteria

*Main discoveries*

The introduction of biological electron microscopy has allowed insights into cellular and cell wall structures. Based on this methodology the "concept of prokaryotes" has been developed (Stanier & Van Niel, 1962).

Further progress was achieved by Jost (1965), who developed the model of the four layered structure of the cyanobacterial cell wall, while fibrilar structures wound helically around the trichomes of filamentous cyanobacteria were first identified by Halfen and Castenholz (1970). Cell surface structures of cyanobacteria have also been the subject of ultrastructural analysis by TEM. For example, Vaara (1982) analyzed the surface structures of chroococcacean cyanobacteria and Karlsson et al. (1983) resolved the three-dimensional structure of the surface layer of *Synechocystis* sp. strain CLII by combination of electron microscopy and Fourier reconstruction. An other example of the use of TEM applied on

cyanobacteria is the reconstruction of the three-dimensional structure of an unicellular cyanobacterium (Nierzwicki-Bauer et al., 1983).

A further step in the identification of ultrastructural characters of prokaryotes involves the isolation of cell inclusions; this has been successful in several cases. Walsby and Buckland (1969) isolated gas vacuoles, and Shively et al. (1973) isolated polyhedral bodies, and discovered that they contain ribulose diphosphate carboxylase, thus represent the site of $CO_2$-fixation. Consequently, polyhedral bodies were renamed as carboxysomes (Shively et al., 1973). Cell wall structures, such as pore complexes (Hoiczyk & Baumeister, 1998) or surface fibrils (Adams & Bean, unpubl. data) have also been isolated and characterized.

*Use of TEM in taxonomic research*

Whitton (1972) was among the first who suggested the use of TEM for the taxonomic analysis of cyanobacteria and listed characters with potential value for ultrastructural characterization. Despite these proposals, TEM of cyanobacteria has so far concentrated more on the analysis of certain characters than on comparison of a wide range of strains for taxonomic purposes. The work of Jensen (1985), who analyzed the taxonomic relationship of more than 60 cyanobacterial isolates on the basis of cell inclusions, represents a rare exception. Furthermore, despite the many suggestions of Whitton (1972), the choice of ultrastructural characters used for taxonomic comparisons has mainly concentrated on cell inclusions (Jensen, 1985) or cell wall pore pattern (Guglielmi & Cohen-Bazire, 1982). Analysis of other possible characters, such as the arrangements of thylakoids (Tomaselli Feroci et al., 1976) were rather rare.

## 1.5 Physiology of cyanobacteria

### 1.5.1 Heterotrophy

*Dark heterotrophy*

Cyanobacteria are aerobic photosynthetic microorganisms (Staley et al, 1989). Harder, however, showed as early as 1917 that *Nostoc punctiforme* (Kütz.) Hariot isolated from *Gunnera* is capable of growing heterotrophically in the dark. Despite this conclusive proof some later reviews stated that cyanobacteria are obligate autotrophs (Holm-Hansen, 1968).

Khoja and Whitton (1971) were the first who screened a wide range of cyanobacterial species for their ability to grow in the dark on sugars. Among the 24 species (from 12 genera) tested only seven did not grow in the dark on sucrose.

*Photoheterotrophy*

Analysis of the ability of cyanobacteria to grow photoheterotrophically can be tested by inhibition of photosynthesis with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in the presence of an organic carbon source. The use of DCMU as an inhibitor of photosynthesis has been described for the first time by Bishop (1958). Using the green alga *Scenedesmus* sp., Bishop (1958) found that 0.5 μM DCMU reduces photosynthesis by 50% and 3 μM DCMU leads to complete inhibition of photosynthesis in this algae. Nultsch (1973), however, warned that the concentration of DCMU necessary to inhibit photosynthesis has to be determined for each species. The presence or absence of sheath surrounding the cyanobacterial cell may require increased concentrations of DCMU to inhibit photosynthesis completely (Nultsch, 1973). Nultsch (1973) determined, for example, that a concentration of 20μM DCMU was necessary to inhibit photokinetics in members of the *Nostocaceae*. Generally, however, 5 or 10 μM (final) concentrations of DCMU are used to inhibit photosynthesis of cyanobacteria, including members of the genus *Nostoc*, without tests of its inhibitory capacity (50 references checked, eg Bastia et al., 1993; Kis et al., 1998; Summers et al., 1995).

Kenyon et al. (1972) screened 29 filamentous strains for photoheterotrophy and found that 18 strains were able to grow under light in the presence of DCMU at the expense of glucose and that 15 of those also grew in the dark on glucose, confirming that heterotrophy is a rather wide spread character of cyanobacteria.

*Reasons for lack of ability to grow heterotrophically*

There seems to be no obvious reason for the lack of the ability for aerobic dark heterotrophic growth of cyanobacteria. The necessary pre-requisites for growth, like the presence of a functional tricarboxylic acid pathway (Pearce and Carr, 1969) and of NADH oxidase (Leach and Carr, 1970), cell wall permeability for the organic substrate (Stanier, 1973) and sufficient ATP supply through oxidative phosphorylation (Pelroy and Bassham, 1973) are provided in cyanobacteria unable to grow dark heterotrophically. However, lethality could emerge from a toxic effect of fructose similar to that observed for

*Synechocystis* strains PCC 6803 and PCC 6714 (Joset et al., 1988; Flores and Schmetterer, 1986). Zhang et al. (1998) showed that providing the obligate photoautotrophic *Synechococcus* strain PCC 7942 with a functional glucose transport protein gene (*glcP*) from *Synechocystis* strain PCC 6803 leads to sensitivity to glucose and cell death. The toxicity may be linked to the intracellular accumulation of the sugar (or a breakdown product(s) of it) to a higher concentration than normally encountered leading to a disequilibrium of metabolic activities (Zhang et al., 1998).

Cyanobacteria are well adapted to regulate their "standard" assimilating machinery for uptake of the usual inorganic carbon source $CO_2$ (Sitte et al., 1991). A similar control mechanism is required when organic carbon molecules are offered in high concentrations. If the cell fails to successfully control the sugar influx, then cell damage may arise. Similar observations have been reported for bacterial chemostat cultures upon supply of glucose (Koch, 1997). Cells were found either to die or to grow immediately faster before adjusting to a lower, but steady growth rate appropriate for the concentration of glucose (Koch, 1997).

*Concentration of the organic carbon source*

A wide range of concentrations of sugars has been used in the past to test cyanobacteria for their ability to grow heterotrophically. While Khoja and Whitton (1975) used glucose, fructose and sucrose in the concentration of 10 mM to screen cyanobacteria for their ability to dark and photoheterotrophic growth, Pelroy et al. (1972) used 27.75 mM (0.5% w/v) and Rippka (1972) 55 mM (1% w/v) glucose. Photoheterotrophic growth of *Arthrospira* strains was often investigated using an even wider range of concentrations. While Marquez et al. (1995) used glucose in a concentration of 2.5 mM, Ogawa and Terui (1972) used 111 mM (2% w/v) glucose. Marquez et al. (1995) showed for a strain of *Arthrospira* (= D0885) that increasing glucose concentrations led to an increase in yield under photoheterotrophic conditions.

*Potential industrial applications*

Heterotrophic growth for the improvement of biomass production is of economical importance in cyanobacterial biotechnology and has been studied in some detail on *Arthrospira*. Photoheterotrophic growth can be used to increase cell density and phycobiliprotein content in *Arthrospira* (Chen & Zhang, 1997). Generally, glucose was used for any detailed studies on dark and photoheterotrophic growth characteristics (Marquez et

al., 1993) or improvement of cell density and cell composition (Marquez et al., 1995; Chen & Zhang, 1997) of *Arthrospira* strains. There is no conclusive proof in the literature on the ability of *Arthrospira* strains to grow on sucrose only, which is a cheaper carbon source than glucose. Balloni et al. (1980) reported on the efficient use of waste waters from a sugar refinery for Spirulina production. The waste water, however, was treated anaerobically with photosynthetic bacteria. Therefore, it is likely that the carbon source utilized by *Arthrospira* sp. was not sucrose but a breakdown product of it. Similarly, Jourdan (1998) reports about increase in biomass production in an outdoor pond containing sucrose supplemented medium. In contrast to the non axenic outdoor trials of Jourdan (1998), Tomaselli et al. (1978) tested axenic cultures of *Spirulina (Arthrospira) maxima* and *Spirulina (Arthrospira) platensis* for their ability to photoheterotrophic growth. The results demonstrate that the strain of *Arthrospira maxima* showed increased growth in the presence of sucrose (no DCMU added) compared to growth in inorganic medium, while *Arthrospira platensis* did not show an increased growth in sucrose supplemented medium.

It was generally found that photoheterotrophic growth exceeds dark heterotrophic growth (Khoja & Whitton, 1971). If, however, dark heterotrophically grown cells contain valuable compounds in higher concentrations then the higher production costs may be compensated by the higher profit margin.

### 1.5.2 Growth on alternative nitrogen and phosphorus sources

Addition of nutrients, such as nitrate and phosphate, to the culture medium in large scale outdoor ponds represent big expenses of *Arthrospira* production. However, *Arthrospira* strains have been shown to be capable of utilizing alternative nitrogen sources, such as ammonium (Boussiba, 1989) and urea (Saxena et al., 1983). Using this knowledge, much effort has been dedicated towards development of growth of outdoor cultures on alternative nutrient sources. Special emphasis has been put on the use of animal waste products (Olguin et al., 1999). This has also the advantage of removal of nitrogen and phosphorus from the animal waste.

Although there have been doubts in the past on the ecological importance of phosphatases in nature (Rigler, 1961), it has been proven only very recently that surface phosphatases hydrolyse efficiently naturally occurring organic molecules containing phosphorus, and thus play an integeral role in the phosphorus turnover in an ecosystem (Jansson et al., 1988).

Phosphatase activity is a widespread physiological character among cyanobacteria growing in P-limited environments (Healey, 1982). Doonan and Jensen (1980) reported that all 18 strains tested showed phosphomonoesterase activity which was inducible in 12 of the strains. Whitton et al. (1991) screened 50 cyanobacterial strains from ten genera for their ability to use organic phosphorus compounds as their sole P-source. The authors found variation between strains which were useful for taxonomic grouping (Whitton et al., 1991).

## 1.6 Biochemical characters

### 1.6.1 Fatty acids

Fatty acids can be regarded as "carboxylic acid derivatives of long-chained aliphatic molecules" (Suzuki et al., 1993). Therefore, a fatty acid molecule is composed of two distinct regions, a long, hydrophobic hydrocarbon chain and a hydrophilic carboxylic acid group (Sitte et al., 1991). The large number of different fatty acids is a consequence of the great variation in the length of the hydrocarbon chain. Generally, however, only a few very abundant $C_{16}$ and $C_{18}$ fatty acids (containing 16 or 18 carbon atoms, respectively) compose the main part (up to over 90%) of the fatty acid fraction of plants and cyanobacteria (Harwood & Russel, 1984).

#### 1.6.1.1 Fatty acids in cyanobacteria

*Function of fatty acids in the cyanobacterial cell*

Cyanobacteria are not known to maintain fat reserves in the form of neutral fatty acids (Murata & Nishida, 1987). Fatty acids are a functional part of the cell membranes, and, therefore, are found mainly in the cytoplasmic and thylakoidal membranes where they form the basis for the lipid bilayer. Within this bilayer the photosynthetic machinery is located. Thus, the fatty acid composition plays an integral role in the function of the photosynthetic apparatus. As a great part of the fatty acids are unsaturated, the membranes are very fluid at physiological temperatures. Fluidity is necessary to allow lateral movement of pigment-protein complexes through the membrane (Lawlor, 1993).

*Evolutionary significance of fatty acids*

The organisation of the membrane lipids is known to be similar to that of chloroplasts (Murata & Nishida, 1987). The evolutionary significance of this similarity has been recognised by Kenyon and Stanier (1970), who compared the fatty acid composition of plant chloroplasts with that of several genera of cyanobacteria and other photosynthetic prokaryotes. Based on this comparison Kenyon and Stanier (1970) concluded that the ability to form polyunsaturated fatty acids, which, among prokaryotes, is confined to cyanobacteria, has been transferred, in association with the machinery of oxygen-evolving photosynthesis, to the eukaryotic cell. Therefore, it is not surprising that the synthesis of fatty acids in plants and cyanobacteria is similar (Murata & Nishida, 1987) as well as the changes in fatty acid composition of plant and cyanobacterial cells in response to environmental changes (Murata & Wada, 1995).

*Fatty acid synthesis and desaturation*

16:0-ACP (acyl-carrier protein) and 18:0-ACP are synthesized in cyanobacterial and plant cells as end products by the fatty acid synthase system (Lem & Stumpf, 1984a, b; Stapelton & Jaworski, 1984a, b). Subsequently, fatty-acyl chains are incorporated into glycerolipids via the stepwise esterification of glycerol 3-phosphate. Enzymes involved in this reaction are glycerol-3-phosphate acyltransferase and 1-acylglycerol-3-phosphate acyltransferase (Roughan & Slack, 1982). Once plant fatty acids have been esterified on the *sn*-1 and *sn*-2 position of the glycerol backbone forming phosphatidic acid, they can generally not be desaturated any further. In contrast to plant cells, the acyl groups of acyl-ACPs in cyanobacterial cells are directly incorporated into glycerolipids by acyltransferases and then converted to unsaturated fatty acids by acyl-lipid desaturases (Murata & Wada, 1995). This is confirmed by the lack of any desaturases other then those of the membrane-bound acyl-lipid desaturase type, which are specific for the biochemical form of their fatty acid substrate (Murata et al., 1996; Nishida & Murata, 1996). Therefore, the levels of unsaturated fatty acids in the membranes of cyanobacterial cells are determined exclusively by acyl-lipid desaturases which are specific to fatty acids esterified to glycerolipids (Nishida and Murata, 1996). Acyl-lipid desaturases are referred to as desaturases here.

Each desaturase is catalyzing the formation of a carbon-carbon double bond in the hydrocarbon chain at the specific delta (counting from the carboxyl group) or omega

(counting from the methyl end) position. Furthermore, the desaturases are also specific for the *sn* position of the acyl-chain on the glycerol backbone (Nishida & Murata, 1996).

In cyanobacteria the desaturation of $C_{18}$ fatty acids has been studied in some detail and in *Synechocystis* sp. PCC 6803, all desaturases have been cloned and sequenced. The first step in desaturation of stearic acid is catalyzed by the delta 9 desaturase which is encoded by the *desC* gene. The product of this reaction, oleic acid, is then further desaturated to linoleic acid by the delta 12 desaturase which is encoded by the *desA* gene. In *Synechocystis* PCC 6803 linoleic acid can then be transferred into either γ-linolenic acid by the introduction of a carbon-carbon double bond at the delta 6 position, or into α-linolenic acid by introducing the carbon-carbon double bond at the delta 15 (omega 3) position of the fatty-acyl chain. The reactions are catalyzed by the delta 6 and the omega 3 desaturase, respectively. Unlike γ-linolenic acid, α-linolenic acid is not necessarily the endproduct of the desaturation of the $C_{18}$ fatty-acyl chain, but can be transferred into cis-6, 9, 12, 15-octadecatetraenoic (18:4) acid by the introduction of a carbon-carbon double bond at the delta 6 position (Harwood & Russel, 1984; Lawlor, 1993; Murata et al., 1996; Nishida & Murata, 1996; Suzuki et al., 1993).

*Arthrospira* has also been subject to extensive analysis of the fatty acid deaturation machinery and the *desA* and *desD* genes have been cloned and characterized (Murata et al., 1996). In contrast to *Synechocystis* PCC6803, *Arthrospira* strains are not able to form α-linolenic acid and, therefore, also lack 18:4 (Murata et al., 1996).

As the desaturation of fatty acids occurs as last step in the fatty acid synthesis in cyanobacteria it may seem easy to investigate factors controlling the desaturase gene expression and enzyme activity. However, despite extensive research and increasing knowledge there are still uncertainties about the precise molecular and biochemical factors controlling the desaturase gene expression (Kis et al., 1998) or mRNA turnover (Sakamoto & Bryant, 1997).

*Influence of environmental factors on the fatty acid composition*

The response to an environmental change that is best understood today, is the low-temperature-induced variation in the fatty acid composition which has been studied during the last two decades in some detail. The changes in the fatty acid composition of membrane lipids of plants and cyanobacteria to alterations in the ambient growth

temperature were found to be similar and an adaptive response (Murata et al., 1979; Wada & Murata, 1990). Temperature decrease generally leads to an increase in fatty acid desaturation of membrane lipids (Somerville, 1995). Such temperature-induced changes of the unsaturation of fatty acids are responsible for the maintenance of membrane fluidity, which is necessary for the proper functioning of biological membranes (Cossins, 1994, Lawlor, 1993).

Due to the lack of an extensive data set it is difficult to generalize the effect of light intensity on the fatty acid composition of *Arthrospira*, and cyanobacteria in general. The studies of Cohen et al. (1987) showed that light intensity does not affect much the desaturation of fatty acids but the overall fatty acid content of *Arthrospira* cells: the higher the light intensity the lower the fatty acid content. Similarly, Kis et al. (1998) showed that the level of unsaturation of the fatty acid fraction of *Synechocystis* PCC 6803 does not change when the light intensity is increased from 70 to 2000 µmol photon $m^{-2}$ $s^{-1}$.

Some studies on the influence of light limitations on the fatty acid composition of cyanobacteria have been carried out in the past using *Arthrospira* strains as a model system. Tanticharoen et al. (1994) reported an increase in overall fatty acid content as well as in fatty acid desaturation in cultures grown under a light-dark cycle, while Hirano et al. (1990) observed a 50% increase in γ-linolenic acid content when a photoautotrophic *Arthrospira* culture was kept in the dark for seven days in inorganic medium. In contrast to *Arthrospira* strains, Kis et al. (1998) reported that cultures of *Synechocystis* PCC 6803 grown under light-activated (white light pulse of 10 µmol photon $m^{-2}$ $s^{-1}$ for 10 min each day) dark-heterotrophic condition on glucose show a decrease in fatty acid desaturation.

### 1.6.1.2 Fatty acid composition and chemotaxonomy

Fatty acids have been used in the past to classify cyanobacteria at the genus level. Kenyon (1972) and Kenyon et al. (1972) developed a system that divides cyanobacteria into four groups. These are: cyanobacteria lacking any polyunsaturated fatty acids but containing only unsaturated and monounsaturated fatty acids. The second and third group are characterized by the presence of either cis-9,12,15-octadecadienoic acid (α-linolenic acid) or cis-6,9,12-octadecatrienoic acid (γ-linolenic acid), respectively, while members of group four contain in addition to all those fatty acids also cis-6,9,12,15-octadecatretaenoic

acid (Kenyon et al., 1972). Furthermore, according to Kenyon et al. (1972) an additional characterization can be based on the unicellular versus filamentous nature of cyanobacteria. While unicellular cyanobacteria belong to groups one and three, filamentous cyanobacteria can be found in groups two, three and four (Kenyon et al., 1972). Murata et al. (1992) confirmed later the chemotaxonomic grouping of cyanobacteria into four groups but also showed that the filamentous and unicellular character does not correlate to this grouping but that filamentous and unicellular species are distributed among the four groups.

#### 1.6.1.3 Economic value of polyunsaturated fatty acids

Polyunsaturated long-chained fatty acids have long been known to have diverse biochemical effects on the human metabolism. Among these, γ-linolenic acid was recognised as one of the most important components leading to reduction of the levels of serum triglycerides and low density lipoproteins (Leaf and Weber, 1988) and stimulation of the immune system (Wu & Meydani, 1996).

### 1.6.2 Lectin-binding to cell surface structures

Lectins are carbohydrate-binding proteins of non-immune origin which specifically conjugate with monosaccharides or simple polysaccharides (Goldstein et al., 1980). Due to the presence of membrane proteoglycans and glycolipids the investigation of lectin-binding represents a useful tool for the characterization of cell surfaces (Slifkin & Doyle, 1990).

The application of immunofluorescently of labelled cell probes as a tool in studies of the systematics and biogeography of freshwater and marine phytoplankton originated three decades ago, when Bernhard et al. (1969) first attempted to differentiate phytoplankton species using antibodies. Since then, lectins have also been used for the identification of eukaryotic algae and cyanobacteria.

Lectins allowed the differentiation of the toxic dinoflatellate *Gymnodinium catenatum* from morphological similar non-toxic *Gymnodinium* species (Costas & López-Rodas, 1994). Furthermore, several species belonging to the dinophyceae and conjugatophyceae were successfully identified using lectins (Costas et al., 1993). Costas et al. (1993) were also able to differentiate between two morphological similar species of the cyanobacterium

*Chroococcus* based on their lectin-binding ability only, while López-Rodas and Costas (1997) used a combination of lectins and polyclonal antibodies to identify three species of *Microcystis*. In all reports lectins appeared to be a useful tool not only for differentiation between genera and species, but also between subclones of the same species. Moreover, López-Rodas and Costas (1997) could identify geographic differentiation within the same species. Differences in lectin-binding were moderate among geographically close catchments and intensified among areas more distant from one another (López-Rodas & Costas, 1997).

## 1.7 Pyrolysis Mass Spectrometry

Pyrolysis is the thermal breakdown of complex organic compounds in an inert atmosphere (Magee, 1993). The fragments resulting from the thermal breakdown can be resolved by gas chromatography (PyGC) or mass spectrometry (PyMS), thus providing a characteristic whole-cell fingerprint (Goodfellow & O'Donnell, 1993).

PyMS has been identified as a useful technique in bacterial systematics (Shute et al., 1985), and has also been applied on cyanobacteria (West et al., 1999). The great advantage of pyrolysis techniques over other whole-cell fingerprint techniques lies in the rapid and low cost analysis; furthermore, the data are reproducible (Goodfellow & O'Donnell, 1993).

The complex data obtained from pyrolysis require analysis by multivariate statistical methods (Goodfellow & O'Donnell, 1993). However, since the development of fully automated pyrolysis mass spectrometers with improved software for data analysis PyMS has become an alternative to standard molecular procedures such as 16S rDNA or ITS sequence comparison or RAPDs and RFLP. The methodology of PyMS involves in essence (Magee, 1993): i) spreading a sample of an isolate onto a metal foil; ii) pyrolysis, which results in a mixture of low molecular weight, volatile organic compounds (the pyrolysate); iii) mass spectrometry, where the pyrolysate is ionized and the resulting ions separated by a mass spectrometer on the basis of their mass-to-charge ratio; iv) data analysis of the pyrolysis mass spectra of all samples which can then be compared statistically for relatedness.

## 1.8 Numerical taxonomy

Numerical taxonomy is the grouping of taxonomic units into taxa based on shared characters (Goodfellow & O'Donnell, 1993).

### 1.8.1 Characters

Phenotypic characters used for taxonomic purposes should not be included more than once in a numerical analysis (O'Brien & Colwell, 1987). In general, it is thought that the number of phenotypic characters used for numerical analysis should be greater than sixty (Trüper & Schleifer, 1992). This number of tests has, however, only been achieved for strains from more than one genus of prokaryotes, other than cyanobacteria (eg actinomyces: Williams et al., 1983). In the case of cyanobacteria, however, such a high number of tests has never been carried out (eg Kirkby & Whitton, 1976 and McGuire, 1984 tested 26 and 30 characters, respectively, for strains from two genera). Up to now, characters included in a numerical analysis of cyanobacteria were always based on morphological criteria (Kirkby & Whitton, 1976; McGuire, 1984). In contrast, the majority of tests for actinomyces is based on tests which are not applicable for cyanobacteria (eg degradation of substrates, resistance to antibiotics: eg Williams et al., 1983), or provide only a few characters for cyanobacteria (eg growth on sole carbon sources; Section 1.5).

The numerical analysis of the data set of phenotypic characters can be performed using either a hierarchical or non-hierarchical method.

### 1.8.2 Hierarchical methods

A hierarchical method for the analysis of taxonomic relationship leads to the representation in form of a dendrogram or phenogram. The structure of the hierarchy in a dendrogram or phenogram can be calculated using a variety of methods.

Due to the limited availability of algorithms able to deal with multivariate characters, the data set is usually transferred into a binary data set, where "1" states the presence, and "0" the absence of a character. Using a binary code, it is, however, often difficult to judge which state resembles the presence or absence of a character (Sneath & Sokal, 1973; Sackin & Jones, 1993). This has to be considered when a algorithm is to be selected as being the most

appropriate for the data set. The most commonly used algorithms for phenotypic data sets are the simple matching coefficient ($S_{SM}$) and the Jaccard coefficient ($S_J$) (A. Ward, pers. comm.). While the $S_{SM}$ resembles the proportion of characters with the same state, both positive or negative, of the two OTU's (operational taxonomic unit, eg a strain or species) that are being compared, $S_J$ represents only the proportion of shared positive character states (ie stated as "1"), and negative states are neglected (Sackin & Jones, 1993).

The matrices obtained from the $S_{SM}$ and $S_J$ are subsequently used to compute the relationship of OTU's by cluster analysis. There is a great variety of clustering methods, which are mainly a result of the definition of similarity between OTUs or groups (Sackin & Jones, 1993). While single-linkage defines the similarity between two groups as the similarity between the two most similar OTUs, one in each group, average linkage takes the average of all similarities across the groups (Sackin & Sokal, 1993). In contrast to the average linkage methods, single-linkage is highly influenced by OTUs or groups of OTUs that are intermediate between groups. These intermediates can cause neighbouring groups to join very closely, thus masking their actual integrity (Sackin & Sokal, 1993). The two average linkage methods available (weighted and unweighted average linkage or, also called, weighted and unweighted pair group method with arithmetic averages (WPGMA or UPGMA, respectively)), have been shown to give very similar results (Sackin & Sokal, 1993). UPGMA, however, has been shown to yield the highest cophenetic correlation coefficient ($r_{cs}$; see below), and is, therefore, most commonly used in numerical taxonomy (Sackin & Jones, 1993). A further advantage of UPGMA is, that it does not require any weighing of the characters, which is, in practice, generally difficult to assess.

A general scenario of the computation involves finding the pair of closest OTUs, thus resulting in the formation of a group or cluster (Sackin & Jones, 1993). Subsequently, the similarity of this group and the remaining OTUs are computed. This process is repeated with the set of OTUs, in which the newly formed group is regarded as a single OTU. After t-1 repeats (where t = number of OTUs) all OTUs have been analyzed for their position in the clusters.

To answer the question as to how well the phenogram produced represents the actual similarity (or distance) matrix calculated, the cophenetic correlations have been developed by Sokal and Rohlf (1962). The cophenetic value between any OTUs is the maximal similarity between the same OTUs implied by the phenogram. The $r_{cs}$ is the 'product-moment' correlation coefficient between the values of the similarity matrix and the cophenetic values

(Sneath & Sokal, 1973). In practise, it is a measure on how well the phenogram resembles the actual relatedness of the OTUs, which is expressed in the similarity matrix (or the distance matrix). Generally, the $r_{cs}$ has been found to vary between 0.6 and 0.95 (Sneath & Sokal, 1973). A $r_{cs}$ above about 0.8 is thought to be reasonably good; but below 0.7 "limited credence" should be given to the hierarchical structure, "though there may yet be some structure showing through the 'noise', including structure that the investigator may have anticipated" (Sackin & Jones, 1993).

### 1.8.3 Non-hierarchical methods

Although short comings of a hierarchical method can be tested by, for example, the use of different methods for clustering, non-hierarchical methods do not encounter these problems as they do not force group structure on the data, but represent the relationship in form of a multidimensional plot. The most commonly used methods in bacteriology are multivariate analyses, which analyse the contribution of each single character to the classification of the OTUs. While principal component analysis (PCA) is based on the analysis of a (rectangular) data matrix, ie characters versus OTUs, principal coordinates analysis analyzes a (triangular) distance matrix (Sackin & Jones, 1993). Although there are several possibilities in the representation of the results from multivariate analyses (eg two- or three-dimensional plots), taxonomic hierarchies are more easily interpretable in phenograms than in plots.

## 1.9 The research project

### 1.9.1 Background

The increasing use of Spirulina as a food supplement and other purposes has led to a large number of strains being held in different culture collections, though often under conditions likely to differ considerably from those encountered in nature. The isolated strains are not only often morphologically highly variable, but in a number of cases marked morphological changes appear to have taken place, especially the partial or almost complete loss of the trichome helix.

In spite of earlier reports, classification of *Arthrospira* strains is still often based alone on trichome morphology. Considering the high degree of morphological similarities among

*Arthrospira* strains and their morphological changes only a comprehensive characterization including phenotypic and genotypic characters will allow the classification of strains.

### 1.9.2 Aims

In order to clarify the taxonomy of *Arthrospira* spp., Earthrise Farms (California), one of the biggest producers of Spirulina, has initiated a collaborative project between the Universities of Durham and Liège (Dr A. Wilmotte), with the former establishing a database for phenotypic characters of *Arthrospira* strains and the latter conducting a molecular characterization of the same set of strains (Scheldeman et al., 1999; Appendix C).

For this purpose, a culture collection of a large set of *Arthrospira* strains of different geographic origin should be assembled (Chapter 2) and screened for phenotypic characters (Chapters 5-9). In particular, morphological (Chapter 5), ultrastructural (Chapter 6), physiological (Chapter 7) and biochemical (Chapter 8) characters as well as the whole-cell composition (Chapter 9) of *Arthrospira* strains should be investigated. More detailed studies should be carried out, where necessary, to assess whether a character can be used for taxonomic purposes. The data from the screening program should finally be analyzed using numerical methods (Chapter 10) and compared with the molecular data of Scheldeman et al. (1999) (Chapter 11).

As the analysis of physiological and biochemical characters require axenic cultures, all growing in the same medium, the set of strains was planned to be purified from contaminants, and a medium, which supports growth of all strains, was to be identified (Chapter 4). To avoid genetic drift leading to different geno- and phenotypes, it was also aimed to develop a method for long-term preservation of the strain (Chapter 4).

# CHAPTER 2 CULTURE COLLECTION OF *ARTHROSPIRA* AND *SPIRULINA* STRAINS

## 2.1 *Arthrospira* strains

Subcultures of *Arthrospira* strains were obtained from culture collections, other academic institutions and private sources. In total 40 *Arthrospira* strains were received, of which five are duplicate strains (clones of apparently the same strain obtained from different sources). A Durham Culture Collection number was given to each strain received, whether or not there is an apparent duplication. The numbers, origins, sources and other information on their history are summarized in Table 2.1. The cultures represent four different botanical species (*Arthrospira platensis, A. maxima, A. fusiformis, A. indica*) and originate from four continents (Africa, America, Asia, Europe).

At the point of receiving subcultures of strains, the cultures of three strains (D0872, D0885, D0910) proved to contain two different morphological types of trichomes. While strains D0872 and D0910 contained helical and straight trichomes (Fig. 2.1A), strain D0885 contained trichomes with differences in cell width (Fig. 2.1B; Section 5.2). During maintenance of the strains cultures of further two strains (D0914, D0918) as well as a culture of strain D0885/H2 (morphotype of strain D0885 with thinner trichome) and strain D0906, presumably a duplicate strain of D0880, were found to contain straight trichomes. Clonal, axenic cultures of all different morphotypes were produced (Section 4.5.3). A letter was added to their Durham strain number to indicate their morphology (/H = helical, /S = straight).

During the period of the project all cultures of helical and straight morphotype of the same clone remained stable, ie no further occurrence of straight trichomes within cultures containing helical trichomes only or vice versa. Strain D0885/S, however, showed in some of its subcultures trichomes which were in some cases helical or part straight and part helical (Section 5.2).

In total, the Durham culture collection contains in addition to two strains (D0881, D0882), which were obtained as only straight trichomes, a further five different strains with straight morphotypes, as well as straight morphotypes of further two strains (D0887, D0906/S), which are duplicates of strains included in the set of 35 *Arthrospira* strains. The culture history of helical and straight morphotypes is summarized in Fig. 2.1.

Table 2.1 Strains, source, origin and culture history of the *Arthrospira/Spirulina* collection at Durham University

Table 2.1A List of 35 *Arthrospira* strains held in the Durham Culture Collection.

| Durham number | Source | Strain | Name | Origin | Clones in other culture collections |
|---|---|---|---|---|---|
| D0867 | CCAP | 1406/2 | *Arthrospira maxima* | Lake Naivasha; Kenya | |
| D0872/H [1)] | CCAP | 1475/8 | *Arthrospira fusiformis* | Lake Chitu, Ethiopia | |
| D0872/S [1)] | CCAP | 1475/8 | *Arthrospira fusiformis* | Lake Chitu, Ethiopia | |
| D0873 | CCAP | 1475/9 | *Arthrospira maxima* | Lake Chad, Chad | ATCC 53871; M 132/1; SAG 84.79; UTEX 2342; DIC-Sp-6; D0879 |
| D0880 | SAG | 85.79 | *Spirulina platensis* | natron lake; Chad | M 132/2b; NIVA CYA 120; UTEX 2340; DIC-Sp-4; D0887; D0906 |
| D0881 | SAG | 257.80 | *Spirulina platensis* | Laguna Huaca-china; Ica, Peru | |
| D0882 | SAG | 86.79 | *Spirulina platensis* | Lake Chad; Chad | Trebon Compère; 86.79; D0905 |
| D0884 | unknown | Sp-1 | *Spirulina platensis* | Lake Texcoco; Mexico | NIES-46; IAM M-185 |
| D0885/H1[2)] | unknown | Sp-2 | *Spirulina platensis* | Lake Chad; Chad | NIES-39; IAM M-135 |
| D0885/H2[2)] | unknown | Sp-2 | *Spirulina platensis* | Lake Chad; Chad | NIES-39; IAM M-135 |
| D0885/S [3)] | unknown | Sp-2 | *Spirulina platensis* | Lake Chad; Chad | NIES-39; IAM M-135 |
| D0890 | unknown | Sp-7 | *Spirulina/Arthrospira* sp. | Lake Texcoco; Mexico | |
| D0891 | unknown | Sp-8 | *Spirulina/Arthrospira* sp. | Lake Simbi; Kenya | |
| D0895 | unknown | Sp-12 | *Spirulina/ Arthrospira* sp. | | |
| D0896 | unknown | Sp-13 | *Spirulina/Arthrospira* sp. | | |
| D0897 | unknown | Sp-14 | *Spirulina/Arthrospira* sp. | | |
| D0899 | unknown | Sp-16 | *Spirulina/Arthrospira* | | |
| D0900 | unknown | Sp-17 | *Spirulina/Arthrospira* sp. | | |
| D0904 | Trebon | Compère, 1968; 3786 | *Arthrospira* sp. *('platensis')* | Lake Bodon; Chad | |
| D0905 | Trebon | Compère; 86.79 | *Arthrospira* sp. *('platensis')* | Lake Chad; Chad | SAG 86.79; D0882 |

| | | | | | |
|---|---|---|---|---|---|
| D0907 | Trebon | Leonard and Wouters, 1968 | *Arthrospira* sp. *('platensis')* | Kanem, Lake Bodon; Chad | |
| D0909 | Trebon | Hindák, 1985; 1 | *Arthrospira fusiformis* | Ethiopia | |
| D0910/H [1)] | Trebon | Hegewald 1976; 83 | *Arthrospira fusiformis* | Lake Naguru; Kenya | |
| D0910/S1 [1)] | Trebon | Hegewald 1976; 83 | *Arthrospira fusiformis* | Lake Naguru; Kenya | |
| D0910/S2 [1)] | Trebon | Hegewald 1976; 83 | *Arthrospira fusiformis* | Lake Naguru; Kenya | |
| D0911 | PCC | 7345 | *Arthrospira platensis* | Del Mar Slough, San Diego Co., CA-USA | ATCC 29408; UTEX 1926; UTEX 1928; DIC-3; DIC-Sp-5; D0875; D0876 |
| D0913 | PCC | 7940 | *Arthrospira* sp. | India, Kenya, Mexico or Peru | records lost at PCC |
| D0914/H | PCC | 8005 | *Arthrospira* sp. | India, Kenya, Mexico or Peru | records lost at PCC |
| D0914/S | PCC | 8005 | *Arthrospira* sp. | India, Kenya, Mexico or Peru | records lost at PCC |
| D0915 | PCC | 8006 | *Arthrospira* sp. | India, Kenya, Mexico or Peru | records lost at PCC |
| D0916 | PCC | 9108 | *Arthrospira* sp. | Commercial culture facility, Cheng-Hai, Yunnan; China | records lost at PCC |
| D0918/H | A.M. Sanangelantoni | | *Spirulina* sp. ( C1) | Lake Chad; Chad | |
| D0918/S | A.M. Sanangelantoni | | *Spirulina* sp. ( C1) | Lake Chad; Chad | |
| D0919 | R. Fox | | *Arthrospira* var. *'crater'* | Lake in volcano crater; Mexico | |
| D0920 | R. Fox | | *Arthrospira* var. *'lanar'* | Lake Lonar; Maharashtra, India | |
| D0921 | R. Fox | | *Arthrospira* var. *'orovilca* | Lake Orovilca; Ica, Peru | |
| D0922 | R. Fox | | *Arthrospira* var. *'titicaca'* | Lake Titicaca; Peru | |
| D0923 [4)] | Earthrise Farms | | *Arthrospira* sp. | | |
| D0925 [4)] | Earthrise Farms | | *Arthrospira* sp. | | |
| D0929 | Jeeji Bai | | *Arthrospira indica* | | |
| D0930 | Jeeji Bai | | *Arthrospira platensis* | Berhampur | |
| D0933 | PCC | 9223 | *Arthrospira* sp. | Spain | |

1) two types of filaments (straight and helical) were observed and clonal, axenic cultures were obtained of each; both trichome types count together as one strain but will be investigated separately (/H = helical, /S = straight)
2) a thicker and thinner type of filament occurred within the culture; both types were isolated and clonal, axenic culture were produced

3) straight filament occurring in culture of strain D0885/H2 was isolated and clonal, axenic culture was produced
4) sample from ponds from Earthrise Farms, Calipatria; clonal, axenic culture was produced from single filaments
ATCC = American Type Culture Collection, Rockville, Maryland, USA
CCAP = Culture Collection of Algae and Protozoa, Ambleside, Cumbria, England, UK
CCALA = Culture Collection of Algal Laboratory, Institute of Botany, Trebon, Czech Republic
CCMEE = Culture Collection of Microorganisms from Extreme Environments, Florida State University/Florida A&M University, Tallahassee, Florida, USA
IAM = Institute of Applied Microbiology, University of Tokyo, Japan
NIES = National Institute for Environmental Studies Collection, Tsukuba, Ibaraki, Japan
NIVA = Norwegian Institute for Water Research, Oslo, Norway
PCC = Pasteur Culture Collection of Cyanobacterial Strains, Paris, France
SAG = Sammlung von Algenkulturen der Universität Göttingen, Germany
UTEX = Culture Collection of Algae at the University of Texas at Austin, Austin, Texas, USA

Table 2.1B List of duplicates of *Arthrospira* strains held at the Durham Culture Collection. Subclones of the same strain included in this project are indicated in bold letters and those subclones, which were included in the set of 35 strains, are indicated in italics. (For guide to culture collection acronyms, see Table 2.1A.)

| Durham number | Source | Strain | Name | Origin | Clones in other culture collections |
|---|---|---|---|---|---|
| D0875 | UTEX | 1926 | *Spirulina platensis* | Del Mar Slough, San Diego Co., USA | PCC 7345; UTEX 1928; ATCC 29408; DIC-Sp-3; DIC-Sp-5; **D0876*; D0911*** |
| D0876 | UTEX | 1928 | *Spirulina platensis* | | PCC 7345; UTEX 1926; ATCC 29408; DIC-Sp-3; DIC-Sp-5; **D0875*; D0911*** |
| D0879 | SAG | 84.79 | *Spirulina maxima* | Lake Chad; Chad | ATCC 53871; CCAP 1475/9; M 132/1; DIC-Sp-6; UTEX 2342; ***D0873*** |
| D0887 | unknown | Sp-4 | *Spirulina platensis* | natron lake; Chad | M 132/2b; NIVA CYA 120; SAG 85.79; UTEX 2340; ***D0880;*** **D0906** |
| D0906/H 1) | Trebon | Laporte, 1963; M-132/2b | *Arthrospira* sp. *('platensis')* | Lake Chad; Chad | DIC-4; NIVA CYA 120; SAG 85.79; UTEX 2340; ***D0880;*** **D0887** |
| D0906/S 1) | Trebon | Laporte, 1963; M-132/2b | *Arthrospira* sp. *('platensis')* | Lake Chad; Chad | DIC-4; NIVA CYA 120; SAG 85.79; UTEX 2340; ***D0880;*** **D0887** |

1) two types of filaments were observed and clonal cultures were obtained of either; both trichome types count together as one strain, but are investigated separately

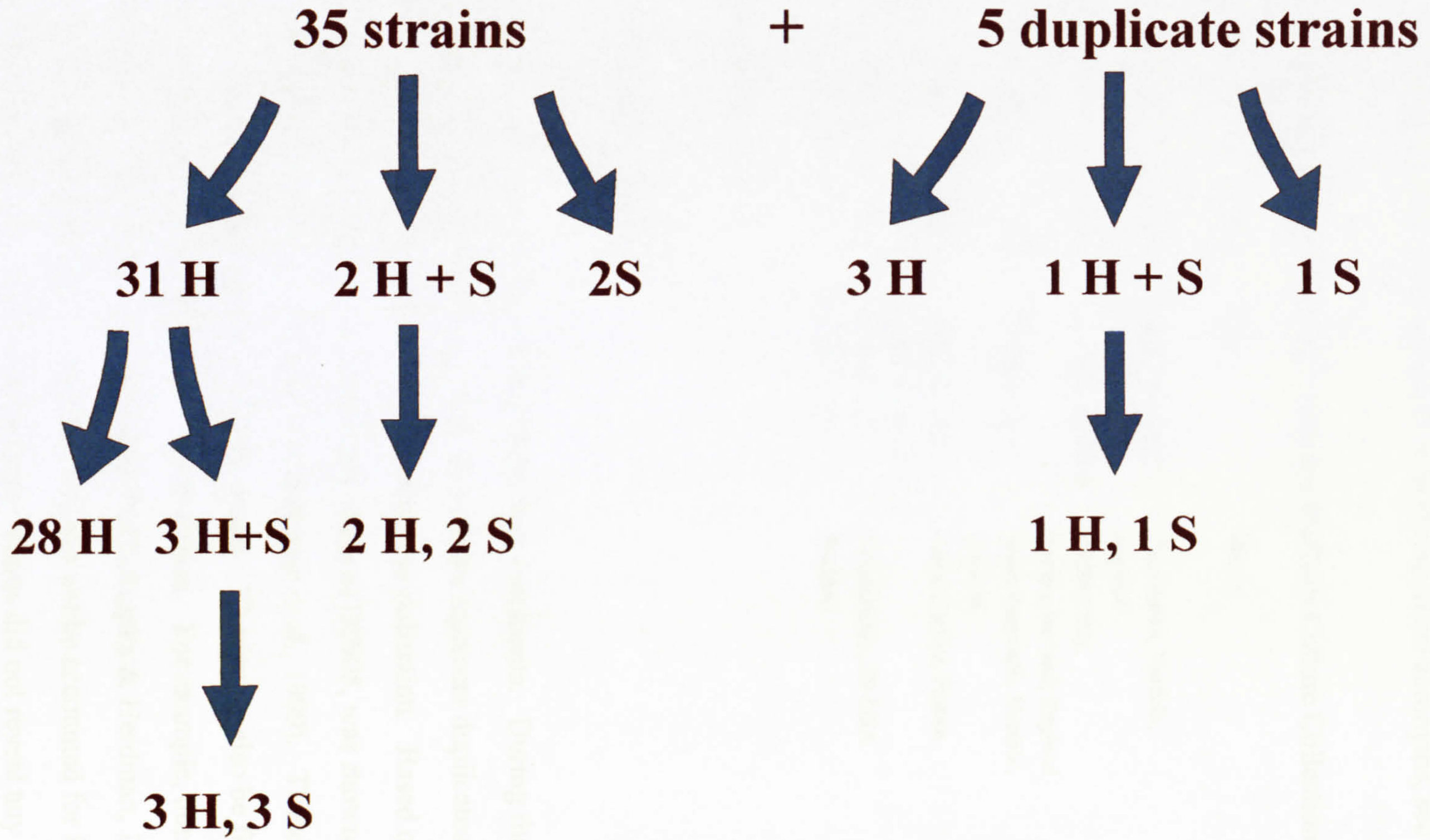



Fig. 2.1 Culture history of helical and straight morphotypes hold at Durham culture collection. For details see text.

## 2.2 *Spirulina* strains

The collection of *Spirulina* strains resulted in seven strains from three continents resembling at least two different botanical species (*Spirulina subsalsa, S. major*). Their origin and strain history are summarized in Table 2.2. All but one strain (D0917) represent marine isolates. (For guide to culture collection acronyms, see Table 2.1A.)

Table 2.2 List of *Spirulina* strains held in the Durham Culture Collection.

| Durham Number | Source | Strain | Name | Origin | Clones in other culture collections |
|---|---|---|---|---|---|
| D0868 | CCAP | 1475/1 | *Spirulina subsalsa* | Hunstanton, Norfolk, England | |
| D0869 | CCAP | 1475/2 | *Spirulina subsalsa* | Whitley Bay, Northumberland, England | UTEX 1318, SAG 1475/2 |
| D0870 | CCAP | 1475/3 | *Spirulina major* | Great Yarmouth, Norfolk, England | UTEX 552 |
| D0871 | CCAP | 1475/7 | *Spirulina subsalsa* | Calvi, Corsica, France | |
| D0877 | UTEX | 1954 | *Spirulina subsalsa* | | |
| D0878 | UTEX | 770 | *Spirulina* sp. | Bloomington, IN-USA | |
| D0917 | D.G. Adams | | *Spirulina* sp. | Pakistan | |

## 2.3 Discussion

### 2.3.1 *Arthrospira* strains

40 *Arthrospira* strains were collected from four continents. During the course of the project, however, it became apparent that five strains represent duplicates of others, ie subcultures of the same clone hold at another culture collection. Based on ARDRA of the ITS region, D0882, thought to be a duplicate strain of D0905, was shown to be likely the result of a wrong labelling of the strain (Scheldeman et al., 1999). These two strains are, therefore, regarded in this work as different clones. There may also be further "hidden" but not obvious duplicates within the set of 35 strains. For example, due to the loss of the records for most of the strains obtained from PCC (Rippka & Herdman, 1992) there is a potential of further duplicates. This, however, can not be accounted for in this work and studies on the molecular taxonomy of the same strains did not reveal any further obvious duplicate strains (Scheldeman et al., 1999).

Although five strains were duplicates of other strains the culture collection of 35 different (as judged by their history), clonal and axenic strains represents the largest collection of *Arthrospira* strains reported so far, exceeding those of all major culture or research group collections.

Maintenance of cultures, and selection of strains for particular studies, is complicated by the fact that a number of morphological shifts have appeared during subculture. Especially the production of new clonal isolates leads, presumably, each time to a potential loss of genetic information. The helical morphotype is in each case chosen for inclusion in the 35 'standard' strains, so the standard set consists of 33 helical and 2 straight forms. For D0885, morphotype /H1 is used as the 'standard'.

### 2.3.2 *Spirulina* strains

All but one (D0917) of the *Spirulina* strains were received from culture collections where they had been identified as "true" *Spirulina* strains by main stream taxonomists based on their morphology. Strain D0917, a freshwater isolate from Pakistan, was also determined as *Spirulina* strain based on its morphology (D.G. Adams, pers. comm.).

## 2.4 Summary

i) A culture collection of 35 *Arthrospira* and five duplicate strains of the former was established. The collection holds also morphologically different subclones of six of the strains.

ii) Additionally, seven *Spirulina* strains were assembled.

# CHAPTER 3 MATERIALS AND METHODS

## 3.1 Materials

Most of the chemicals and solvents used in this research project were supplied by BDH Chemicals Ltd, Poole, Dorset, UK, and SIGMA Chemical Co., Poole, Dorset, UK, and were of the purest grade available.

Other reagents, kits and consumable suppliers are as listed below:

Agar (bacteriological): Unipath Ltd, Basingstoke, England, U.K

Cryovials: Nalgene, Rochester, NY, USA

Chloramphenicol: SIGMA, Poole, UK

3-(3,4-dichlorphenyl)-1,1-dimethylurea (DCMU): SIGMA, Poole, UK

Filter paper: Whatman Ltd, Maidstone, UK

GC63 and GC65 methylester standard mix: Nu-Chek Prep Inc,

Glutaraldehyde: Agar Scientific, Stansted, UK

HCl (3M) in MeOH ($H_2O$-free): SIGMA, Poole, UK

Heptadecaneoic acid methylester: SIGMA, Poole, UK

Lectins from *Glycine max, Lens culinaris, Helix pomposa, Triticum vulgare, Tetragonopulus*: SIGMA, Poole, UK

Methylester standards GC63: SIGMA, Poole, UK

Non-absorbent cotton wool: BDH, Poole, UK

Paraformaldehyde: Agar Scientific, Stansted, UK

Ruthenium red: BDH, Poole, UK

Silver enhancement Kit: Amersham International plc., Little Chalfont, England, UK

50 and 75 Square mesh copper grids: Agar Scientific, Stansted, UK

## 3.2 Sterilization of media and consumables

To avoid cross-contamination of strains or (bacterial) contamination of axenic cultures, all media and materials used for handling cultures were autoclaved at 15 lb $in^{-2}$ for 20 minutes,

unless heat sensitive (eg sugars and other organic compounds), in which case they were filter sterilised by passing through a 0.2 μm nitrocellulose membrane filter.

## 3.3 Culture

### 3.3.1 Media and strain maintenance

*Arthrospira*

*Medium*

All *Arthrospira* strains were maintained in a modified Zarrouk's medium (1966). The modifications of the medium were replacement of the heavy metal composition of Zarrouk's medium (Zarrouk, 1966) with the one included in BG11 (Waterbury & Stanier, 1981) plus the addition of Ni (0.049 g $L^{-1}$ $NiSO_4 \cdot 7H_2O$).

The modified Zarrouk's medium consisted (per L) of: $NaHCO_3$ (18.0 g), $K_2HPO_4$ (0.5 g), $NaN0_3$ (2.5 g), $K_2SO_4$ (1.0 g), NaCl (1.0 g), $MgSO_4.7H_2O$ (0.2 g), $CaCl_2.6H_2O$ (0.06 g), $FeSO_4.7H_20$ (0.01 g), EDTA (disodium salt) (0.08 g), trace element solution* (1 mL).

*Trace element solution (per L) $H_3BO_3$ (2.86 g), $MnCl_2$ (1.8 g), $ZnSO_4.7H_2O$ (0.22 g), $(NH_4)_6Mo_7O_{24}$ (0.018 g), $CuSO_4.5H_2O$ (0.08 g).

Care was taken that the pH of the medium after autoclaving was 9.5. To achieve this, the pH of the medium was adjusted to 8.5 which resulted in a pH of 9.5 after autoclaving and "recovery" of the medium.

In the following this medium is referred to as Zarrouk's medium, unless further modifications were made.

*Maintenance*

For maintenance of cultures all strains were subcultured into fresh medium. Throughout the project two sets of stock cultures were maintained at different locations within the department (research laboratory, 30 °C room) in case of loss during emergency. Stock cultures (10 - 15 mL) in 50-mL boiling tubes stored at 30 °C under permanent irradiance of 5-10 μmol photon $m^{-2}$ $s^{-1}$ were subcultured each 50 days, while the stock cultures in 100-mL flasks kept in the laboratory at room temperature (approx. 17-25 °C) and under a natural light

dark cycle (10-30 µmol photon $m^{-2}$ $s^{-1}$) were subcultured each 90 days or occasionally sooner, depending on the state of the cultures.

*Modifications to Zarrouk's medium to lower N and P*

Cultures of five *Arthrospira* strains (D0867, D0872/H, D0873, D0885/H1, D0891; no replicates) were washed with Zarrouk's medium minus P and N and subcultured in Zarrouk's medium, but containing much lower concentrations of P (1 mg $L^{-1}$ $PO_4$-P) and N (8 mg $L^{-1}$ $N0_3$-N). The cultures were incubated at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$.

A second approach involved a stepwise decrease of P and N. Cultures of the five strains were washed with Zarrouk's medium minus P and N and subcultured in Zarrouk's medium containing P and N in the concentration of 24 mg $L^{-1}$ ($PO_4$-P) and 192 mg $L^{-1}$ ($NO_3$-N). The subcultures were incubated at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$ for 20 days. Subsequently, an aliquot of each of the subcultures of the strains was used to inoculate Zarrouk's medium containing P and N in the concentration of 12 mg $L^{-1}$ ($PO_4$-P) and 96 mg $L^{-1}$ ($N0_3$-N) followed by incubation at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$ for 20 days. Aliquots of these subcultures were then used to inoculate Zarrouk's medium containing P and N at 6 mg $L^{-1}$ ($PO_4$-P) and 48 mg $L^{-1}$ ($N0_3$-N), followed by incubation as above. After 10 days, aliquots of these subcultures were used to inoculate Zarrouk's medium containing P and N at 1 mg $L^{-1}$ ($PO_4$-P) and 8 mg $L^{-1}$ ($N0_3$-N) followed by incubation as above.

*Spirulina*

*Medium*

*Spirulina* strains were originally cultured in ASW:BG medium recommended by CCAP (CCAP, 1998). This medium is made up in two parts, which are autoclaved separately and mixed aseptically (1:1), when cool. Part 1 consists (per L) of: tricine (0.5 g), $NaNO_3$ (0.113 g), $Na_2HPO_4$ (0.0045 g), $K_2HPO_4$ (0.0038 g), soil extract (25 mL) in filtered sea water. The pH of part 1 of ASW:BG medium was adjusted to pH 7.7.

Soil extract was prepared from sieved wood soil, thus free of invertebrates and roots. The soil was air dried, followed by autoclaving in twice its volume of distilled water for 2 h at 121 °C and 15 lb $in^{-2}$. After cooling the soil extract was filtered through filter paper (Whatman) before used for preparation of part 1 of ASW:BG medium.

Part 2 consists (per L) of: $NaNO_3$ (1.5 g), $K_2HPO_4$ (0.04 g), $MgSO_2.7H_2O$ (0.075 g), $Ca_2CO_3$ (0.02 g), $CaCl_2.6H_2O$ (0.06 g), citric acid (0.006 g), ferric ammonium citrate (0.006 g), EDTA (disodium salt) (0.001 g), trace element solution (same as for *Arthrospira* strains) (1 mL). The pH was adjusted to 7.4.

*Maintenance*

Cultures of *Spirulina* strains were maintained within the research laboratory at room temperature (approx. 17-25 °C) and under a natural light dark cycle (10 to 30 µmol photon $m^{-2}$ $s^{-1}$). Subculturing into fresh medium was carried out each 90 days or occasionally sooner, depending on the state of culture.

### 3.3.2 Production of clonal, axenic cultures

*Growth and test media*

Zarrouk's medium was used for the isolation procedure and the growth of cultures from single filaments.

Five heterotrophic growth media were employed in solidified form as test plates for the purity of the cultures.

Table 3.1 Composition of enriched media used for testing growth of contaminants.

| Medium | Nutrient broth | Agar | Glucose | Tryptone | Yeast | Bacto-peptone | Water | Zarrouk's medium |
|---|---|---|---|---|---|---|---|---|
| EZ | 10 g | 10 g | 10 g | | 5 g | | | 1 L |
| NB | 25 g | 10 g | | | | | 1 L | |
| PG | | 10 g | 1 g | | | 1 g | 1 L | |
| SST | | 10 g | 10 g | 10 g | | | 1 L | |
| Y | | 10 g | | | 5 g | | 1 L | |

The pH of both Zarrouk's medium and enriched Zarrouk's medium (EZ) was adjusted to pH 9.5 using 5M NaOH. For solid media double-strength agar and double-strength nutrient solution were prepared, sterilized separately and then combined aseptically. EZ was sterilized by autoclaving for 30 min at 10 lb $in^{-2}$, as autoclaving at higher pressures led to caramelization of, presumably, glucose.

*Isolation and purification of single filaments*

All microscopic manipulations were carried out aseptically in a 'Microflow Pathfinder' laminar flow cabinet using a binocular microscope (surface sterilized with 70% ethanol) within the laminar flow cabinet.

A culture in the phase of fast growth was mixed to suspend the filaments. A 100-µl aliquot was taken using a 200-µL Gilson pipette, set at 100 µL volume, and sterile tip. The aliquot was placed as a droplet onto the centre of a petri dish containing EZ. Using a fresh pipette tip, 100 µL Zarrouk's medium was taken. One drop was then removed from the pipette by depressing the plunger. With the plunger still depressed, a single filament was picked from the original droplet in the centre of the agar plate by releasing the plunger. The complete content of the pipette was then deposited as a droplet onto an area around the edge of the same agar plate. This process constitutes one transfer, effectively washing the filament. Using a fresh pipette tip, another 100 µL Zarrouk's medium was taken, one drop removed and the single filament selected was again picked and deposited as an individual droplet at the edge of the plate as before. This process was repeated 20-25 times in a cyclic fashion around the surface of the agar plate. After the final transfer on agar, the now washed single filament was picked up, again using the Gilson pipette, and transferred to a boiling tube containing 15 mL of Zarrouk's medium. The innoculated medium in the boiling tube was then incubated at 30 °C and 10-15 µmol photon $m^{-2}$ $s^{-1}$ until visible growth could be observed. The agar plates used as surface to isolate the single filaments were incubated at 30 °C in the dark for 14 days. After the isolation procedure was established, the increasing purity of the isolated filament was not monitored any longer and sterile plastic petri dishes were used instead of enriched Zarrouk's medium in form of agar plates.

*Tests for axenity*

Besides observation of bacterial growth on the agar plate used for the isolation process (see above), the purity of the cultures grown from the single filaments was also confirmed by the use of the five bacterial test media (EZ, NB, SST, PG, Y; Section 3.3.2). 40-µL aliquots of cultures were mounted on the agar plates followed by incubation at 30 °C in the dark for 14 days. A further test involved light-microscopic examination of DAPI-stained aliquots. Cultures were judged axenic if no contamination was observed by either of the tests.

### 3.3.3 Long-term preservation of cultures

#### 3.3.3.1 Cryopreservation

Care was taken when samples were handled during preparation for or handling after long-term storage. To avoid cellular damage centrifugation was avoided and the biomass was concentrated by filtration where necessary throughout all experiments.

*Use of cryoprotectants*

Cryopreservation in liquid nitrogen was tested using DMSO, glycerol or methanol as cryopreservants. Table 3.2 summarizes the concentrations used for the different cryoprotectants.

Table 3.2 Cryoprotectants and concentrations used for cryopreservation of *Arthrospira* and *Spirulina* strains.

| | Concentration of cryoprotectant | | | | | |
|---|---|---|---|---|---|---|
| Cryoprotectant | 5% | 7.5% | 10% | 15% | 30% | 50% |
| DMSO | + | + | + | + | | |
| Glycerol | | | | | + | + |
| Methanol | + | + | + | + | | |

Standard cryovials (1.8-mL polypropylene tube with screw top to avoid leakage of liquid nitrogen into the cryovial; Nalgene) were used, filled with 1.5 mL of a dense culture in early stationary phase of growth. The tubes were placed directly into a cryogenic storage container filled with liquid nitrogen. Regeneration of a culture from the cryopreserved samples was performed by thawing the liquid culture at room temperature, washing the cyanobacterial sample free of cryoprotectant and inoculating either 30 mL Zarrouk's medium in 100-mL flasks or 15 mL Zarrouk's medium in 50-mL boiling tubes. Subsequently, the cultures were incubated at 20 °C and 5 μmol photon $m^{-2}$ $s^{-1}$ for 10 d followed by incubation at 30 °C and 10 μmol photon $m^{-2}$ $s^{-1}$.

*Slow-freezing procedure*

Attempts were made to establish the improved method for cryopreservation recommended by UTEX (Brand, 1996). The cryovial containing the cyanobacterial

sample and the cryoprotectant was pre-chilled at -20 for 1 h, followed by incubation at -70 °C for 2 h prior to storage in liquid nitrogen. Recovery of the culture was performed by rapid thawing of the samples. Using a Gilson pipette one mL of the medium was taken off carefully without taken filaments and replaced with fresh growth medium. The sample in the cryovial was incubated at 30 °C in the dark for 1-2 days to allow recovery. The lid of the cryovial was kept slightly loosened to allow gas exchange. Subsequently, the samples were placed into growth medium and incubated as described above.

### 3.3.3.2 Storage at low temperature

Liquid medium

*Storage at -20 °C in 50 % glycerol*

10 *Arthrospira* strains (D0867, D0880, D0885/H1, D0896, D0911, D0916, D0918/H, D0920, D0923, D0925) were grown in the following media: Zarrouk's medium, Zarrouk's medium plus 20 mM glucose, Zarrouk's medium plus 75 nM DCMU, Zarrouk's medium plus 20 mM glucose plus 75 nM DCMU. Glycerol was added to the samples to a final concentration of 50% and carefully mixed. The samples were then placed in sterile cryo-vials with screw tops and incubated at 4 °C for 1 h before transfer to -20 °C in the dark.

After an incuation period of 30 days at -20 °C all samples were washed in Zarrouk's medium containing the same addition(s) but not glycerol (glucose, DCMU, glucose+DCMU). The biomass was collected and 15 mL of the same medium in 50-mL boiling tubes were inoculated with the corresponding sample. All cultures were incubated at 30 °C and 5 µmol photon $m^{-2}$ $s^{-1}$ for five days prior to transfer to 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$.

*Storage at low temperature (4 °C) in the dark*

Eight mL Zarrouk's medium in test tubes were inoculated (no replicates) with aliquots of the 35 *Arthrospira* strains and the five duplicate strains; the tubes were then incubated in the dark (cardboard box) in a cold room maintained at 4 °C. Although humidity in the 4 ° C room was not measured, it was probably close to saturation. After seven months of

storage at 4 °C the cultures were incubated at 30 °C and 5 µmol photon $m^{-2}$ $s^{-1}$ for five days prior to transfer to 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$.

*Storage at low temperature (10 °, 15 °C) under low light or in the dark*

Two sets of cultures each composed of two replicates of each of the 35 *Arthrospira* strains and the five duplicate strains were grown at 20 °C and 15 µmol photon $m^{-2}$ $s^{-1}$ for ten days in test tubes containing 10 mL of Zarrouk's medium. Subsequently, one set was stored at low temperature and low light (10 °C and 5 µmol photon $m^{-2}$ $s^{-1}$) and the other set at low temperature (15 °C) in the dark. To compensate for loss of medium in cultures at 15 °C, sterile distilled water was added after 90 days. The light intensity was kept low (approx. 20 µmol photon $m^{-2}$ $s^{-1}$), while adding the distilled water to the cultures otherwise stored in the dark. No water was added to the cultures at low temperature and low light. After six months of incubation the two sets of cultures were incubated at 30 °C and 5 µmol photon $m^{-2}$ $s^{-1}$ for five days followed by incubation at 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$.

Solidified medium

*Storage at low temperature (4-5 °C) in the dark on agar slopes*

Test tubes containing 5 mL solidified Zarrouk's medium (1 % (w/v) agar) were inoculated with the 35 *Arthrospira* strains and the five duplicate strains and incubated for seven days at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$, followed by storage at 4-5 °C and 2 - 5 µmol photon $m^{-2}$ $s^{-1}$ for twelve months. Incubation in this case was carried out in a refrigerator and most of the agar slopes dried out completely during the storage period. After 12 months of incubation five mL of Zarrouk's medium were added to those tubes which still contained agar (and some green biomass) that was not completely dried out. Incubation was carried out at 30 °C and 5 µmol photon $m^{-2}$ $s^{-1}$ for 5 days, followed by incubation at 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$.

3.3.3.3 Drying under a natural light-dark cycle

Cultures of ten strains (D0867, D0885/H1, D0890, D0891, D0895, D0896, D0904, D0905, D0910/H1, D0916) were grown on solidified Zarrouk's medium at room

temperature (approx. 17-25 °C) under a natural light-dark cycle (10-30 µmol photon $m^{-2}$ $s^{-1}$). After three and five months incubation 30 mL Zarrouk's medium in 100-mL flasks were inoculated with aliquots of the cultures on the agar plates. The cultures were incubated at 30 °C under 10-15 µmol photon $m^{-2}$ $s^{-1}$.

## 3.4 Microscopy

### 3.4.1 Light microscopy

Light microscopy was carried out either on a Nikon 6003 microscope (upright) with a Nikon AFM camera system attached to it or a Nikon Optiphot II (upright) with a Nikon FX-35 camera and a mercury lamp.

FITC-labelled lectins were observed using the Nikon Optiphot II microscope under UV-light provided by a Super High Pressure Mercury Lamp (Nikon) using an FITC-filter set (450-490 nm excitation). Gold-labelled lectins were visualised using the same microscope and UV-light in combination with an IGS-excitation filter, which produces polarised light.

For standard light microscopy photographs were generally taken using a FUJICHROME 64 Tungsten slide film. Samples observed under UV-light (eg lectin-binding) were photographed using a FUJI 400 Daylight slide film to compensate for the lower light intensity and different wavelength.

### 3.4.2 Confocal microscopy

Confocal microscopy was carried out on either a Zeiss LSM 410 Laser Scanning Confocal microscope using a 543 helium laser or on a Nikon Optiphot II light microscope with a Biorad MRC-600 Krypton/Argon laser attached to it. A laser beam of 543 nm (Zeiss LSM) or 488 nm (Biorad MRC-600) was used to excite autofluorescence of the cyanobacterial pigments. Depending on the sample, up to 40 one-µm-sections were analyzed and processed to a three-dimensional picture using the Cosmos 7.0A software package on an Elonex 486 PC or the Confocal Assistant 3.0 software on a Pentium 200MHz.

### 3.4.3 Transmission electron microscopy (TEM)

*Fixation and embedding*

Cultures were grown at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$ for ten days. Samples were prepared for TEM using standard procedures (Hawes, 1994). The samples were prefixed in 1.5% (w/v) paraformaldehyde, 2.5% (w/v) glutaraldehyde in Zarrouk's medium (*Arthrospira*) or ASW:BG (*Spirulina*) for 4 h. The samples were subsequently washed once with growth medium and once with distilled water prior to fixation (4 h) in 1% (w/v) aqueous osmium tetroxide.

To improve the preservation of the cell surface structure, the ruthenium red-lysine fixation method of Fassel *et al.* (1993) was adopted in a modified form.

The samples were pre-fixed first for 40 min in Zarrouk's medium (*Arthrospira*) or ASW:BG (*Spirulina*) containing 75 mM lysine, 0.075% (w/v) ruthenium red, 2.5% (v/v) glutaraldehyde and 1.5% (w/v) paraformaldehyde. The second pre-fixation was carried out in 0.075% (w/v) ruthenium red, 2.5% (v/v) glutaraldehyde and 1.5% (w/v) paraformaldehyde in the respective growth medium for 4 h, followed (after washing of the samples as above) by fixation (4 h) in aqueous 1% (w/v) osmium tetroxide.

Fixed samples were dehydrated in an increasing ethanol series (5, 10, 15, 20, 25, 30, 40, 50, 60, 80, 90, 100% (v/v) ethanol) with 30 min in each concentration. Incubation in absolute ethanol was repeated twice.

Embedding of the samples in resin was carried out by incubation of the dehydrated samples over night in 10% (v/v) resin in absolute ethanol. Subsequently, samples were incubated in an increasing series of ethanolic resin solution (20, 30, ... 90, 100% (v/v)). Incubation time at each of the concentrations of resin was 2 h. The last incubation step was repeated twice with fresh resin (2 h first time and over night the second time) before incubated over night at 60 °C (Spurr resin) or 35 °C (LR White resin) for polymerization.

*Sectioning of resin embedded material*

Spurr or LR White resin embedded material was sectioned using glass knives on a C. Reichert (Austria) Om U3 ultra microtome. The sections were floated onto a reservoir of water, which was created on the glass knives using water-resistant surgical tape.

Ultra thin 90 nm sections were collected onto 50 or 75 mesh copper grids from the reservoir. Prior to TEM the sections were stained for 10 min in 1% (w/v) uranyl acetate

solution (70% ethanol). After washing twice in distilled water the sections were stained in 33 mM lead citrate solution for 10 min followed by rinsing in distilled water. When LRWhite resin was used staining was carried out in a saturated uranyl acetate solution instead of 1% (w/v) uranyl acetate. Subsequently the sections were dried on filter paper (Whatman). TEM was carried out on a Philips EM 400T transmission electron microscope.

The microanalysis-system (Link Systems QX 200) attached to the transmission electron microscope was used on ultra-thin sections (collected on nickel grids) for the analysis of main components of inclusion bodies.

### 3.4.4 Scanning electron microscopy (SEM)

Biomass was harvested from cultures in the phase of fast growth. After washing with distilled water an aliquot was attached to aluminium stubs. When dried the sample was sputter-coated with gold (Edwards, UK), and examined using a JEOL JSM 848 scanning electron microscope. The images taken were transferred to a Pentium 200 MHz and processed using the Adobe Photoshop software package (Adobe).

## 3.5 Morphological investigations

### 3.5.1 Measurement of morphological characters

Morphological characters of *Arthrospira* and *Spirulina* strains were analysed by light microscopy using a calibrated scale in one of the two eyepieces. The length of an eyepiece unit was determined by the magnification: 10 µm at 100x, 2.5 µm at 400x, 1 µm at 1000x. The calibration of the light microscope was checked regularly using a calibration slide (Zeiss, Jena). The cultures used for the analysis of morphological characters were in the phase of fast growth, grown at 30 °C (± 0.5) and 25 µmol photon $m^{-2}$ $s^{-1}$ for generally four to seven days or until they had reached the phase of fast growth.

### 3.5.2 Influence of environmental factors on helical morphology

*Growth temperature*

Assays to test the influence of growth temperature on the trichome morphology were done with 30 mL cultures (Zarrouk's medium) in 100-mL flasks. Three replicates were used for each of the ten strains tested (D0885/H1, D0891, D0895, D0913, D0918/H, D0920, D0921, D0922, D0923, D0925) and sampling occasion. The inoculum was taken from cultures grown at 30 (± 0.5) °C and 50 µmol $m^{-2}$ $s^{-1}$, unless specified otherwise. Irradiance was provided by cool-white fluorescent tubes. The influence of temperature on helix orientation was assessed using a temperature gradient plate covering the range 10 to 45 °C, with approximately 2.5 (± 0.5) °C between neighbouring flasks, thus providing test temperatures for each strain. Irradiance at the surface of the flasks on the plate was 50 (± 3) µmol $m^{-2}$ $s^{-1}$. The cultures were shaken manually briefly once a day. After 14 days of incubation the cultures were analyzed by light microscopy for changes in helix orientation.

The influence of elevated temperature over a shorter period (short-term high-temperature pulse) was also tested on strains D0918/H, D0923 and D0925. This was done at 32 (± 0.5) °C and 50 µmol photon $m^{-2}$ $s^{-1}$, and in the case of D0925 additionally at 34° (± 0.5) C and 50 µmol $m^{-2}$ $s^{-1}$. Controls were maintained at 30 °C (± 0.5) and 50 µmol photon $m^{-2}$ $s^{-1}$. The time periods of the high-temperature pulse tested were 1, 2, 3,…,7 days. At the end of the period at higher temperature, the cultures were checked by light microscopy for changes in orientation. Subsequently, the cultures were incubated under standard growth conditions (30 (± 0.5) °C and 50 µmol photon $m^{-2}$ $s^{-1}$) and checked each day for changes in helix orientation by examining the helix orientation of 100 trichomes. The culture was scored as having reversed helix orientation if more than one-third of the trichomes had changed. The standard deviation was calculated from the results of the three replicate cultures (n = 3).

Chloramphenicol (SIGMA, St. Louis) was used at a final concentration of 40 µM in experiments where growth inhibition was required.

*Shaking*

Studies on the impact of mechanical forces on helix conformation were carried out in a Grant SS40-2 water bath (Grant Instruments (Cambridge) Ltd). Cultures were shaken at 120 strokes $min^{-1}$.

*Osmosis*

The impact of osmosis on trichome morphology of four strains (D0867, D0873, D0880, D0905) was investigated using NaCl as osmoticum added to Zarrouk's medium (17 mM NaCl) resulting in the final concentrations 0.25M, 0.5M and 0.75M. The latter two concentrations were achieved by incubation of the inoculum in 0.25M NaCl for two days followed by increasing the NaCl concentration to 0.5M and after two more days of incubation to 0.75M NaCl. Growth experiments were carried out in 100-mL flasks containing 30 mL Zarrouk's medium. Three replicates of each strain were used for the experiment. After seven days of incubation at 30 °C and 25 µmol photon $m^{-2}$ $s^{-1}$ in the final NaCl-concentration changes in trichome morphology were determined by measuring (light microscope) the diameter of the helix and the length between two turns (pitch) of 50 trichomes.

### 3.5.3 Test for motility on solid medium

Cultures of the 35 strains, the duplicate strains and different morphotypes (two replicates for each strain) were grown on solidified (1% (w/v) agar) Zarrouk's medium. Inoculation of the agar plates was carried out by mounting an 1-mL aliquot in the centre of the agar plate followed by distributing the inoculum equally by rotating the agar plate. Subsequently, the plates were incubated at 30 °C and 25 µmol photon $m^{-2}$ $s^{-1}$. Ability to glide was scored 15 days after inoculation.

## 3.6 Physiological tests

### 3.6.1 Heterotrophy

All experiments on the ability to either dark- or photoheterotrophic growth were carried out at 30 (± 0.5) °C.

*Dark heterotrophy*

Dark heterotrophic cultures were grown in four replicates in 100-mL pyrex flasks containing 30 mL of Zarrouk's medium and an organic carbon source. Cotton wool (BDH) bungs were used to seal the flasks as cotton wool has been shown in the past to allow a better aeration of the flasks compared to cell culture bungs (B.A. Whitton, pers. comm.). Carbohydrates tested were glucose, fructose, lactose, galactose, maltose, sucrose (all in 20 mM final concentration), starch (0.05% w/v). A control culture lacked any organic carbon source. In all experiments the cultures were incubated in the dark (cardboard box) for 40 days unless otherwise stated. Aeration of the cultures was achieved by blowing air through the cardboard box every second day for 20 min unless otherwise stated; additionally, the cultures were shaken three times a day for one min each. After the end of the incubation period all dark heterotrophic cultures were tested for bacterial contamination on five bacterial test media (Section 3.2.3.1) and the pH of the medium of each of the cultures was determined.

To optimise environmental conditions a permanent aeration system was set up to provide continuous aeration of the cultures under axenic conditions in the dark. Growth assays were carried out in 100 mL Zarrouk's medium in 250-mL pyrex flasks containing an organic carbon source (20 mM). Air was sterilised by passing it through a 0.3 µm air filter (Whatman) in autoclavable silicone tubing (Cole-Parmer). Eight replicates were analysed for each of the strains tested in this system.

*Photoheterotrophy*

Assays for photoheterotrophic growth were carried out in 50-mL boiling tubes containing 18 mL (final volume) Zarrouk's medium with a carbohydrate as carbon source in a final concentration of 20mM or with 20mM carbohydrate plus 10 µM DCMU, which inhibits photosynthetic electron transport (Bishop, 1951). Cultures in Zarrouk's medium with or without 10 µM DCMU were used as controls.

As autoclaving of medium led often to evaporation of some of the liquid from the boiling tubes, thus altering the volume, Zarrouk's medium and boiling tubes were autoclaved separately and 16 or 15.1 mL (if sugar (0.9 mL of 0.4 M stock solution) was to be added) medium were added with sterile pyrex glass pipettes. Care was also taken that the volume (2 mL) of the inoculum was the same for all replicates and environments by calibration of pasteur pipettes used for inoculation.

Depending on the final concentration of DCMU different concentrations of stock were prepared in order to avoid to high a concentration of ethanol to the growth medium. The concentrations used were 1, 10, 100, 500 µM, 1, 5 mM resulting in an addition of ethanolic DCMU solution of 36 µL (0.18% ethanol final concentration) or less for all cultures screened for their ability to grow photoheterotrophically.

A screening program of 20 *Arthrospira* strains for their ability to grow photo-heterotrophically on sucrose was carried out in 100-mL pyrex flasks. Two replicates were used for controls without sucrose and four replicates for cultures containing sucrose. Irradiance was provided by cool-white fluorescence bulbs. The large number of flasks meant that it was impossible to provide all strains with the same irradiance in the growth room. However, care was taken to ensure that all replicates of the same strain were exposed to the same irradiance. The overall range for this study was 30 to 70 µmol photon $m^{-2}$ $s^{-1}$.

### 3.6.2. Growth on alternative nitrogen and phosphorus sources

*Nitrogen*

Cultures of all 35 *Arthrospira* strains in the phase of fast growth were twice washed free of any contaminating combined N with Zarrouk's medium minus $NaNO_3$ and resuspended in the same medium. 2-µL aliquots of these stock cultures were used to inoculate three variations of Zarrouk's medium (30 mL in 100-mL flasks), each containing a different nitrogen source. Nitrogen sources tested were potassium nitrate, ammonium chloride or urea (all 5 mg N $L^{-1}$). The cultures (no replicates) were incubated at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$. Control was N-free Zarrouk's medium. On days 4, 6, 8, and 10 additional nitrogen was added (in the same form and concentration, 5 mg N $L^{-1}$) to allow continued growth. After 10 days of incubation the biomass was determined in form of its ash-free dry weight (dried at 105 °C over night).

*Phosphorus*

10 strains were washed free of phosphate using Zarrouk's medium minus $K_2HPO_4$ followed by resuspension in P-free medium. 2-µL aliquots of these stock cultures were used to inoculate 16 mL Zarrouk's medium in 50-mL boiling tubes containing 1 mg P $L^{-1}$

of one of the following: $K_2HPO_4$, sodium β-glycerophosphate (Na-β-GP), p-nitrophenyl phosphate (pNPP), bis-p-nitrophenyl phosphate (bis-pNPP), DNA, phytic acid (phytate). Additional P (1 mg $L^{-1}$) was added after each seven days. A control culture lacked P. Three replicates were analysed for each of the ten strains. Cultures were maintained for three weeks at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$.

### 3.6.3 Surface phosphatase activity

*Assay medium*

The assay medium was different from the culture medium in that no phosphorus was present so that the organic phosphorus substrate was the only external source of phosphorus. The strains under investigation were grown for seven days in assay medium prior to the phosphatase assays.

*Method*

The method for phosphatase activity using pNPP was based on Grainger *et al.* (1989). 1.5 mL of a culture was added to 1.4 mL of assay medium in a snap cap vial. The vials were transferred to a water bath at 30 °C and allowed to equilibrate for 10 min. 0.1 mL of a 6 mM substrate solution were added, giving a final concentration of 200 µmol pNPP. After 2.5 h incubation the assay was terminated by filtering through a GF/C (Whatman) filter and addition of 0.25 mL of a 5M NaOH solution to 2.5 mL of the assay. The increase in pH of the medium inactivates enzyme activities and facilitates the maximum colour development (yellow) of the substrate (pNP) (N. Ellwood, pers. comm.). The absorbance of the product (pNP) was read at 405 nm on a Shimadzu Double-Beam UV-150-02 (Perkin Elmer LS-3B spectrophotometer). Control vials were set up in the same way as the experimental vials, but without organisms present.

*Analysis of results*

Phosphomonoesterase activity was expressed as µmol product µg chl $a^{-1}$ $h^{-1}$, using the formula:

$$\text{Phosphatase activity} = A \times v / Ap \times c \times t$$

where $A$ is absorbance reading after subtraction of control, $v$ is the total assay volume in litres, $Ap$ is the absorbance reading of 1 µM standard product (0.0153; M. Christmas, pers. comm.), $c$ is chlorophyll a in µg and $t$ is the assay time in hours.

The amount of the algal material used for the assay was determined as its chlorophyll a. Chlorophyll a was extracted by incubation of the GF/C filters with the biomass in 4.5 mL chloroform in a snap cap vial followed by incubation for 12 h at 4 °C in the dark or until the algal material went colourless. Material debris was removed by centrifugation and 0.5 mL $H_2O$ were added to give a 90% methanol solution. The absorbance of the solution was determined on a spectrophotometer at 665 nm and at 750 nm, previously blanked on 90 % methanol. The following equation was adopted for the determination of chlorophyll a in each sample (Marker *et al.*, 1980):

$$\text{Chlorophyll a } (\mu g\ l^{-1}) = K\,(A_{665} - A_{750})\ v/L$$

where $A$ is absorbance reading, $K$ is 1000 x the reciprocal of the specific absorption coefficient for chlorophyll a at 665 nm (the specific absorption coefficient for chlorophyll a in methanol is 13; Marker, 1980), $v$ is the volume of solvent (90 % methanol) used to extract the sample (in mL) and $L$ is the path length of the cuvette (in cm).

## 3.7 Biochemical analysis

### 3.7.1 Fatty acids

*Growth environment*

Cultures were grown in 100-mL pyrex flasks containing 30 mL of Zarrouk's medium (Zarrouk, 1966); three replicates were used for each strain and sampling occasion, unless specified otherwise. Irradiance was provided by cool-white fluorescence bulbs. Growth temperatures were maintained constant within ± 0.5 °C. To achieve high cell density the cultures were harvested at the late phase of fast growth by filtration through a Whatman nitrocellulose membrane filter (0.45 µm pore size). The time period of growth was dependent on the culture condition: 10 d at 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$, 6 d at 30 °C and 70 µmol photon $m^{-2}$ $s^{-1}$, 16 d at 20 °C and 10 µmol photon $m^{-2}$ $s^{-1}$, 12 d at 20 °C and 60 µmol photon $m^{-2}$ $s^{-1}$ and 40 days at dark-heterotrophic conditions on glucose.

*Lipid extraction and transmethylation*

Prior to any experiment all glassware used for lipid extraction was washed with detergents, thoroughly rinsed in distilled water, dried and rinsed in hexane. The harvested biomass was resuspended in 1 mL distilled water. Cell lysis was achieved by addition of 3.75 mL of $CHCl_3$/MeOH (1:2) containing 50 mg $L^{-1}$ butylated hydroxy toluene to prevent oxidation of double-bonds during the isolation procedure. Heptadecanoic acid (17:0) was added (25 μg) as internal standard (its absence in *Arthrospira* was demonstrated prior to this work in 16 of the 35 *Arthrospira* strains; data not shown). The suspension was vortexed and incubated at room temperature until all biomass was lysed, followed by centrifugation (400 g for 10 min) in a swing-out rotor and transfer of the supernatant into a fresh vial. Separation of the aqueous and organic phases was achieved by adding one mL of $CHCl_3$ (containing 50 mg $L^{-1}$ BHT) and 1 mL 0.88% (w/v) KCl. After vortexing and centrifugation (400 g for three min) the organic phase was transferred into a fresh vial and dried at 30 °C under nitrogen. Methyl esters were prepared by incubation of the lipid preparation at 80 °C for 1 h in 1 mL 1M methanolic HCL (SIGMA) under a nitrogen atmosphere. After cooling down 1 mL of NaCl-saturated water was added and the fatty acid methyl esters (FAMES) were extracted with two mL hexane. The volume of the hexane extract was reduced to 200 μL for fatty acid analysis by drying under nitrogen. Two μL of each sample were injected into the gas chromatograph.

A control sample contained distilled water (1 mL) instead of an algal sample and was treated as for isolation and transmethylation of fatty acids.

*Fatty acid analysis*

Analysis of the methyl esters was performed on a Shimadzu GC-14 gas chromatograph with flame ionization detector. The column used was an Alltech AT2335 capillary GC-column (30 m x 0.25 mm ID; stationary phase thickness of 0.25 μm). After 2 min at 140 °C the column was heated up (4 °C $min^{-1}$) to 212 °C and maintained at this temperature for 3 min. Methylesters were injected at 250 °C and detected at 270 °C. A standard mix of fatty acids methyl esters (GC 63 and GC 65, Nu-Chek Prep Inc.) with additionally added γ-linolenic acid was used for the identification of the major fatty acids.

*Biomass determination*

Biomass determination was carried out by determining the ash-free dry weight (dried at 105 °C for 12 h) of an aliquot of known volume of an equally suspended culture and correlating it to the volume of the aliquot that was used for the lipid extraction and the amount of 17:0 added to the sample prior to the fatty acid extraction.

### 3.7.2 Lectin-binding

Aliquots of clonal, axenic cultures in the early phase of fast growth were harvested and collected by centrifugation (10000 x g, 5 min, room temperature) in a microtube centrifuge (Hettich, Germany). The biomass was washed with distilled water, followed by washing with PBS-buffer ($KH_2PO_4$ 0.2 g $L^{-1}$, $Na_2HPO_4$ 1.15 g $L^{-1}$, KCl 0.2 g $L^{-1}$, NaCl 8 g $L^{-1}$; pH 7.4). 40 µL biomass was resuspended in a solution (1:100) of lectins in PBS-buffer and incubated for 2 h at room temperature under constant agitation. The lectins tested (Table 3.3) were obtained from SIGMA.

Table 3.3 Binding specificity of lectins to polysaccharides. Gal = + galactose; GalNAc = N-acetylgalactosamine; GlcNAc = N-acetylglucosamine. Taken from Slifkin and Doyle (1990) or SIGMA product catalogue (SIGMA, 1998).

| Lectin | Abbreviation | Label | Specificity |
|---|---|---|---|
| *Glycine max* | G | Gold | GalNAc, Gal |
| *Helix pomatia* | He | Gold | GalNAc, Gal, GlcNAc |
| *Triticum vulgare* | T | Gold | GlcNAc |
| *Lens culinaris* | L | FITC | α-D-glucosyl or α-D-mannosyl residues |
| *Tetragonolobus purpureas* | Te | FITC | α-L-fucosyl residues |

After incubation the biomass was collected by centrifugation as above and washed in distilled water. Samples treated with FITC-labelled lectins were examined directly under UV-light (Section 3.2.5.1). Samples treated with gold-labelled lectins were silver enhanced in darkness using IntenSE M Silver Enhancement Kit (Amersham) following manufacturers instructions with the exception that the exposure time to the silver-enhancement solution was extended from 10-12 min to 15-20 min. The samples were washed twice in distilled water and observed under UV-light in combination with an IGS-

filter set (Section 3.2.5.1). The experiment was repeated twice independently testing one replicate each time.

In addition, the influence of environmental factors on the formation of surface polysaccharides, appropriate for lectins to bind to, was investigated on strains D0918/H, D0923 and D0925. Environments tested were: 20 °C and 30 µmol photon $m^{-2}$ $s^{-1}$, 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$, 30 °C and 70 µmol photon $m^{-2}$ $s^{-1}$, laboratory conditions (17-25 °C with approx. 10-30 µmol photon $m^{-2}$ $s^{-1}$ and a natural light-dark cycle). Furthermore, the dilution of the lectin stock was tested on the same set of strains using 1:50, 1:100 and 1:200 dilutions. Ability to lectin-binding was tested as described above.

## 3.8 Pyrolysis Mass Spectrometry

*Strains and growth conditions*

To obtain standardized stock cultures, cultures of the 35 strains, the five duplicate strains and the eight morphological different subclones were grown at 30 °C and 25 µmol photon $m^{-2}$ $s^{-1}$ in 30 mL Zarrouk's medium (in 100-mL flasks). After seven days incubation, aliquots of these stock cultures were used to inoculate solidified (1% (w/v) agar) Zarrouk's medium. These cultures were grown at 30 °C and 25 µmol photon $m^{-2}$ $s^{-1}$ for 10 days. The growth temperature was maintained constant at 30 (± 0.5) °C, and all cultures experienced exactly the same changes within this temperature range. To compensate for slight differences in the light intensity at different areas on the agar plates each plate was rotated clockwise 90° each day. In addition, *Spirulina* sp. strain D0917 was grown on both ASW:BG and Zarrouk's medium under the same environmental conditions.

*Pyrolysis*

PyMS samples were prepared by taking three aliquots from each of the two subcultures of a strain. The samples of the cultures were taken directly from the plates and mounted onto a Ni-Fe pyrolysis foils (Horizon Instruments) using a wire loop. The foils were dried at 80 °C for 5 min and pyrolysed in a Horizon Instruments 200X pyrolysis mass spectrometer at 530 °C for 4 s. To reduce the vast amount of data prior to analysis and to select only the most discriminating and reproducible data, a window was set for the selection of mass ions for analysis. Therefore, mass ions of below 50 and above 200 mass

units were excluded from the analysis. The resulting mass spectra containing counts of the mass ions within the range of 50-200 mass units, together with the total ion count of each sample were recorded.

*Data analysis*

The data obtained were transferred onto a Pentium 200MHz computer and analysed using the Genstat 5 software package applying principles and techniques described in some detail elsewhere (Gutteridge et al., 1985; Aries et al., 1986; Magee, 1993). In brief, the analysis of the data involves the following steps.

To compensate for the weight differences between samples analyzed, the spectra are normalised using the Genstat 5 software package. Furthermore, to select for reliable data, only data of those samples, which produced a total ion count of the spectra within the range of 0.5-6.0 x $10^6$, were included in the analysis.

The data were analyzed using principal component and canonical variate analysis to compare ion intensities as well as patterns within spectra. The combined results from principal component and canonical multivariate analyses were plotted in form of two- or three-dimensional ordination diagrams. The diagram showing the maximum variation was selected for the determination of the Mahalanobis distances (Magee, 1993).

Using the Mahalanobis distances from the PC and CV analysis a distance matrix was produced which then was converted to a percentage similarity matrix by using Gowers coefficient (Magee, 1993). Subsequently, a dendrogram was constructed from the similarity matrix applying the unweighted pair group method with arithmetic averages (UPGMA; Sneath and Sokal, 1973).

## 3.9 Database and computer-aided numerical analysis

### 3.9.1 Storage and coding of the data

All data, except the one from PyMS, were stored in form of an Excel spreadsheet and subsequently transferred in to a binary code. The average of the range formed the basis for scoring of quantitative characters. For example, the trichome width of 7.5 - 8.5 µm was scored as '0' as the criterion is '> 8.0 µm', but the average of the range is 8.0 µm.

### 3.9.2 Numerical and principal component analysis

*Numerical analysis*

The data were analyzed using the MVSP (Multivariate Statistics Package; Version 2.1) software (Kovach Computing Services. Pentraeth, UK) on a PC 200MHz computer. A similarity matrix was calculated using the simple matching coefficient ($S_{SM}$; Sokal & Michener, 1958), which includes both positive and negative matches. The $S_{SM}$ of two OTUs (i, j) is calculated as:

$$S_{SM}(i,j) = \frac{a + d}{a + b + c + d}$$

;

| | | OTU i | |
|---|---|---|---|
| | | Presence | Absence |
| with OTU j | Presence | a | b |
| | Absence | c | d |

Clustering was achieved using the NTSYS software package (Exeter Software, New York, USA), applying the unweighted pair group method with arithmetic averages (UPGMA; Sneath & Sokal, 1973). A phenogram was drawn and a $r_{cs}$ was calculated between the implied similarity values of the phenogram and the $S_{SM}$ of the OTUs using NTSYS.

# CHAPTER 4 PRODUCTION AND MAINTENANCE OF CLONAL, AXENIC CULTURES OF *ARTHROSPIRA* AND *SPIRULINA* STRAINS

## 4.1 Introduction

Most of the subcultures of the *Arthrospira* and *Spirulina* strains received were contaminated (except those from PCC). To produce clonal, axenic culture of *Arthrospira* and *Spirulina* strains, it was planned to test whether the methods of differential filtration (Heaney & Jaworski, 1977; Torzillo et al., 1985), or micromanipulation (Bowyer & Skerman, 1968) and repeated transfer of cells (Vaara et al., 1979) allow the production of a large number of clonal, axenic cultures of *Arthrospira* strains. If none of the methods proved to be appropriate, then a new procedure was planned to be developed that would allow the production of such a large number of strains in a reasonable time period.

Furthermore, the strains assembled were cultured in different media at their sources. Therefore, it was planned to identify a growth medium which allows the culture of all *Arthrospira* strains over a relatively long time period thus enabling long-term experiments without the need for fed-batch culture. The P concentration of the medium, however, should be low, thus allowing analysis of enzyme activities, such as surface phosphatase activity.

To avoid genetic drift of phenotypic characters through subsequent subculturing, attempts were undertaken to establish a method for long-term storage of cultures which should be applicable to all *Arthrospira* strains.

## 4.2 Media

### 4.2.1 *Arthrospira* strains

*Zarrouk's medium*

The composition of the modified version of Zarrouk's medium ("Zarrouk's medium") used in this project is given in Table 1. The modifications concern the replacement of the trace element solution of Zarrouk's medium (1966) with that of BG-11. Furthermore, Ni (0.049 g $L^{-1}$ $NiSO_4.7H_2O$) has been added to the trace element solution.

Table 4.1 Zarrouk's medium, based on concentrations of elements.

| | Na | K | Mg | Ca | Mn | Fe | Co | Ni |
|---|---|---|---|---|---|---|---|---|
| (mg $L^{-1}$) | 5996 | 671.5 | 19.71 | 11.0 | 0.50 | 2.00 | 0.01 | 0.01 |

| | Cu | Zn | Mo | $NO_3$-N | $PO_4$-P | B |
|---|---|---|---|---|---|---|
| (mg $L^{-1}$) | 0.02 | 0.05 | 0.155 | 411.8 | 88.9 | 0.50 |

*Attempts to lower P and N concentrations of Zarrouks' medium*

An initial attempt to grow five *Arthrospira* strains (D0867, D0872/H, D0873, D0885/H1, D0891) in Zarrouk's medium containing P and N in the concentrations of 1 mg $L^{-1}$ ($PO_4$-P) and 8 mg $L^{-1}$ ($NO_3$-N) failed. All strains tested behaved similarly. After an initial phase of growth during the first seven days of incubation the cultures of the five strains showed loss of pigmentation during the following 10 days and finally lysed.

An approach involving a stepwise decrease of P and N concentrations of the Zarrouk's medium did also fail as it did not allow to culture *Arthrospira* strains without regular (once every seven days) addition of P and N. Generally the cultures survived 20 d incubation under a P and N concentration of 12 mg $L^{-1}$ ($PO_4$-P) and 96 mg $L^{-1}$ ($NO_3$-N). Cultures grown in Zarrouk's medium containing only 6 mg $L^{-1}$ $PO_4$-P and 48 mg $L^{-1}$ $NO_3$-N or 1 mg $L^{-1}$ $PO_4$-P and 8 mg $L^{-1}$ $NO_3$-N showed loss of pigmentation earlier (after 15-18 days or 8-12 days of incubation, respectively).

### 4.2.2 *Spirulina* strains

*Spirulina* strains were cultured in a modified ASW:BG medium. Modification of the medium concerned the addition of 0.049g $L^{-1}$ $NiSO_4.7H_2O$ to part 1 of the medium. A second modification involved omission of the aqueous extract of soil. No obvious problems in growth were observed in the medium without the soil extract when compared to growth on ASW:BG medium (plus 0.048g $L^{-1}$ $NiSO_4.7H_2O$ ).

## 4.3 Production of clonal, axenic cultures

The method for the production of clonal, axenic cultures based on serial washing of a filament proved to be successful for all *Arthrospira* strains. Carrying out the isolation and purification procedure on solidified (1% (w/v) agar) enriched Zarrouk's medium allowed monitoring of the purification process. After incubation, the agar plates used for the isolation process show a distinctive pattern of contaminant bacterial growth. Areas of the plate that correspond to the first two or three droplet transfers are heavily contaminated. Successive transfers of a single filament with 100µl sterile Zarrouk's medium correspond with areas of decreasing contamination until no bacterial growth can be observed (usually after about ten transfers). Filaments washed beyond this point could be considered to be potentially axenic.

After inoculation and incubation of an isolated and purified filament in growth medium a culture suitable in purity and size for any further study was obtained after two to four weeks depending on the growth potential of the strain. Generally, all filaments isolated grew to a culture and of the six attempts carried out for each strain, five to six cultures proved to be axenic. Therefore, the yield of axenic cultures obtained by this method was in excess of 80% of the culture tubes inoculated with a single filament.

In contrast, all attempts to obtain clonal, axenic cultures of *Spirulina* strains failed as the isolated filaments did not grow to a culture.

## 4.4 Long-term preservation of *Arthrospira* and *Spirulina* strains

### 4.4.1 Cryopreservation

Cryopreservation of six *Arthrospira* and four *Spirulina* strains by storage in liquid nitrogen was tested using DMSO, glycerol or methanol as cryopreservants. Ten strains were tested for one week. All attempts to cryopreserve *Arthrospira* and *Spirulina* strains by placing samples of the cultures directly into liquid nitrogen failed.

Using the modified slow-freezing method of Brand (1996) no *Arthrospira* strain was successfully cryopreserved, independent on the cryoprotectant used (DMSO, glycerol, methanol). One strain (D0867) showed some indication that it had survived (Table 4.2).

However, only a few filaments appeared to have survived, which eventually died during subsequent incubation under photoautotrophic conditions.

In contrast to *Arthrospira* strains, the method for cryopreservation recommended by UTEX (Brand, 1996) proved to allow cryo-preservation of all *Spirulina* strains. Best strain recovery was achieved using DMSO as cryoprotectant in a concentration of 7.5-15% (v/v).

Table 4.2 Results of cryopreservation of *Arthrospira* and *Spirulina* strains using cryoprotectants.

| Method | Strains investigated | Results |
|---|---|---|
| Cryopreservation in liquid nitrogen (without stepwise cooling of the samples) | *Arthrospira* D0867, D0871, D0872,D0880, D0882, D0896<br>*Spirulina* D0868, D0869, D0870, D0871 | No viability of any *Spirulina* or *Arthrospira* strains, independent of use of DMSO, glycerol or methanol and the concentrations of those used |
| Cryopreservation in liquid nitrogen after Brand (1996) | *Arthrospira* D0867, D0871, D0872,D0880, D0882, D0896 | D0867 only *Arthrospira* strain that potentially retained viable, but culture died after 2 weeks; no other strain showed viability |
| | *Spirulina* D0868, D0869, D0870, D0871 | All *Spirulina* strains tested retain viable; growth to culture after 10-20 days |

### 4.4.2 Storage at low temperature

*Storage at -20 °C in 50 % glycerol*

Despite all precautions taken to avoid cell lysis, cells were lysed as judged by the presence of phycocyanin in the samples after 14 days of incubation. Although some trichomes of a sample showed still auto-fluorescence when observed under UV-light the majority proved to have lost their auto-fluorescence, indicating potential loss of viability. Inoculation of growth medium with or without glucose and/or DCMU did not lead to growth of any of the samples tested.

*Storage at low temperature (4 °C) in the dark*

Another approach was to store cultures in liquid medium at 4 °C in the dark for seven months followed by incubation under moderate temperature and irradiance conditions (20 °C and 5 μmol photon $m^{-2}$ $s^{-1}$) for five days, before transfer to more normal growth conditions

(30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$). After 15 to 20 days at the higher temperature, 11 of the 35 *Arthrospira* strains tested showed growth (Table 4.3).

Table 4.3 Viability of *Arthrospira* strains after storage at 4 °C in the dark. The 35 strains and the five duplicate strains were stored for seven months in liquid medium in the dark at 4 °C. 11 strains retained viable during the storage period.

| | | | |
|---|---|---|---|
| D0882 | D0890 | D0899 | D0916 |
| D0884 | D0895 | D0904 | D0919 |
| D0885/H1 | D0896 | D0909 | |

*Storage at low temperature (10 °, 15 °C) under low light or dark*

As some *Arthrospira* strains survive storage at 4 °C under low light conditions (Section 4.4.2.2) or in the dark (see above) tests were carried out to investigate whether more cultures would survive at slightly higher temperatures, eg at 10 °C under low light or 15 °C in the dark.

None of the 35 strains and five duplicate strains survived under either of the two environments tested. As judged by the loss of blue-green pigmentation, most cultures lost their viability during the first months at low temperature. Microscopy of the cultures containing bright-green biomass after six months of incubation showed that they consisted of lysed or damaged cells only, which was also confirmed by the lack of auto-fluorescence.

*Storage at low temperature (4-5 °C) in the dark on agar slopes*

Long-term preservation was also tested by storing cultures for 12 months on solidified (1% (w/v) agar) medium at 4-5 °C and low irradiance. Of the 35 strains and five duplicate strains tested (no replicates) 18 cultures consisted still of green biomass while the agar of the other cultures were dried out resulting in loss of the biomass, presumably due to desiccation. Of the 18 cultures that were still looking viable on agar after seven month incubation at 4 °C six grew to cultures (Table 4.4). Cultures of these six strains retained their viability also after storage for seven months in liquid medium at 4-5 °C in the dark (Section 4.4.2.1; Table 4.3).

Table 4.4. Viability of *Arthrospira* strains stored on agar at 4-5 °C. The 35 strains and five duplicate strains were stored for twelve months at 4-5 °C. Of the following 18 samples, which consisted of agar plus biomass that was not dried out completely, those shown in bold and italics were still viable.

| | | | |
|---|---|---|---|
| D0867 | D0879 | ***D0885/H1*** | ***D0896*** |
| D0872/H+S | D0880 | D0887 | D0897 |
| D0873 | D0881 | ***D0890*** | D0925 |
| D0875 | ***D0882*** | D0891 | |
| D0876 | ***D0884*** | ***D0895*** | |

### 4.4.3 Drying under a natural light-dark cycle

Studies were undertaken to test whether drying of cultures can be used as means of long-term storage. Ten strains were grown on agar plates (1% w/v) at room temperature (approx. 17-25 °C) under a natural light-dark cycle. After three months incubation the agar plates started to become dry. Therefore, the viability of the cultures was tested by inoculation of Zarrouk's medium with aliquots of the biomass on the agar plates. Of the ten strains tested all grew. However, the drying out of the agar plates during extended incubation proved to restrict preservation by this method up to a maximum of three to four months. Drying led to loss of viability independent of whether or not the cultures grew into the agar (see Section 5.2.3.1). This revealed by inoculation of Zarrouk's medium with samples of the ten strains after further two months. None of the ten strains tested grew to a culture.

## 4.5 Discussion

### 4.5.1 Media

*Arthrospira*

Comparison of the versions of Zarrouk's medium used in various culture collections for the culture of *Arthrospira* strains reveals that a wide range of modifications have been made to the trace metal content. PCC uses the same trace element concentrations as used

in this work (Tables 4.1), apart from the absence of Ni. Ni in the form of $NiSO_4.7H_2O$ was added because this is an essential element for use of urea, a likely source of combined N for cyanobacteria in some environments (Daday et al., 1988; Singh & Rai, 1990). All *Arthrospira* strains were able to grow on urea as sole N-source (Section 7.3). Vanadium, chromium, tungsten and titanium are apparently unnecessary, and were omitted.

The high N and P contents of Zarrouk's medium mean that it is probably far removed from the natural environment of many of the strains isolated. These strains are therefore likely to have been under strong selective pressure to adapt to these high concentrations.

Attempts were made to develop less nutrient-rich media and media which permit growth to either N- or P-limitation in order to check morphological characters and make a range of comparisons of enzyme activity. The P to N ratio of 1 to 8 as this has been shown to be most favoured of cyanobacteria. However, despite stepwise adaptation to lower P and N concentrations, all modifications tested required fed-batch culture or short-time periods between subsequent subculturing, thus increasing the risk of contamination or selecting mutants.

Strains which underwent a stepwise decrease of available inorganic P and N showed loss of pigmentation earlier then cultures which were directly exposed to Zarrouk's medium containing 1 mg P $L^{-1}$ and 8 mg N $L^{-1}$. It seems that preceding culturing at lower concentrations of P and N than Zarrouk's medium led to utilization of internal P (polyphosphate granules) and N (cyanophycin granules) reserves.

It is difficult to estimate the P and N content of the natural environment. Except for the marine isolates, it must be assumed that the natural environment of *Arthrospira* strains undergoes changes due to rainfall and drought. Therefore, *Arthrospira* strains presumably have regularly a period during which they accumulate P and N reserves between periods of low P and N environments.

*Spirulina*

The medium used to culture *Spirulina* strains is based on seawater mainly enriched with P, N, $Mg^{2+}$ and $Ca^{2+}$, but also other (trace) elements. The soil extract, a constituent of several of the media used at CCAP, proved to be not necessary for the growth of *Spirulina* strains and was omitted. Due to the addition of sea water, the precise composition of the modified ASW:BG medium is unknown.

### 4.5.2 Production of clonal, axenic cultures

An attempt to use differential filtration based on Heaney and Jaworski (1977) and Torzillo *et al.* (1985), or micromanipulation (Bowyer & Skerman, 1968) and repeated transfer of cells (Vaara et al., 1979) proved to be time-consuming, without leading to much success, mainly due to the large number of contaminants present in many cases or due to lack of obtaining cultures from isolated filaments. Therefore, it was necessary to develop a procedure, which allowed a high number of repeats of the washing step in a shorter time than the method of Torzillo *et al.* (1985).

*A method for the production of clonal, axenic cultures of* Arthrospira *spp.*

Isolation of a single filament followed by a series of washing steps proved to be an appropriate procedure for obtaining clonal, axenic cultures of *Arthrospira* spp. and may be applicable for other filamentous cyanobacteria of suitable size.

The use of cyanobacterial cultures in the phase of fast growth proved to be of more advantage than using a culture in the stationary phase, as relatively fewer contaminants are present. Applying the method established in the presented work (Section 3.2.3) not only a serial dilution is being carried out but also a series of steps which effectively contribute to purify the cyanobacterial filament from tightly associated bacteria. Especially pressing out medium containing the single filament from the pipette tip in a strong fashion leads to the removal of many bacteria, which are tightly attached to the cell surface. At average, this procedure led to axenic cultures after approximately ten repeats of the washing step as it revealed from the agar plate on which the isolation steps were performed. In practice at least 20 transfers were carried out per filament to ensure the removal of all contaminant bacteria. Furthermore, only short filaments or fragments of filaments present in the culture were chosen for the isolation. By transferring the smallest fragment of filament possible the risk of retaining contamination is significantly reduced.

*High yield production of axenic cultures*

Inoculation and incubation of potentially axenic filaments for two to four weeks led to cultures of high cell density. Generally all of the boiling tubes inoculated with a single filament showed growth. Performing a "rougher" purification procedure using repeated washing of the *Spirulina* (*Arthrospira*) cultures on nylon net followed by mechanical

fragmentation, centrifugation in order to pellet the filament fragments and subsequent washing of the fragments prior to inoculation, Torzillo et al. (1985) observed growth only in 34% of the inoculated test tubes. In general, five to six out of six cultures grown from single filaments proved to be bacteria-free resembling an efficiency of more than 80%. Torzillo et al. (1985) reached an efficiency of 28% of axenic *Spirulina* (*Arthrospira*) *maxima* and *S.* (*A.*) *platensis* cultures.

However, despite the success of the method applied on *Arthrospira* strains inoculation of medium with a single filament of the seven *Spirulina* strains did not result in cultures, despite incubation for up to six months. The reason for this behaviour is unclear.

Although the production of axenic cultures is laborious and time-consuming the attempts to produce bacteria-free cultures allowed the production of 43 axenic cultures. Together with 5 clonal, axenic cultures obtained from PCC an *Arthrospira* culture collection was established containing clonal, axenic cultures of 35 *Arthrospira* strains and five duplicate strains of those as well as eight different morphotypes. Furthermore, the potential to isolate particular trichomes (eg different morphotypes of a strain), represents a great advantage over other methods, such as streaking techniques. The potential that is offered by this method is resembled in the production of clonal cultures of eight morphologically different subclones of six strains as well as two morphological different filaments from a sample from the out door ponds of Earthrise Farms (Calipatria). The latter two proved later to be genetically different (Scheldeman et al., 1999).

### 4.5.3 Long-term preservation

To avoid genetic drift affecting phenotypic characters of *Arthrospira* strains and to maintain the large set of strains much effort was allocated towards the development of a method for long-term storage of *Arthrospira* strains. A main criterion for a successful method was that it would be applicable for all or most of the *Arthrospira* strains held at Durham. A wide range of techniques has been employed.

*Cryopreservation*

All attempts to cryoprotect *Arthrospira* strains have been unsuccessful. However, there is no known procedure that guarantees post-cryopreservation viability of *Arthrospira* strains. Despite the use of controlled cooling (eg 1 °C $min^{-1}$) of *Arthrospira* strains no

great success has been achieved at culture collections (Brand, 1996). For example, of the four *Arthrospira* strains at UTEX only two strains showed viability after cryopreservation, but only when placed on agar slants and after several weeks of incubation (J. Brand, pers. comm.). The reason for lack of viability is unknown. The fact that the great majority of cyanobacteria without gas-vacuoles survive in liquid nitrogen (Whitton and Potts, 2000), however, indicates that the gas-vacuoles are responsible for the failure to cryopreserve *Arthrospira* strains. This is also confirmed by the fact that *Spirulina* strains, which lack gas vacuoles (Section 6.2), retain their viability after cryopreservation.

*Storage at -20 °C in 50 % glycerol*

Experiments were carried out to investigate whether cell lysis through freezing can be avoided by storage at -20 °C in 50% (v/v) glycerol. The high glycerol content prevents freezing of samples stored at -20 °C. Studies were also carried out to improve the growth conditions after thawing. As the photosystem may be stressed during low-temperature storage or thawing of the sample, the effects of photoheterotrophic growth on glucose and/or addition of subinhibitory concentration of DCMU (75 nM), which proved to stabilize the photosystem (Section 7.5.1.3 A), were tested.

The results proved that this approach did also not lead to any success. Reasons for the lack of viability after 14 d incubation at -20 °C are uncertain. The high content of glycerol, however, may either have been toxic or have led to too high an osmotic pressure resulting in cell lysis.

*Storage at low temperature and low light or dark*

The tests at 4 °C and low irradiance showed that all of the strains, which survived for 12 months on agar also, survived for seven months in liquid. This suggests that some strains have a better ability than others to survive long periods at low temperatures. Increase in yield of survivors, however, was not achieved by increasing the storage temperature to 10 °C or 15 °C. Therefore, it seems that this approach does also not provide a generally applicable and reliable method for long-term storage of a large set of *Arthrospira* strains.

*Drying of cultures*

Drying of cyanobacteria on cotton wool proved to be a technique successful for some cyanobacterial strains (B.A. Whitton, pers. comm.). Drying of *Arthrospira* strains, however, led to loss of viability independent of whether or not the cultures grew into the agar (Section 5.2.3).

These observations also suggest that *Arthrospira* cells are not desiccation tolerant in the natural environment. Whether or not fully dried out lakes need to be "re-inoculated" with a fresh *Arthrospira* sample, is, however, unclear, but feasible, as some lakes, eg Lake Chad (Chad), have no record of having totally dried out (Sili et al., 1999).

## 4.6 Summary

i) A modified version of Zarrouk's medium was the most appropriate for all strains. Lowering the N and P content of the medium reduced the culture period.

ii) A method for the production of clonal, axenic cultures of *Arthrospira* strains has been established and successfully applied to all 35 strains and the five duplicate strains. The method also allowed the isolation and production of clonal, axenic cultures of eight different morphotypes of six of the strains or their duplicates.

iii) Due to the lack of single trichomes to grow to a culture, clonal, axenic *Spirulina* cultures were not obtained.

iv) Neither cryopreservation nor storage at low temperatures and light intensity or darkness permitted long-term storage of all *Arthrospira* strains. *Arthrospira* strains are not desiccation tolerant.

# CHAPTER 5 MORPHOLOGY

## 5.1 Introduction

It was planned to produce a database containing morphological characters of all strains, duplicate strains and different morphotypes. Because the helical trichome morphology has been the main morphological character for the classification of *Arthrospira* strains, an aim of this study was to investigate factors that influence it.

Many filamentous cyanobacteria are able to glide on substrate. It was planned to test whether motility provides a useful character for taxonomic purposes within the genus *Arthrospira*.

## 5.2 Morphology of *Arthrospira* strains

### 5.2.1 Morphological markers

All morphological characters were examined by light-microscopy. To obtain a representative average, each morphological character was examined at three different occasions during the period of one year. Results of measurements (20 trichomes were measured at each of the three occasions) are given in the form of a size range. Unless otherwise stated, all data were collected from young (in the phase of fast growth) cultures grown at 30 °C and 25 µmol photon $m^{-2}$ $s^{-1}$.

Measurements were carried out on trichomes whose morphology was not affected by the preparation of the slides; ie the trichome helix was maintained, but not diminished by pressure from the cover slide. This was ensured by maintaining a liquid layer between the slide and the cover slide which was slightly bigger than the diameter of the trichome helix, but did not allow "floating" of the trichomes. The 17 morphological characters tested and their character states are described in Table 5.1 and the data collected are summarized in Appendix A.

Table 5.1 List of morphological characters and character states. The raw data is given in Appendix A. Data from experimental studies (Section 5.2.3) are not included in the list.

1. Tendency to lose helical trichome morphology

Strains that showed straight trichomes in any of their subcultures, even if those represent only a very minor fraction of the culture, are regarded as showing the tendency to lose the helical trichome morphology. Strains that were received as subcultures with straight trichomes only are considered as having derived from a stock culture with helical trichomes, thus also showing the tendency to lose the helical trichome morphology.

Observed

Not observed

2. Orientation of coiling at 30 °C

The orientation of the helix of the trichomes (Fig. 5.2.2.3) is determined for the 'standard' growth temperature of 30 °C. In those cases, in which a few trichomes of a culture (up to 2% of the population; Section 5.2.3.3) showed opposite helix orientation, the helix orientation of the major part of the population is scored. The orientation of the helix was determined by slowly focussing through a helix and scored at the "top" layer of the helix (Fig. 5.1A).

clockwise (right-handed)

anti-clockwise (left-handed)

3. Trichome length

The trichome length was determined independent of the morphology, ie straight or helical. The length is given in form of a range which represents the trichome length of the major part (ca. 80-90%) of cultures grown at 30 °C. Outliers at the lower end of length size were neglected as it was not possible to determine whether those were very "young" trichomes, ie trichomes observed shortly after breakage or hormogonium formation of the "mother" trichome.

(Min)

(Max)

4. Width of trichome

The width of the trichome (= filament without sheath) is measured at the mid of the trichome (point of largest width).

(Min)

(Max)

5. Cell length

The cell length of cells at the centre of the trichome is measured. The measurement of the cell length of helical trichomes is carried out by measuring the cell length at the convex (outer) site of the helix.

(Min)

(Max)

6. Cell length relative to width

The ratio is determined for cells at the middle of the trichome.

(Min)

(Max)

7. Diameter of trichome end

The cell width of the end cell is determined. In the case of capitate end cells the widest point of the cell is measured.

(Min)

(Max)

8. Attenuation of trichome

The attenuation of trichome width is given as the ratio of the trichome width at the mid of trichome to the width of end cell.

(Min)

(Max)

9. Length of pitch

The distance between two neighbouring turns in the helix is determined at the centre of the trichome, independent on the type of helix, which the trichome forms (ie dumbbell-shape/fusiform, barrel-shaped, regular helix).

(Min)

(Max)

10. Diameter of trichome helix

The helix-diameter is determined at the mid of the trichome and includes the trichome, ie measured from the outer site of the trichome to the outer site of the trichome after a turn of 180 °.

(Min)

(Max)

11. Ratio of length of pitch to helix-diameter

The ratio is determined at the mid of the trichome, independent on the type of helix, which the trichome forms (ie dumbbell-shape/fusiform, barrel-shaped, regular helix).

(Min)

(Max)

12. Variation in helix dimensions

Variation in helix dimensions is determined as the ratio between lowest and highest value of character 11 (ratio of length of pitch to helix diameter)

(number)

13. Type of trichome helix

Several strains have helical trichome form, which has been described as fusiform (helix diameter smaller at mid of trichome than towards the apices; eg Fig 5.1A), dumbbell-shaped or irregular. The dumbell shape is formed from the fusiform trichome helix when the helix pitch is very small (eg Fig. 5.1B). Strains showing a barrel-shaped helix form (helix diameter much wider at mid of trichome than towards the apices) were observed to occur also in a fusiform state. Many strains show a regular helix along the whole trichome. This trichome helix appears also relatively "stiff", ie showing less variation between trichomes of the same culture.

Regular

Fusiform/dumbbell-shaped

Barrel-shaped/fusiform

14. Attenuation of helix at apices of trichome

The helix ends towards the apices of the trichome either with slowly diminishing helix diameter over several (approx. 3-8) turns or with fast diminishing diameter over the last 2-4 turns.

Slowly diminishing

Fast diminishing

15. Trichome apical cell (end cell)

The end cells of trichomes exhibit a distinctive cell structure. The specific type of end cell, however, does often not occur in all trichomes of a culture, but often only in a minor part. Therefore, 50 trichomes were examined to determine the type of trichome end cell. A strain was judged as having a calyptrate or capitate endcell if at least three trichomes were found to show this type of end cell. Fig. 5.1B shows an example of a capitate end cell. The calyptra of a end cell is formed by a thicker cell wall at the apical part of the end cell and is visible under the light microscope.

Rounded/conical

Capitate/"knob"-like

Calyptrate

16. Granules at cross-wall

Granules at cross-walls are scored as present only when they are very distinctive.

Distributed

At cross-walls

Absent

17. Gas vacuoles

Gas vacuoles are visible by light microscopy as red to pink "dots".

Distributed

At cross-walls

Absent

### 5.2.2 Two clusters based on helix characters

Two general types of helical trichome morphology were found, an irregular, showing a fusiform, dumbbell-shaped or barrel-shaped helix and a regular helix type. The comparison of these patterns to other helix characters revealed a high correlation, leading to a grouping into two clusters (Table 5.2).

Table 5.2 Comparison of fusiform and regular helix type to other helix parameters. The comparison is based on 33 strains with helical trichomes. Duplicate strains or different morphotypes of a strain are not included in the analysis. Twenty replicates were examined for each strain at three different occasions.

| | | Attenuation of helix towards apices | | Ratio of length of pitch to diameter of helix | | Variation of helical parameters | |
|---|---|---|---|---|---|---|---|
| Helix morphology | Total (out of 33) | Fast diminishing | Slowly diminishing or no attenuation | < 2 | > 2 | </= 0.75 | > 0.75 |
| Irregular [1)] | 17 | 13 | 4 | 15 | 2 | 16 | 1 |
| Regular | 16 | 0 | 16 | 5 | 11 | 3 | 13 |

[1)] ie fusiform or dumbbell- or barrel-shaped

### 5.2.3 Helical growth

#### 5.2.3.1 Loss of helical trichome morphology

In addition to the two strains (D0881, D0882) and one duplicate strain (D0887) which were obtained as subcultures containing only straight trichomes, subcultures of three strains showed a mixture of straight and helical trichomes (Section 2.2; Fig. 2.1A). During the time course of the project, straight trichomes were found in subcultures of further three helical *Arthrospira* strains (D0885/H2, D0914, D0918). Qualitative observations on the mixed cultures showed that subsequent subculturing led to an increase of straight trichomes within the culture.

Experiments were carried out to test whether the straight morphotype of strain D0914 grows faster than the helical one once it occurs in cultures of helical trichomes, thus

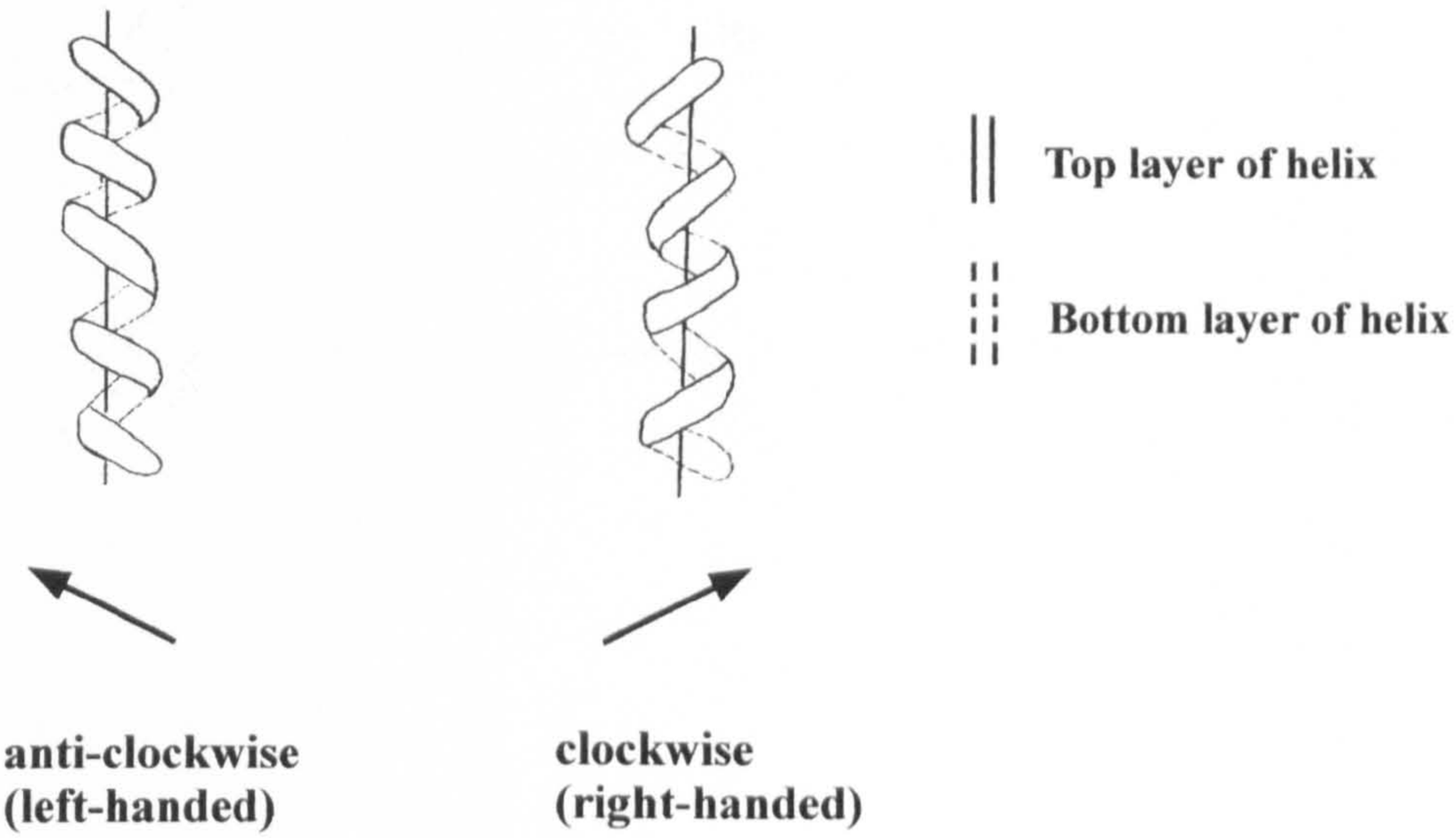
outcompeting the helical morphotype. Five mL of Zarrouk's medium in a test tube (three replicates for each morphotype) were inoculated with a single filament of either straight or helical morphotype of strain D0914 (length approximately similar) followed by incubation at 30 °C and 15 μmol photon $m^{-2}$ $s^{-1}$. The three replicates of the straight mutants showed growth ten days after inoculation while the wild type did not show any signs of growth in any of the replicates (Fig. 5.2). 17 to 20 days after inoculation all replicates of the helical wildtype showed growth, while all replicates of the straight mutant were already grown to dense cultures. Similar results were obtained from the same experiment carried out with the helical wild type of strains D0885/H2 and D0918/H and their straight mutants.

After clonal cultures of the straight and helical morphotype of a strain were newly established, no further loss of helical trichome morphology was observed in the cultures of the helical morphotype. The straight morphotype was stable throughout the project. The straight morphotype of one strain (D0885/S), however, showed in some subcultures trichomes which were either helical or part straight and part helical (Fig. 5.3). The straight morphotype of strain D0885/S was always the most abundant one in any of the subcultures of D0885/S.

Fig. 5.1 Morphological characters.

Determination of helix orientation of *Arthrospira* spp. (A) and capitate endcell (B). The size bar in micrograph B (*Arthrospira* sp. strain D0893) represents 10 μm.

**A)**


Top layer of helix
Bottom layer of helix
anti-clockwise
(left-handed)
clockwise
(right-handed)

**B)**



Fig. 5.2 Comparison between helical and straight morphotype for their ability to grow from a single filament.

Three replicates for each of the two morphotypes were inoculated with a single filament of approximately equal length. 5.2A. Observation on day five of incubation. 5.2B. Observation on day 14.

Fig. 5.3 Back mutation to helical morphotype of strain D0885/S.

5.3A The two helical morphotypes of the subculture of strain D0885 at the point of receiving. Clonal, axenic cultures of both morphotypes were produced. Later, straight trichomes were found within cultures of the thinner trichome type (B to E). Note the partially straight and part helical trichomes (C to E). Sometimes a whole trichome reversed to a regular helix morphology (D).

### 5.2.3.2 Influence of salinity on the helical trichome morphology

The influence of salinity was determined for four strains and four different NaCl concentrations. The results show that increasing salinity leads to a loosening of the helix due to decrease in helix diameter and/or increase of the helix pitch (Fig. 5.4). Despite the high NaCl concentrations of up to 0.75 M no straight trichomes were formed. Subculturing of the cultures into Zarrouk's medium without additional NaCl led to reversal of the straightening of the trichomes.

In a second experiment mannitol (0.1M, 0.2M, 0.3M,...0.7M) was used as an uncharged osmoticum. However, mannitol proved to be toxic to *Arthrospira* cultures leading to cell lysis.

The influence of increasing salinity on *Spirulina subsalsa* (D0869), a marine isolate, was also investigated using the same NaCl concentrations. Although the small size made it difficult to measure helix parameters, no obvious changes in the coiling pattern were observed, neither in length of pitch nor in helix diameter.

**Fig. 5.2**

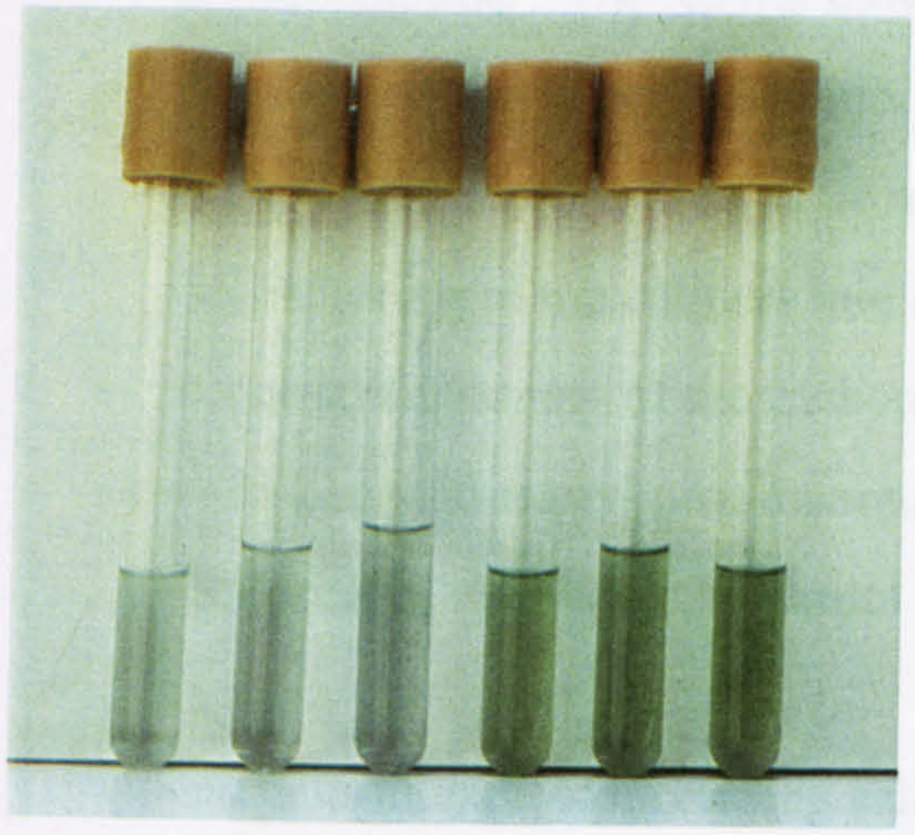


**Fig. 5.3A**



**Fig. 5.3B**

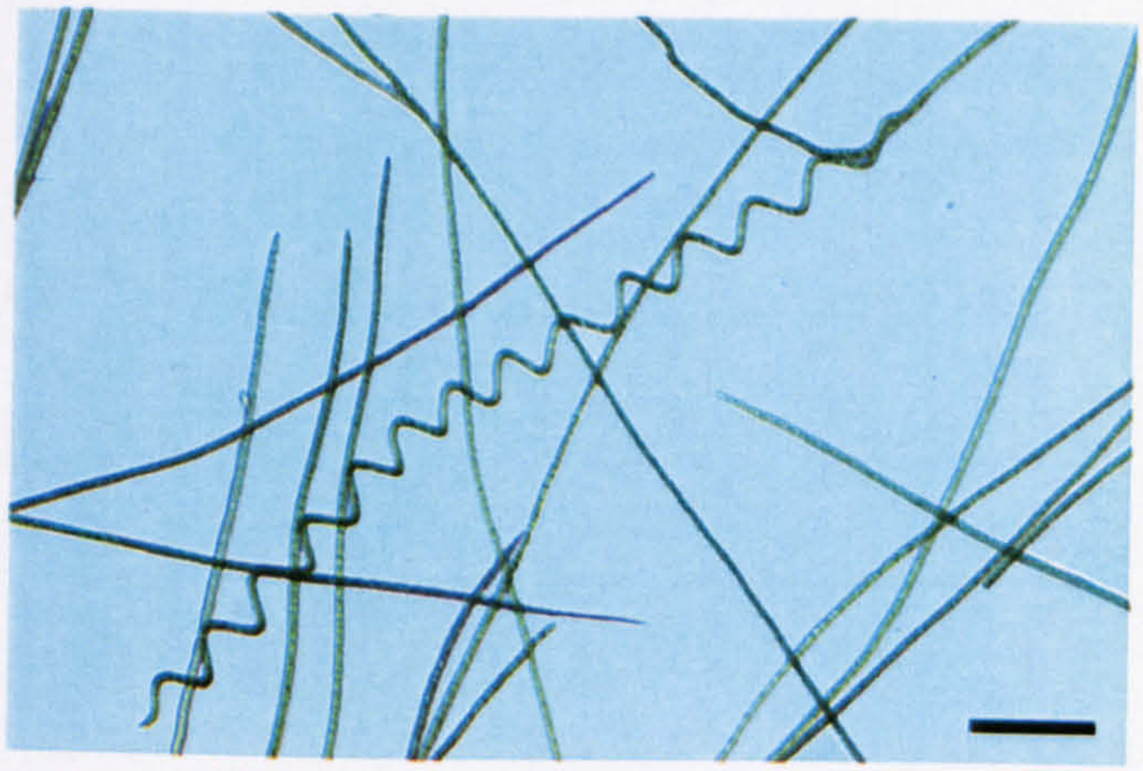


**Fig. 5.3C**

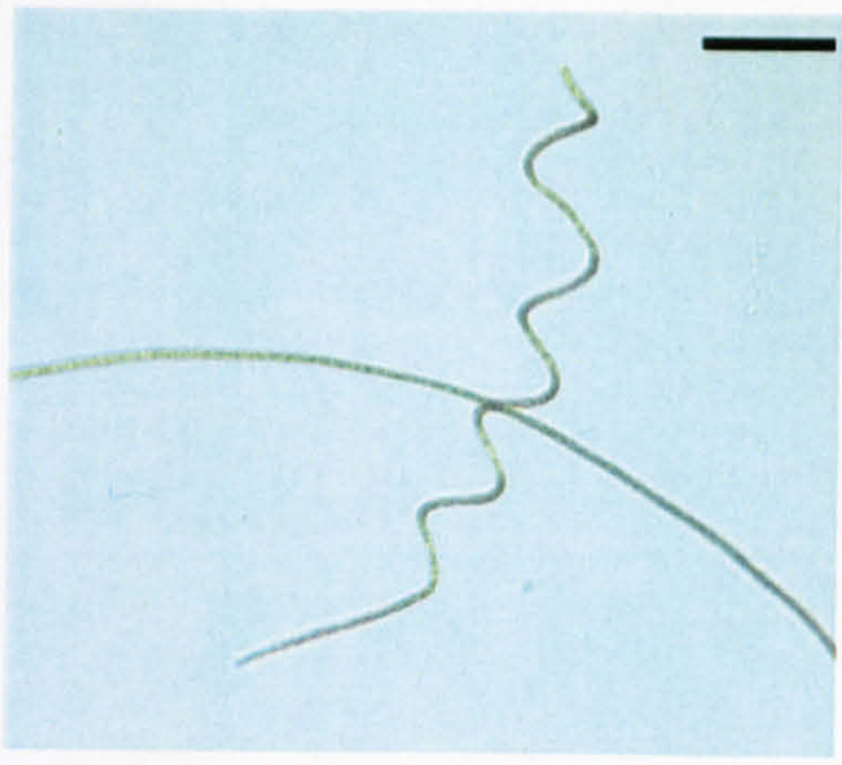


**Fig. 5.3D**

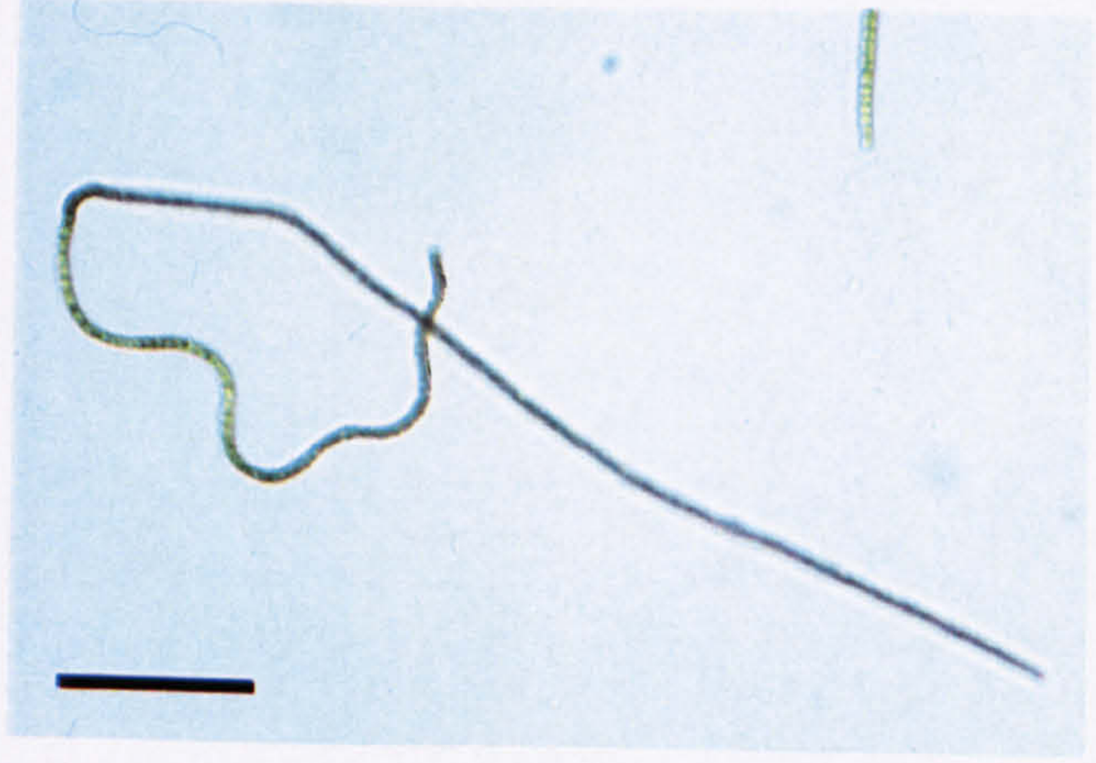
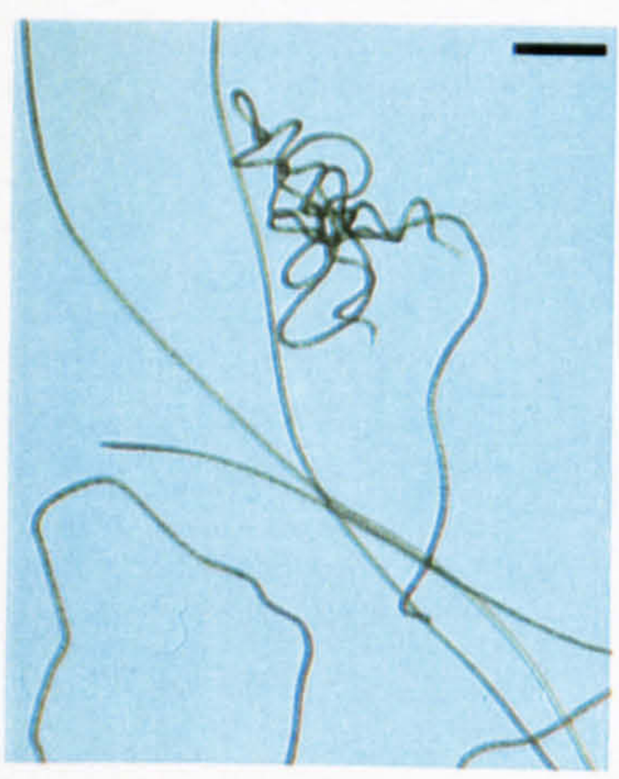


**Fig. 5.3E**



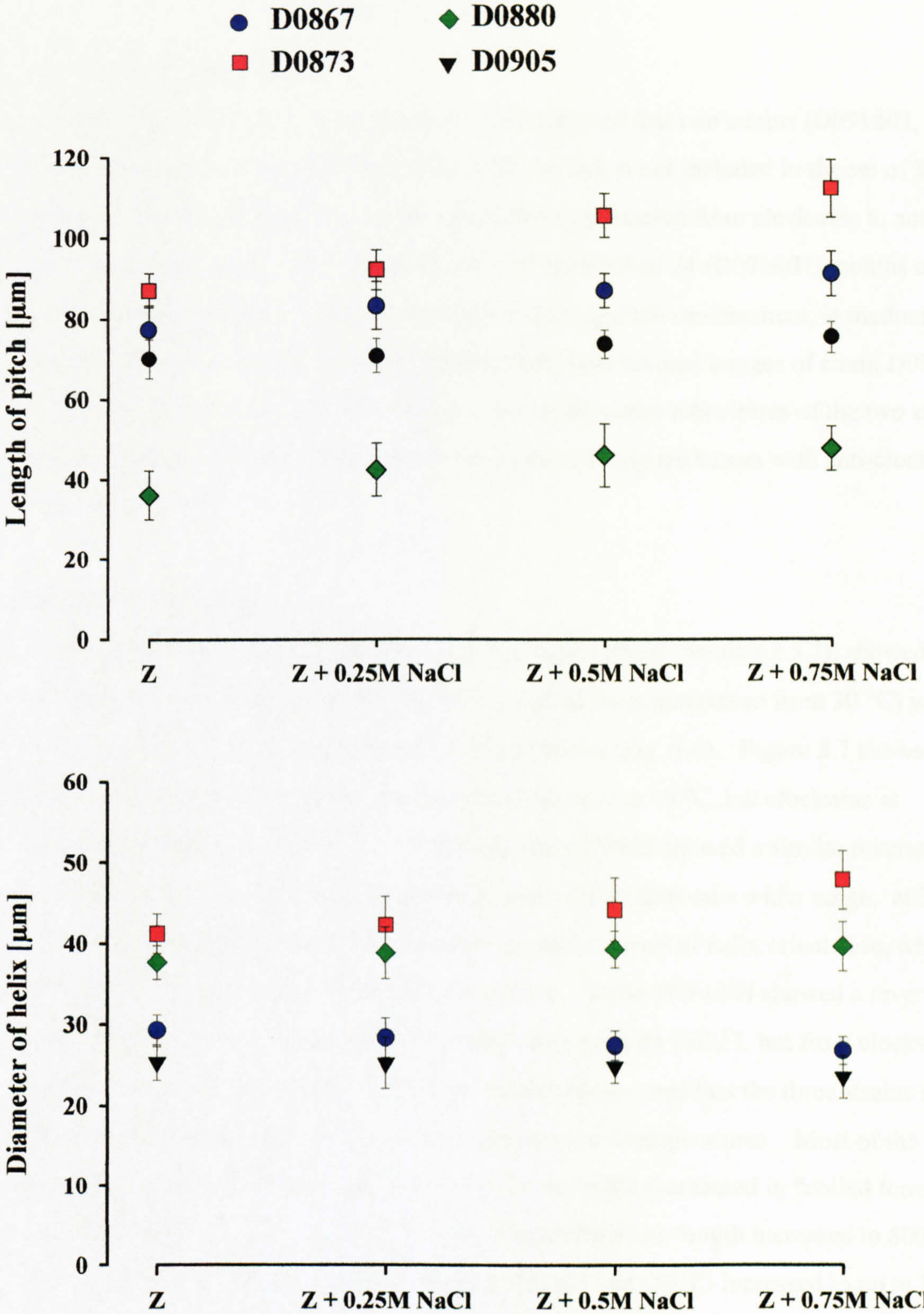



Fig. 5.4 Influence of salinity on helix dimensions.

Cultures of four strains were incubated for seven days at 30 $^{o}$C and 25 µmol photon $m^{-2}$ $s^{-1}$ at the increased salinity. For further details see text.

### 5.2.3.3 Reversal of helix orientation

*Standard culture conditions*

During the time course of the project it was observed that two strains (D0918/H, D0893; the latter strain is an isolate of unknown origin, which is not included in the set of 35 strains or the five duplicate strains) reversed helix orientation from clockwise to anti-clockwise orientation. This was noted after 12 (D0893) or 24 (D0918/H) months of subsequent subculturing without any changes in the growth environment, ie medium, temperature, light intensity. Fig. 5.5 shows three-dimensional images of strain D0893 demonstrating the two helix orientations. Any subsequent subcultures of the two strains examined after the helix reversal occurred, contained only trichomes with anti-clockwise helix orientation.

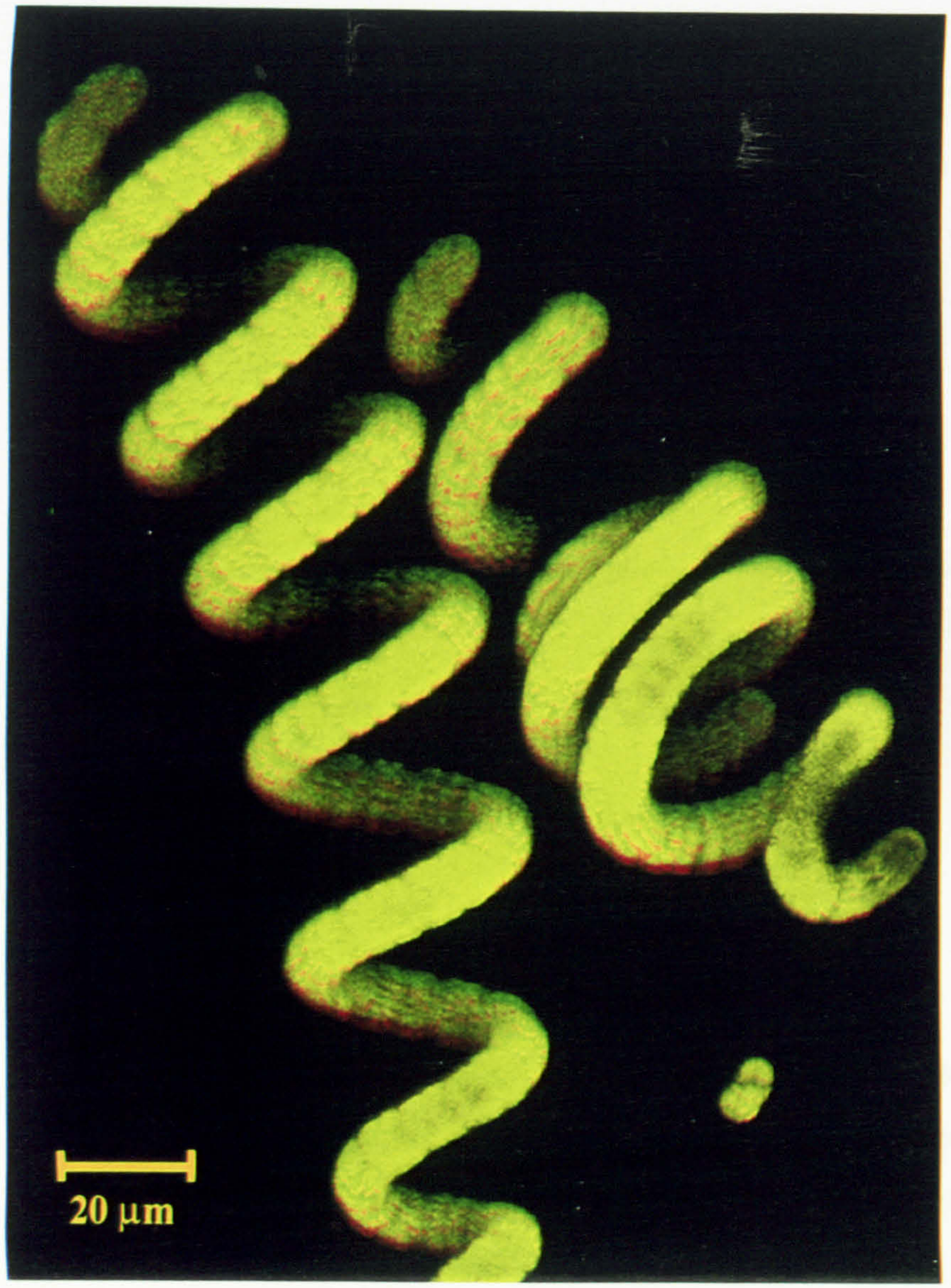
*Influence of temperature*

Three (D0918/H, D0923, D0925) of the ten strains tested (Section 3.5.2), showed a reversal in helix orientation from that in the original inoculum (taken from 30 °C) when grown for 14 days at temperatures of 32 °C and above (Fig. 5.6). Figure 5.7 shows this for strain D0923, where the orientation was anti-clockwise at 30 °C, but clockwise at temperatures above 32 (± 0.5) °C. Although strain D0925 showed a similar reversal, the transition temperature for the helix reversal of this strain showed a wider range: at 32 (± 0.5) °C, approximately 5% of the trichomes showed reversal of helix orientation, whereas at 34 (± 0.5) °C, the value had risen to above 50%. Strain D0918/H showed a reversal of helix orientation at the same transition temperature as strain D0923, but from clockwise to anti-clockwise helix orientation (Fig. 5.6). It should be noted that the three strains showed generally longer trichomes when grown at the increased temperatures. Most of the trichomes of strain D0918/H were up to 500 µm in length (measured in "coiled form") when incubated at 32 °C. At 34 °C, the maximum trichome length increased to 800 µm. The length of most of the trichomes of strains D0923 and D0925 increased to up to 1000 µm when grown at 32 °C and above. Subculturing of the cultures dominated by trichomes with reversed orientation back to standard growth conditions (30 °C) led to the original helix orientation and trichome length being regained.

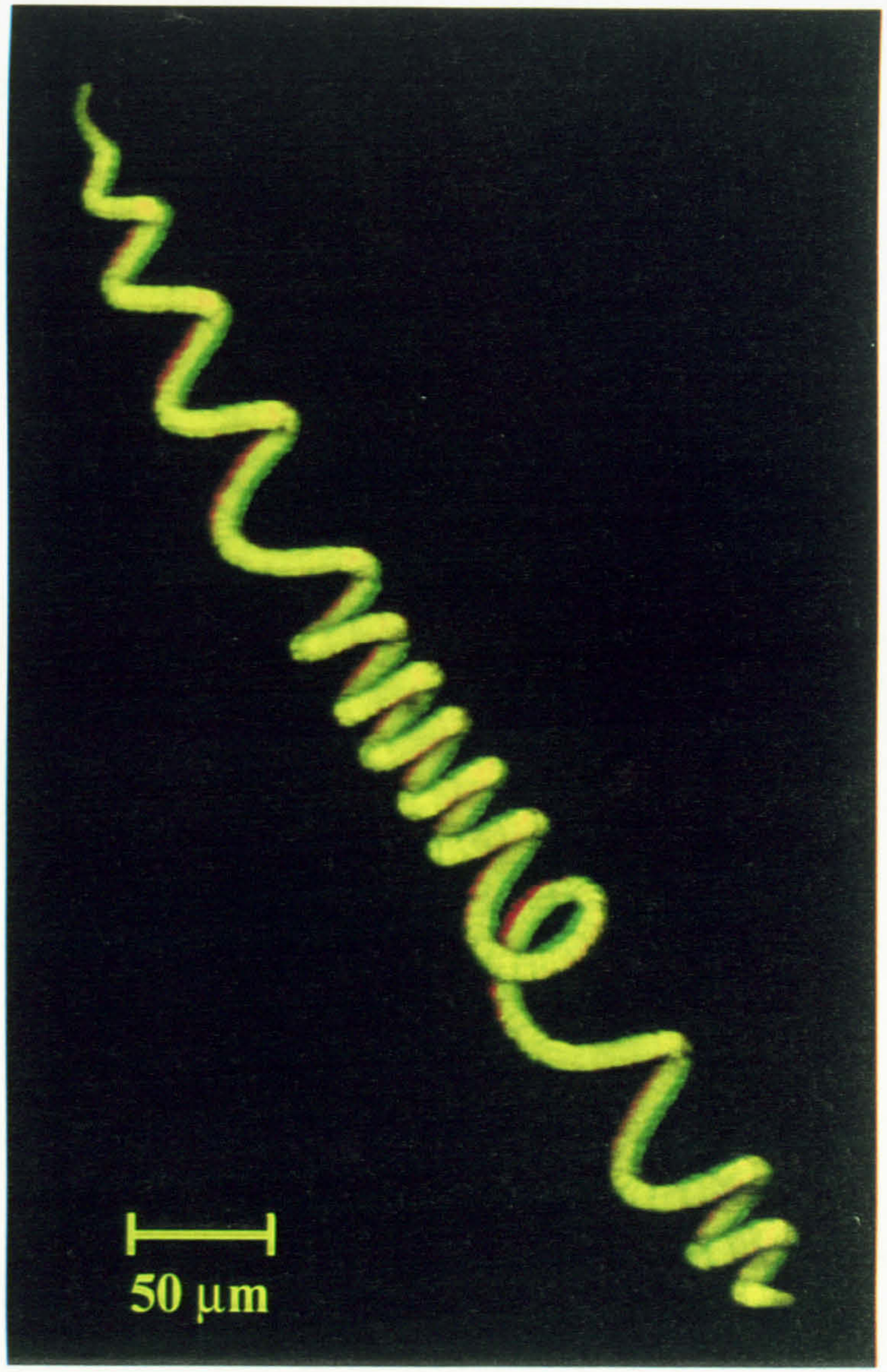
Fig. 5.5 Reversal of helix orientation under standard growth conditions – Strain D0893.

Micrographs of strain D0893 were taken at the start of the project (A) and 24 months later (B). The three-dimensional pictures were produced from 39 (A; clockwise) or 26 (B; anti-clockwise) one-μm sections. Micrograph A was taken by Dr Chris Hawes (Oxford).

A)




B)



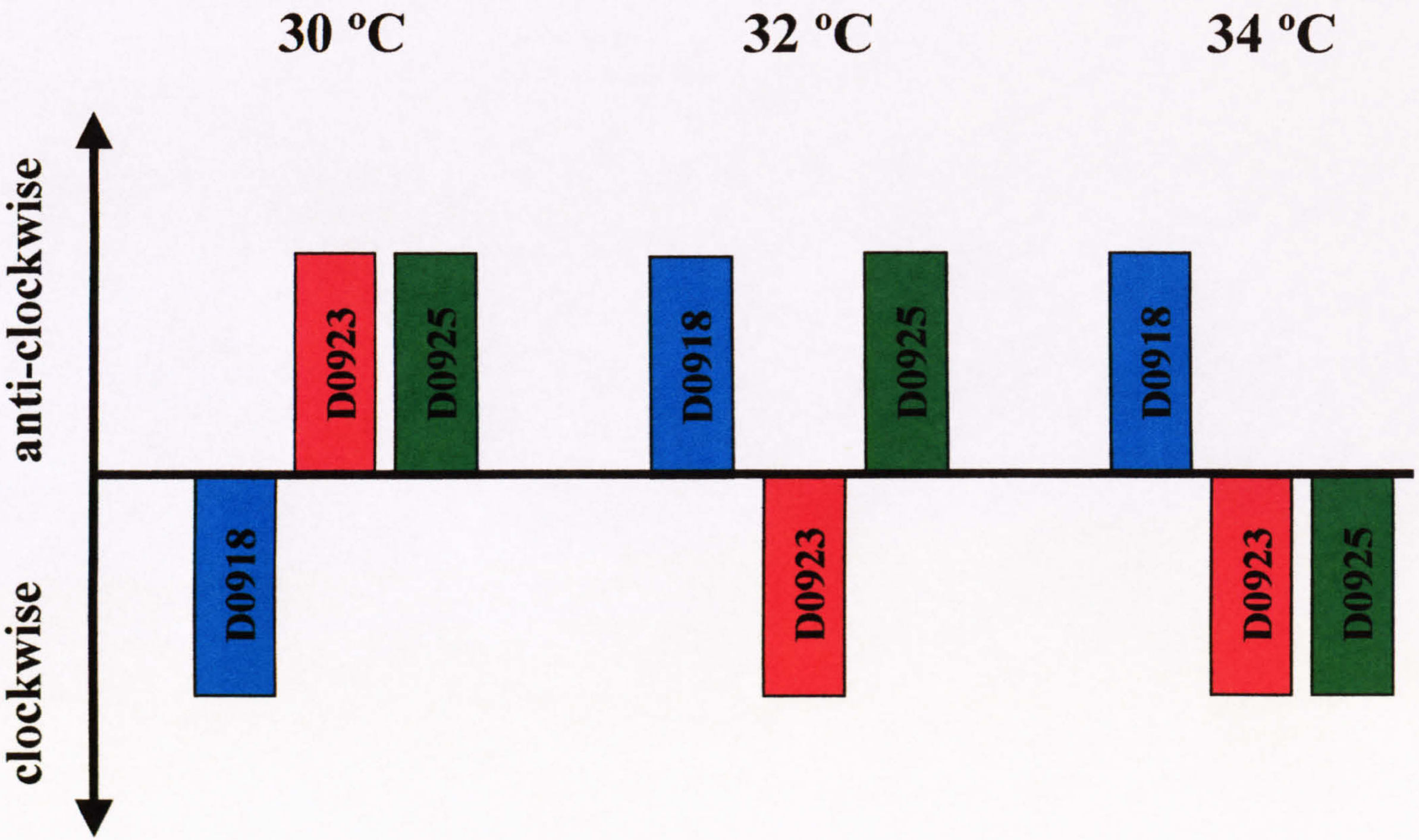


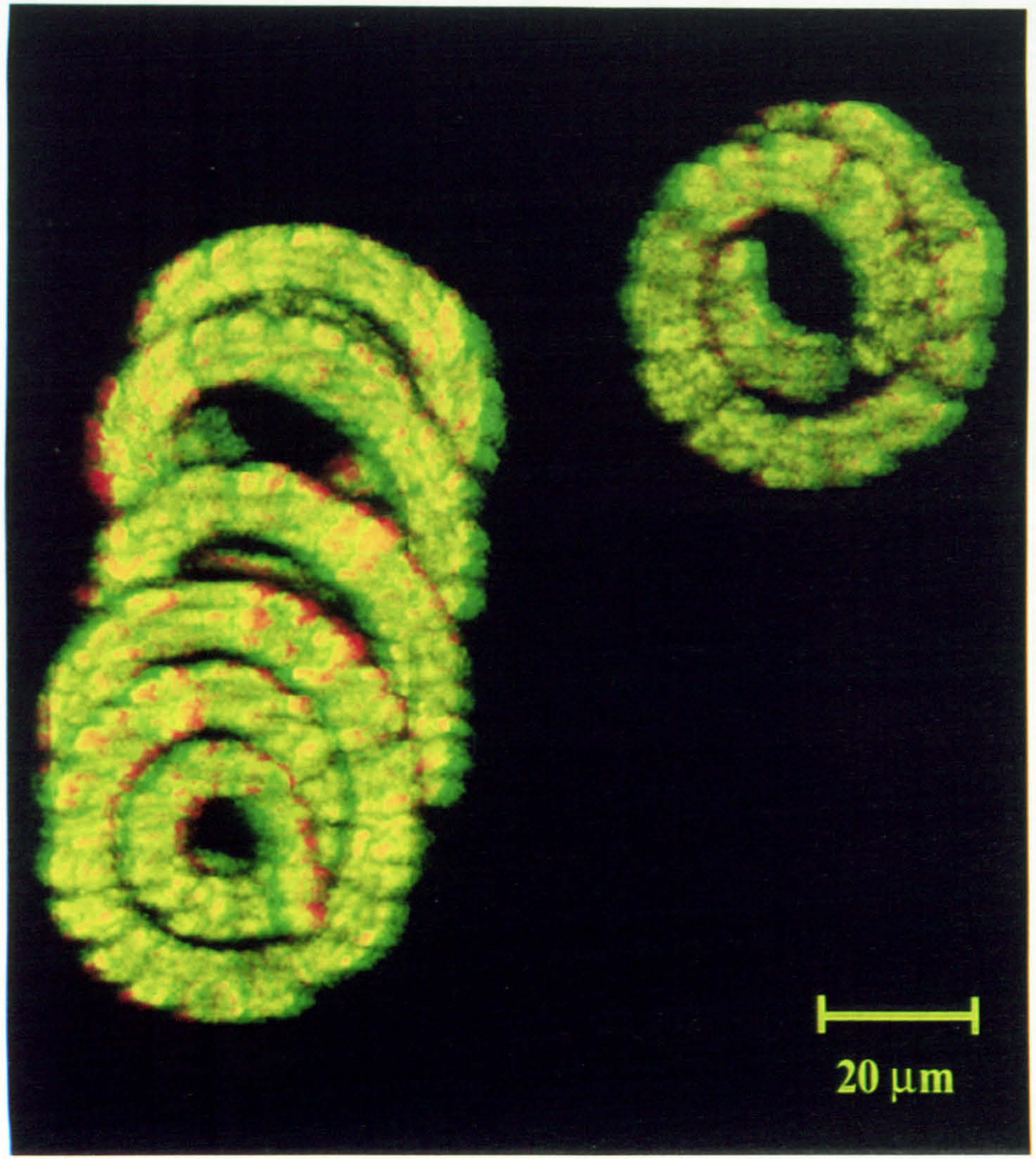

Fig. 5.6 Temperature induced reversal of helix orientation of three *Arthrospira* strains.

After incubation at increased temperatures three strains were found to reverse their helix orientation, ie more than 50% of the trichomes of the culture showed reversed helix orientation.

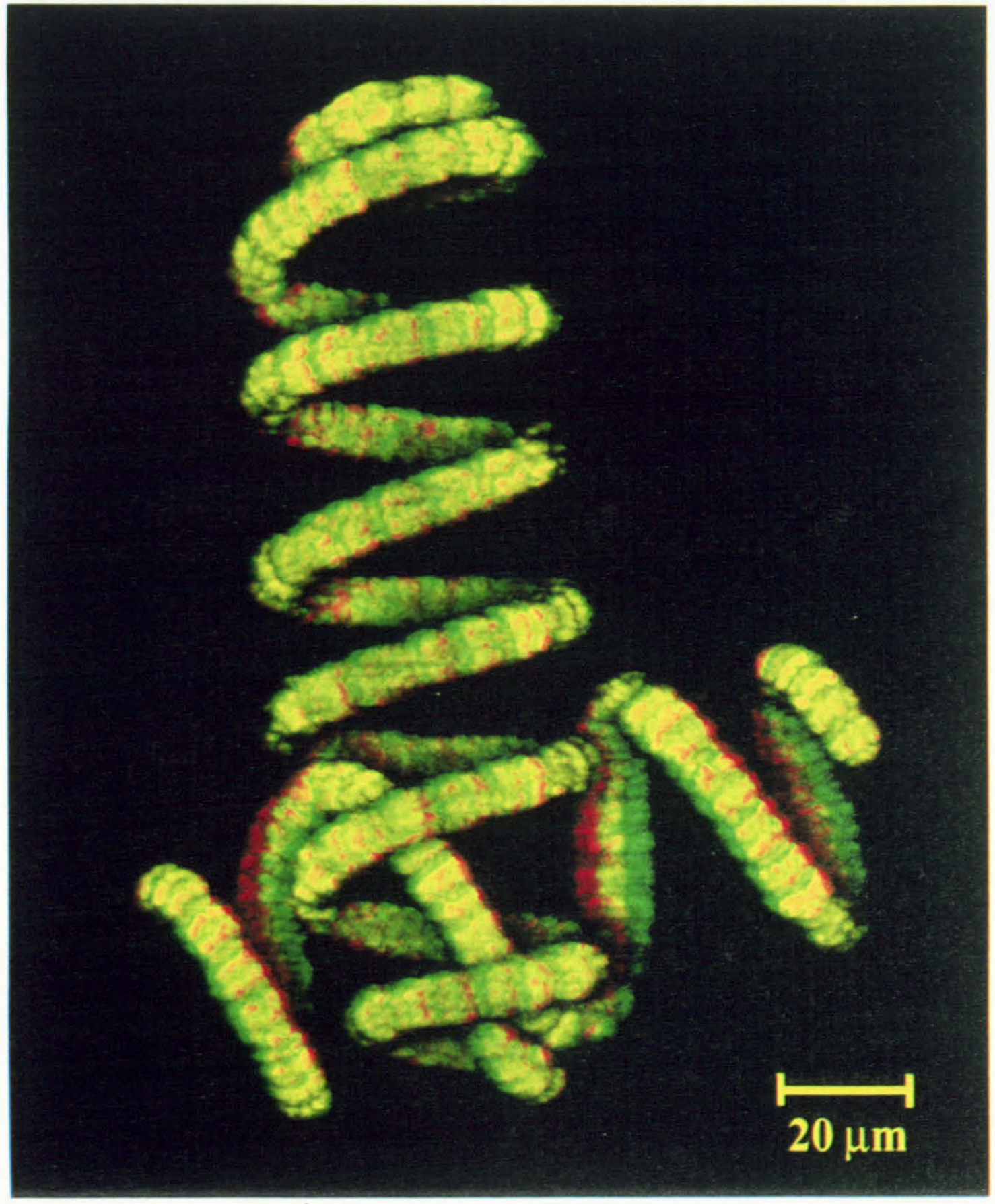
Fig. 5.7 Temperature induced reversal of helix orientation – Strain D0923.

Confocal micrographs of *Arthrospira* sp. strain D0923 grown at 30 °C (A) and 32 °C (B). Note the helix orientation being anti-clockwise at 30 °C and clockwise at 32 °C. The three-dimensional pictures were produced from 24 (A; anti-clockwise) or 40 (B; clockwise) one-µm sections.

A)




B)



The observations of reversal of helix orientation in only three of the ten strains tested led to a thorough check of the helix orientation of all ten strains when growing at 30 °C. The three strains which showed reversal of orientation at the increased temperature were all found to have a few (< 2%) trichomes showing the atypical orientation for the growth temperature of 30 °C, whether or not they had been subcultured once at a higher temperature.

Short-term high-temperature pulse experiments on those *Arthrospira* strains that reversed helix orientation dependent on growth temperature showed that the reversal of helix orientation can be induced also by short-time exposure to the elevated temperatures. In these experiments the time period of the high-temperature pulse was shorter than the time necessary to lead to a reversal of helix orientation at the same increased growth temperature. For example, strain D0923 showed reversal of helix orientation after five to six days of growth at 32 °C (Fig.5.8). A high-temperature (32 °C) pulse of three days followed by incubation at 30 °C led also to helix reversal, but only after incubation for six to seven days at 30 °C (Fig.5.8). However, the yield of trichomes with reversed helix orientation was found to be lower, never reaching 50% of the culture. Therefore, a culture was judged in these experiments as containing trichomes with reversed helix orientation, if more than 30% of the trichomes had reversed their helix orientation. After further incubation at 30 °C the part of the trichomes with reversed helix orientation diminished within the culture and subsequent subcultures did not show trichomes with reversed helix orientation (except a few (< 2%); see above).

There was always a lag between the end of the short-term high-temperature pulse and the initiation of change in orientation to that characteristic for the strain at the increased temperature. However, the longer the temperature pulse at elevated temperature was, the shorter was the time period required to observe the reversal of helix orientation (Fig. 5.8).

Due to changes in helix orientation from clockwise to anti-clockwise of trichomes in the control cultures during incubation (see above), no data are available for strain D0918/H. However, the results with strain D0925 were similar to those of strain D0923. But strain D0925 required higher temperatures (34 °C) for changing helix orientation within the same time period than that required by strain D0923. When grown at 32 °C, only some (<5%) of the trichomes of a culture showed reversal of helix orientation.

To investigate whether newly synthesized proteins are necessary for the reversal of helix orientation, protein synthesis was inhibited using chloramphenicol (40 µM) in Zarrouk's medium. Untreated cultures of strain D0923 changed helix orientation from left to right

after five to six days of growth at 32 °C, while no trichomes of cultures with chloramphenicol added reversed the helix orientation. Chloramphenicol added to cultures of strain D0925 grown at 34 °C led to cell lysis after five to six days of growth. Therefore, no data are available on this strain.

*Influence of mechanical forces*

Culturing of strain D0925 under permanent shaking (120 strokes $min^{-1}$) in a water bath at 30 °C and above led to trichomes showing both helix orientations: clockwise and anti-clockwise with a snag at the point of the helix where the orientation is reversed (Fig. 5.9). No such state was found either in similarly treated cultures of strains D0918/H and D0923 or in cultures of strain D0925 grown without continuous shaking at 32 °C and above. Trichomes showing both helix orientations were always found within aggregates of about 10-100 trichomes. The reversal of orientation leads to a mechanical strain within the trichome helix. It was observed by light-microscopy that this mechanical strain within a trichome is released by breakage of the trichome at the site of the snag leading to two trichomes of opposite helix orientation.

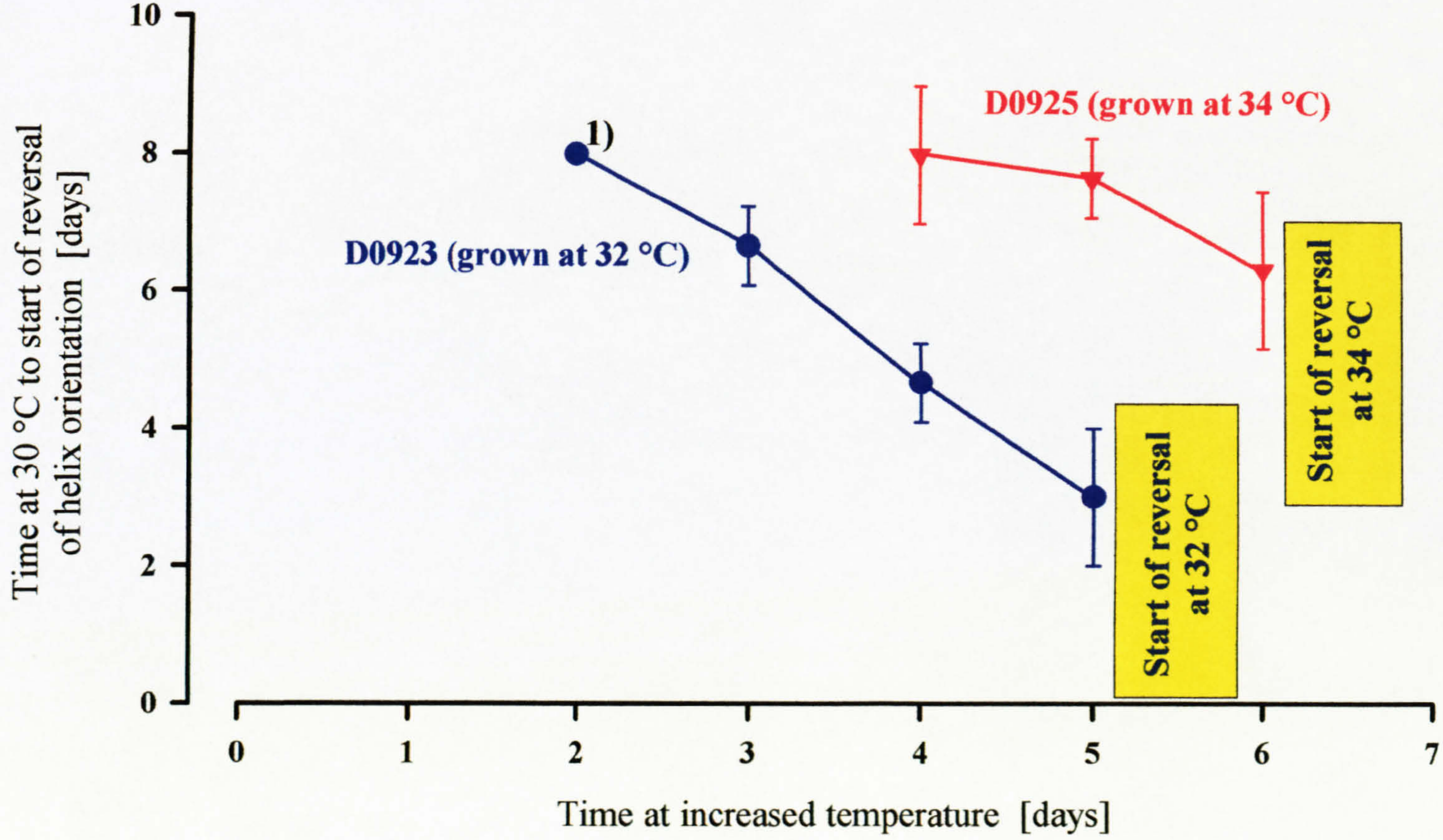



Fig. 5.8 High-temperature pulse induced reversal of helix orientation.

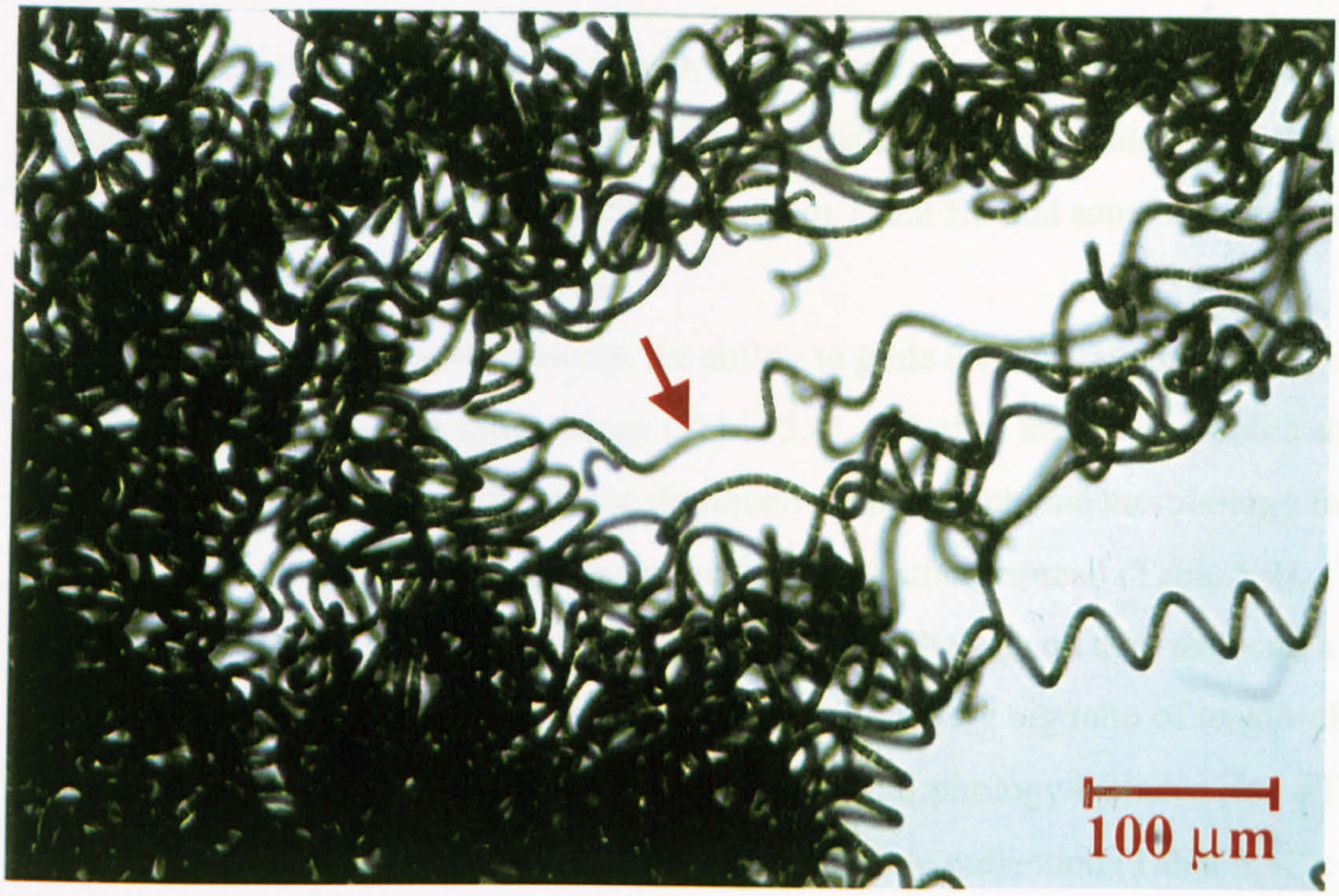
Cultures were exposed to short-term high-temperature pulses (x-axis), followed by incubation at 30 °C (y-axis). The light intensity at which the cultures were grown was 50 µmol photon $m^{-2}$ $s^{-1}$. A culture was judged having reversed helix orientation when more than 30% of the trichomes had reversed helix orientation compared to that at 30 °C. The standard deviation is calculated from the three replicates tested for each day of incubation at increased temperature.

[1)] Only a minor fraction (5-30%) of the population of any of the three replicates showed reversed helix orientation.

Fig. 5.9 Reversal of helix orientation by mechanical forces.

Cultures of strain D0925 were grown in a water bath at 30 °C and 50 μmol photon $m^{-2}$ $s^{-1}$, continuous shaking at 120 strokes $min^{-1}$ for ten days. The arrow (A) indicates the point of the trichomes where the helix orientation reverses ('snag'). B. Snag at higher magnification.

A)




B)



## 5.3 Motility

### 5.3.1 Motility on solidified medium

The macroscopic appearance of growth morphology on agar was found to occur in three major types and, thus, can be scored as one of: (distinct, 'round') colony (14 strains), lawn (13), feathery spread (8). The results are given in Table 5.3 and some examples of the growth pattern are shown in Fig. 5.10.

There is a high correlation between the ability to glide on substrate or to grow into the substrate and the trichome morphology (Table 5.4). Most of the strains, which show a closely coiled and irregular helical (eg dumbbell-shaped) trichome morphology form colonies, the rest grows as a lawn, but none forms a feathery spread (Table 5.4). The eight helical strains which developed a 'feathery spread' morphology on agar show all a regular trichome helix with a wide pitch as shown by their relatively big ratio of length of pitch to diameter of helix (Table 5.4). The 'feathery spread' morphology includes also all nine of the straight morphotypes available at the Durham culture collection (Table 5.3C).

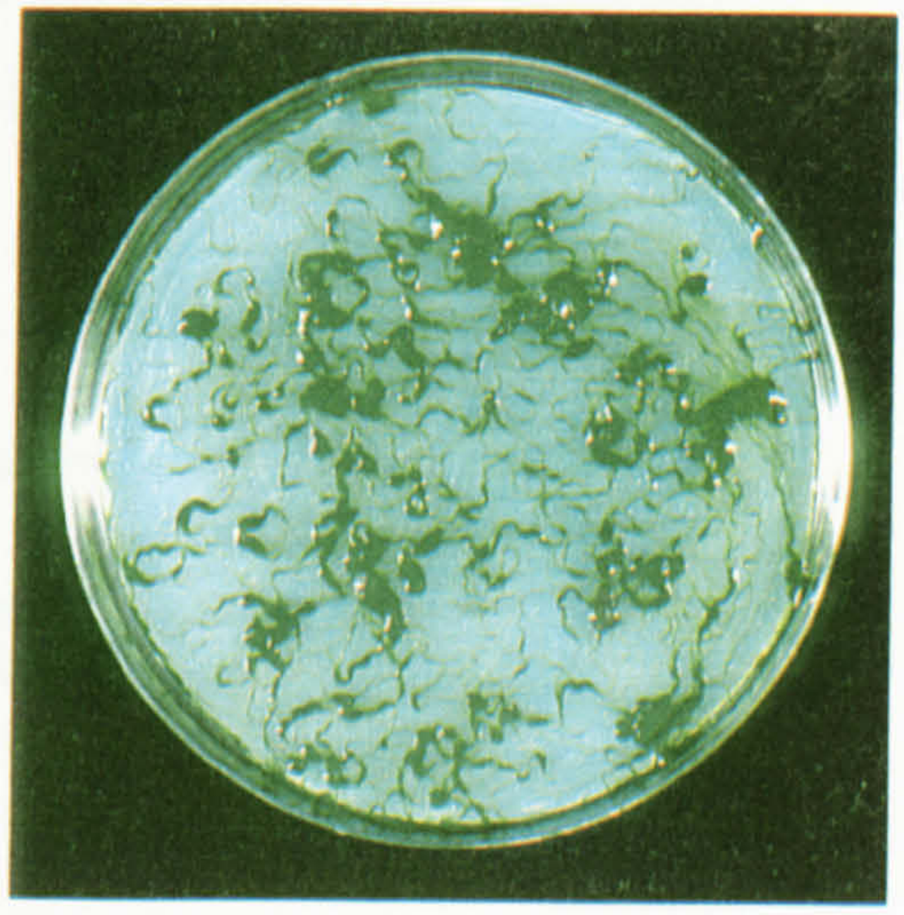
Fig. 5.10 Growth patterns of *Arthrospira* strains on solidified medium.

Cultures were grown on solidified (1% (w/v) agar) 30 °C and 50 μmol photon $m^{-2}$ $s^{-1}$. The macroscopic appearance of growth morphology was found to occur in three major types: (distinct) colony (D, D0884), lawn (C, D0916) or feathery spread (E, D0881). The straight and helical morphotypes of a strain showed different growth patterns (A, D0918/H; B, D0918/S). Some strains grew into the agar (F, D0881, photograph taken from the bottom of the petri dish). Photographs were taken 15 days after inoculation at 30 °C and 25 μmol photon $m^{-2}$ $s^{-1}$.

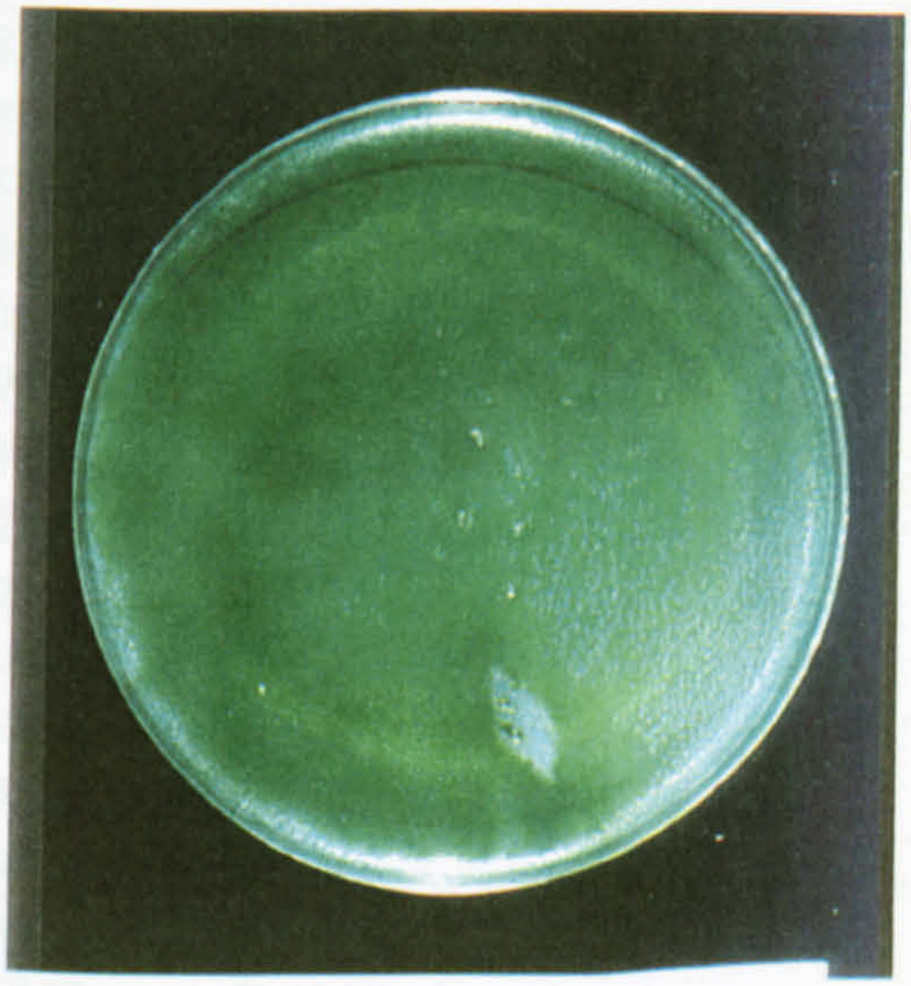
A)



B)



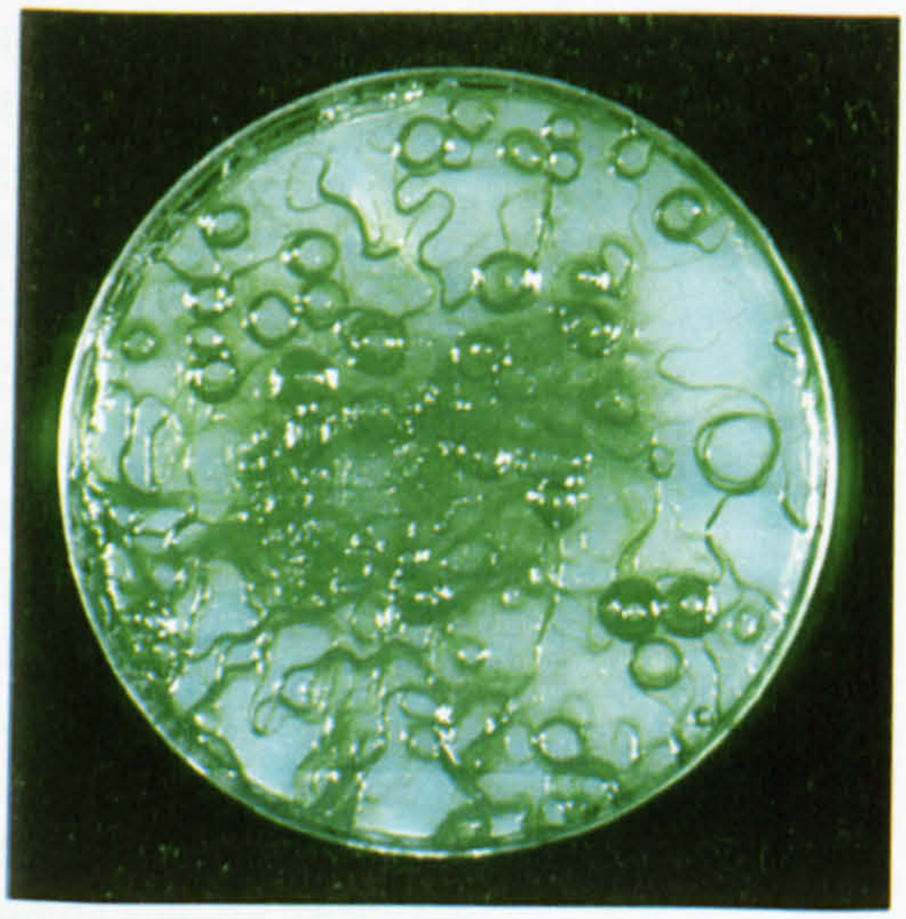
C)



D)



E)



F)



Table 5.3 Results of screening for motility of *Arthrospira* strains on solidified medium. Cultures were grown on Zarrouk's medium (1% (w/v) agar) at 30 °C and 25 µmol photon $m^{-2}$ $s^{-1}$ for 15 days. Two replicates were investigated and the results were confirmed by an independent repeat experiment with two replicates. (Abbreviations: C = colony, L = lawn, FS = feathery spread.)

5.3A Variation between strains.

| | Trichome morphology | Helix | Type of helix attenuation | | Growth on solidified (1% (w/v) agar) medium | |
|---|---|---|---|---|---|---|
| *Strains* | Helical or straight | Fusi-form | Fast diminishing | Slowly diminishing or no attenuation [1)] | Growth pattern | Growth into agar |
| D0867 | H | - | + | - | L | + |
| D0872/H | H | + | + | - | C | - |
| D0873 | H | + | - | + | L | - |
| D0880 | H | + | - | + | L | - |
| D0881 | S | - | - | + | FS | + |
| D0882 | S | - | - | + | FS | + |
| D0884 | H | + | + | - | C | - |
| D0885/H1 | H | - | - | + | FS | + |
| D0890 | H | - | - | + | L | - |
| D0891 | H | + | + | - | C | - |
| D0895 | H | - | - | + | L | + |
| D0896 | H | - | - | + | FS | + |
| D0897 | H | + | + | - | C | - |
| D0899 | H | + | + | - | L | - |
| D0900 | H | - | - | + | C | - |
| D0904 | H | - | - | + | FS | - |
| D0905 | H | - | - | + | FS | + |
| D0907 | H | - | - | + | L | + |
| D0909 | H | - | + | - | C | - |
| D0910/H | H | - | - | + | C | - |
| D0911 | H | - | - | + | C | - |
| D0913 | H | + | - | + | L | - |
| D0914 | H | + | + | - | L | - |
| D0915 | H | - | - | + | L | - |
| D0916 | H | - | - | + | L | + |
| D0918/H | H | + | + | - | C | - |
| D0919 | H | + | + | - | C | - |
| D0920 | H | + | + | - | C | - |
| D0921 | H | - | - | + | FS | + |
| D0922 | H | + | + | - | L | - |
| D0923 | H | + | + | - | C | - |
| D0925 | H | + | + | - | C | - |
| D0929 | H | + | - | + | C | - |
| D0930 | H | - | - | + | FS | + |
| D0933 | H | - | - | + | L | - |

[1)] including straight trichomes

Table 5.3B Comparison between duplicate strains. The strain of each set of duplicates, that is included in the set of 35 strains (Table 5.3A), is indicated in bold letters.

| | Trichome morphology | Helix | Type of helix attenuation | | Growth on solidified (1% (w/v) agar) medium | |
|---|---|---|---|---|---|---|
| *Strains* | Helical or straight | Fusi-form | Fast diminishing | Slowly diminishing or no attenuation [1)] | Growth pattern | Growth into agar |
| **D0873** | **H** | **-** | **-** | **+** | **L** | **-** |
| D0879 | H | - | - | + | L | - |
| **D0880** | **H** | **+** | **-** | **+** | **L** | **-** |
| D0887 | S | - | - | - | FS | + |
| D0906/H | H | + | + | + | L | - |
| D0875 | H | - | - | + | C | - |
| D0876 | H | - | - | + | C | - |
| **D0911** | **H** | **-** | **-** | **+** | **C** | **-** |

[1)] including straight trichomes

Table 5.3C Variation between different morphotypes. The strain of each set of duplicates, that is included in the set of 35 strains (Table 5.3A), is indicated in bold letters.

| | Trichome morphology | Helix | Type of helix attenuation | | Growth on solidified (1% (w/v) agar) medium | |
|---|---|---|---|---|---|---|
| *Strains* | Helical or straight | Fusi-form | Fast diminishing | Slowly diminishing or no attenuation [1)] | Growth pattern | Growth into agar |
| **D0872/H** | **H** | **+** | **+** | **-** | **C** | **-** |
| D0872/S | S | - | - | - | FS | + |
| **D0885/H1** | **H** | **-** | **-** | **+** | **FS** | **+** |
| D0885/H2 | H | - | - | - | FS | + |
| D0885/S | S | - | - | - | FS | + |
| D0906/H | H | + | - | + | C | - |
| D0906/S | S | - | - | - | FS | + |
| **D0910/H** | **H** | **-** | **-** | **+** | **C** | **-** |
| D0910/S1 | S | - | - | - | FS | + |
| D0910/S2 | S | - | - | - | FS | - |
| **D0914/H** | **H** | **+** | **+** | **-** | **L** | **-** |
| D0914/S | S | - | - | - | FS | + |
| **D0918/H** | **H** | **+** | **+** | **-** | **C** | **-** |
| D0918/S | S | - | - | - | FS | - |

[1)] including straight trichomes

The comparison of the sets of duplicate strains shows similar results for those morphotypes, which show similar helix morphology. If the duplicate strain, however, shows different helix morphology, the macroscopic appearance also changes, thus confirming the correlation between helix morphology and growth pattern on agar as described above (Table 5.3B).

Table 5.4 Comparison of the macroscopic appearance of growth morphology on solidified medium with characters of the helical trichome morphology. The data is based on the set of 35 *Arthrospira* strains. (Duplicate strains or different morphotypes of a strain are not included in the analysis.)

| | | Trichome morphology | | | Type of helix attenuation | | Ratio of length of pitch to diameter of helix | |
|---|---|---|---|---|---|---|---|---|
| | Total | Growth into agar | Helical | Straight (of 2) | Fusi-form | Fast diminishing | Slowly diminishing or no attenuation [1)] | <2 | >2 |
| Colony | 14 | 0 | 14 | 0 | 10 | 12 | 2 | 12 | 1 |
| Lawn | 13 | 2 | 13 | 0 | 6 | 4 | 9 | 8 | 5 |
| Feathery Spread | 8 | 8 | 6 | 2 | 0 | 8 | 8 | 0 | 8 |

[1)] including straight trichomes

## 5.3.2 Motility in liquid medium

Assessing motility of cyanobacteria in liquid medium is difficult. However, it was observed that two strains (D0904, D0905) formed aggregates in liquid medium under any of the growth conditions tested (5 to 70 µmol photon $m^{-2}$ $s^{-1}$ ; 20 to 30 °C). These aggregates were formed each time after dispersion of the filaments (which proved rather difficult due to the dense clumps) or inoculation of fresh medium with a small aliquot of dispersed filaments. Other strains, which were found to form sometimes aggregates (eg when culture reached stationary phase of growth or was exposed to high light stress), did generally not grow in clumps during the phase of fast growth under 'medium' light (20 to 40 µmol photon $m^{-2}$ $s^{-1}$) and temperature (30 °C) conditions. The two aggregating strains showed always very long trichomes (Section 5.2.1) and high motility on solidified growth medium (Section 5.4.1).

## 5.4 Discussion

### 5.4.1 Morphological characters

#### 5.4.1.1 Characters and character states

The screening of the set of *Arthrospira* strains revealed several morphological characters useful for taxonomic purposes (eg helix characters), while others did not show variation between strains (eg gas vacuoles) or too great a variation within a culture (eg cell length).

Trichome morphology

Members of the genus *Arthrospira* are defined as filamentous cyanobacteria with helical trichome morphology (Section 1.2.1). Therefore, it can be assumed that the *Arthrospira* strains, which were obtained as subcultures containing straight trichomes only, are derived from stocks with originally helical trichomes. Furthermore, the shift of a culture containing only straight trichomes back to a culture containing mainly helical trichomes was not observed (Section 5.2.3.1). Therefore, the tendency of strains to loose the helical trichome morphology represents a stable character.

Helix orientation at 30 °C

The helix orientation of *Arthrospira* strains was found to be affected by genetic drift (Section 5.2.3). However, the clockwise helix orientation of three strains (D0867, D0872/H, D0891) proved to be stable over a time period of three years. Filaments showing the opposite helix orientation were not observed in any of their subcultures. Using the helix orientation as a character, these three strains can be distinguished from the rest of the strains.

Trichome length

Hindák (1985) suggested that trichomes with fusiform helix morphology, which are characteristic for *Arthrospira fusiformis*, are of limited length. Although the comparison of trichome length and fusiform helix morphology does not prove otherwise, it also does not confirm this statement.

Cell dimensions

Ratio of cell length to width of cyanobacterial cells has been thought to be a "notoriously untrustworthy diagnostic feature" (Komárek & Lund, 1990), which is mainly due to the fact that the cell length depends on the stage of the cell cycle. The measurement of a large number of individual cells may not always compensate for this. The cell width (= trichome diameter), however, represents a more reliable character.

Helix characters

Data on helix characters have been analyzed for taxonomic purposes in different ways. Firstly, the values obtained describe the sizes of the helix pitch and diameter. These values varied between trichomes of a culture, for some strains even considerably, and overlapped with values obtained for other strains, though they still discriminate between strains in several cases when used for identification purposes. The ratio of length of pitch to helix diameter provides an other means to describe the size of the trichome helix, also showing variation between strains. Thirdly, the variation of the ratio of helix pitch and diameter (ratio of lowest to highest ratio) provides a value that represents the overall variation in the helical trichome morphology of a strain and can also be used for identification purposes. While the helix dimensions, as well as the ratio of helix pitch to helix diameter have been used previously as morphological markers, the analysis of the variation in the ratio of helix pitch to helix diameter as carried out here, has never been analyzed before for *Arthrospira* strains.

The type of helix attenuation towards the apices shows a high correlation to the type of the helix of a trichome. Fusiform trichome helices generally show a wider helix diameter towards the apices prior to a fast diminishing (attenuating) helix, while regularly helical trichomes show a slowly diminishing helix towards the their apices.

Strain D0918/H is an exception concerning its type of helix it forms. When grown under 30 °C this strain shows generally a trichome length of 50 to 100 µm representing one to three helical turns only, thus does not allow identification of the type of trichome morphology. Trichomes of cultures grown at 32 °C or above, however, show longer trichomes (up to 500 µm) with fusiform and dumbbell-shaped trichome morphology. Therefore, for strain D0918/H the fusiform and dumbbell-shaped morphology is regarded as the morphology under standard condition.

End cell shape

The shape of the end cells of a trichome has generally been used as a taxonomic marker for *Arthrospira* strains (eg Desikachary & Jeeji Bai, 1996). Desikachary and Jeeji Bai (1992) based the introduction of the species *Arthrospira indica* on the presence of a calyptra, which distinguishes this species from *S. fusiformis* Woron. However, care must be taken when using this character, as sometimes only a very small fraction of a culture shows a calyptrate end cell. This may be due to the fact that reproduction mainly takes place via breakage of a longer trichomes into shorter ones without necridium formation. Necridium formation, which is thought to be the pre-requisite of a calyptrate end cell (J. Komárek, pers. comm.), was observed to occur rather rarely, but in any of the strains. Similarly, the formation of a new capitate end cell may require some time. Therefore, many trichomes of a culture may not show capitate end cells as they are in a stage shortly after trichome fragmentation or hormogonium formation.

The description of an end cell as either round or conical may be in many cases a matter of personal judgement. Therefore, the end cell shape is described as either round/conical, capitate or calyptrate.

Gas vacuoles

According to Komárek and Lund (1990), *Arthrospira platensis* and *A. jenneri* are benthic species and do not possess gas vacuoles, a view first described by Fott and Karim (1973) and confirmed by Hindák (1985). Although six of the 35 strains examined here were obtained as the botanical species *Arthrospira* (*Spirulina*) *platensis*, all strains examined in this project have gas vacuoles. There are, however, differences between strains in the location of the majority of gas vacuoles within the cell. While the gas vacuoles of many strains are equally distributed within the cell, several strains have gas vacuoles accumulated at the cross walls.

Granules at cross-walls

Granules as observed by light microscopy represent probably mainly polyphosphate bodies. Their size and abundance is dependent on the culture medium. As, however, all strains were cultured over many subcultures in the same medium, it is assumed that the occurrence of these granules has "stabilized" and represents, therefore, a reliable taxonomic character.

#### 5.4.1.2 Two morphological clusters

Helical trichome characters have been the characters mainly used for species descriptions (eg Gomont, 1892). The morphological data for the 35 *Arthrospira* strains indicates two main taxonomic groups based on the helix characters, one with irregular (fusiform, dumbbell- or barrel-shaped), and one with a regular helix pattern. The fusiform state may be visible only in a few trichomes of a culture in the fast growing phase. The attenuation of the trichome helix is partially a result of the helical morphology, thus showing high correlation with the type of helix.

Generally, the fusiform or dumbbell- or barrel-shaped helix type shows more variation of the dimensions of the helical trichome than does the regular helix type, a feature also attributed to *Spirulina fusiformis* Woron. (Hindák, 1985).

### 5.4.2 Helical trichome morphology

#### 5.4.2.1 Loss of helical trichome morphology

*Influence of osmosis on trichome morphology*

Previous reports have shown that increasing levels of NaCl (from 17 mM (Zarrouk's medium) to 86 mM final concentration) lead to straightening of the helical trichome morphology of *Arthrospira* strains (Watanabe & Ichimura, 1977). To investigate whether higher osmotic pressures lead to straighter trichomes, *Arthrospira* cultures were grown in high NaCl-environments. The results of the study confirm, that osmosis leads to a straightening of trichomes. However, the helix morphology of the trichomes was found to recover to the original one, when cultured again in Zarrouk's medium without additional NaCl. Therefore, osmosis is not responsible for the stable occurrence of straight trichomes in cultures of otherwise helical trichomes. The reason for the straightening of the helical trichomes are unknown, but may be a direct result of osmotic pressure. The accumulation of osmoprotective substances inside the cell upon osmotic up-shock (Warr et al., 1985) may lead to increased mechanical pressure to the cell wall. This may then lead to lower degree of helicity of each individual cell of a trichome, thus resulting in an overall straightening of the trichome helix.

Observations of straightening of trichomes of *Arthrospira* strains were also made in old (stock) cultures. Evaporation of part of the medium and accumulation of potentially osmotic active substances (eg polysaccharides) excreted from the cells may be the responsible factors in those cases.

The influence of increasing NaCl concentrations on a (non-axenic) culture of *Spirulina subsalsa* (D0869) was also investigated. No straightening of the helical trichome was observed. In contrast to *Arthrospira* cultures, which showed decreasing growth with increasing NaCl concentrations, best growth of *Spirulina subsalsa* was observed when 0.25M NaCl (final concentration) was added to the growth medium. Gabbay and Tel-Or (1985) suggested that the marine *Spirulina subsalsa* is "halophilic rather than halotolerant", while increasing salinity was found to decrease the growth of *Arthrospira* strains (Vonshak, 1997).

*Loss of helical trichome morphology by mutation*

In contrast to these environmentally induced, temporary changes of helical trichome morphology, the culture history of *Arthrospira* strains at Durham Culture Collection, indicates that the loss of helical trichome morphology in some strains represents a mutational event. All nine clonal cultures of straight *Arthrospira* mutants, maintained at Durham University, proved stable during a culture period of up to three years. D0885/S was the only strain that showed some degree of reversion to helical trichome morphology through, presumably, back mutation (Fig. 5.3). Most of those trichomes showed both, helical and straight parts. In addition, the helix of the trichome was not regular but distorted. These observations may indicate that steric factors are also important for the formation of a helical trichome. The peptidoglycan is the shape maintaining layer in the prokaryotic cell wall (Höltje, 1998). The "helical type" of peptidoglycan has to be incorporated in the cell wall of sufficient cells of a straight trichome for the reversion to helical morphology to take place. This is thought to be necessary to overcome the steric hindrance of a straight trichome and to cause it (or a part of it) to become helical. It is assumed that the cells incorporating the helical type of peptidoglycan have to be neighbours or the cell with the helical cell wall type has to divide to produce sufficiently neighbours with the same helical cell wall. An alternative explanation would be the assumption that the trichome with the helical cell morphology is part of a hormogonium, in

which case no strong steric forces are to overcome. The latter may explain the presence of a few regularly helical trichomes (Fig. 5.3).

There has been only one report so far that notes the formation of helical *Arthrospira* trichomes from straight ones. Fox (1996) reported that sonication of a culture of straight trichomes followed by exposure to high light intensity resulted in the occurrence of helical trichomes. This observation supports the hypothesis presented above. Sonication leads to fragmentation of trichomes, possibly even resulting in single cells. One or more of these single cells, or cells within short trichome fragments, may posses the helical cell wall type due to back mutation, and subsequently grow to helical trichomes. Sonication, thus increases the likelihood of formation of helical trichomes by supporting factors that help to overcome the steric integrity of a (straight) trichome.

The steric integrity of the trichome may also be responsible for the fact that back mutation of straight to helical trichome morphology is observed less often than the loss of the helical morphology. In addition, the likelihood of the occurrence of helical trichomes in cultures of the straight morphotype of *Arthrospira* strains is also diminished by the faster growth of the straight morphotype compared to the helical one (Section 5.2.3.1).

### 5.4.2.2 Reversal of helix orientation

The observations that some *Arthrospira* strains reverse their helix orientation due to genetic drift, mechanical forces or upon temperature upshift has not been reported previously. It seems likely that there are more environmental factors which influence the helix orientation. However, further research in this area would be beyond the scope of this project.

*Genetic drift*

The reversal of helix orientation under constant growth conditions indicates that the changes in helix morphology were due to genetic drift. The fact that none of the subsequent subcultures of strains D0893 and D0918/H contained trichomes with the original helix orientation strengthens the explanation of genetic drift and indicates that the mutation is stable.

Only four of the 35 *Arthrospira* strains screened originally showed clockwise helix orientation. This, and the fact that the two strains that were found to have changed their

helix orientation (due to genetic drift), reversed their helix orientation from originally clockwise to anti-clockwise, may indicate that there is some selective pressure on the loss of the clockwise helix orientation. Favre et al. (1985) suggested a 'left-hand-twist' protein of the *Bacillus subtilis* mutants was responsible for the helix reversal to clockwise helix orientation when proteases were added to the growth medium. Similarly, a 'right-hand-twist' protein may be necessary in *Arthrospira* strains to maintain clockwise helix orientation, and which is sometimes lost through genetic drift.

*Temperature-induced reversal*

In contrast to the helix reversal by genetic drift, the temperature-induced helix reversal represents a temporary, environmentally induced change, which is reversed again upon exposure to the original growth conditions.

Very few (< 2%) trichomes with opposite helix orientation were observed in any subculture (and stock culture) of those three strains, that showed temperature-induced reversal of helix orientation. In contrast, cultures of any of the other 30 strains with helical trichome morphology never contained trichomes with different helix orientation. Therefore, it can be assumed that strains D0918/H, D0923 and D0925 represent the only strains of the 33 helical *Arthrospira* strains that are able of helix reversal.

*High-temperature pulse induced reversal*

Temperature pulse induced "memory" for helix orientation has been described for several helical filamentous mutants of *Bacillus subtilis* (Favre et al., 1985). Although the two *Arthrospira* strains (D0923, D0925) need longer time periods for changes to take place, the results on high-temperature pulse induced changes in orientation are similar to the results of Favre et al. (1985), and can be interpreted on the basis of the currently accepted model of cell wall synthesis (Fig. 5.11). The shape determining wall layer of prokaryotes is the peptidoglycan layer and in gram-positive bacteria and cyanobacteria this consists of many individual layers (Archibald et al., 1994, Jürgens et al., 1983). During cell growth new layers are added at the side of the cytoplasmatic membrane and old wall layers are being degraded (Doyle et al., 1988). Short-term incubation at increased temperature may cause either molecular changes in the structure of the peptidoglycan

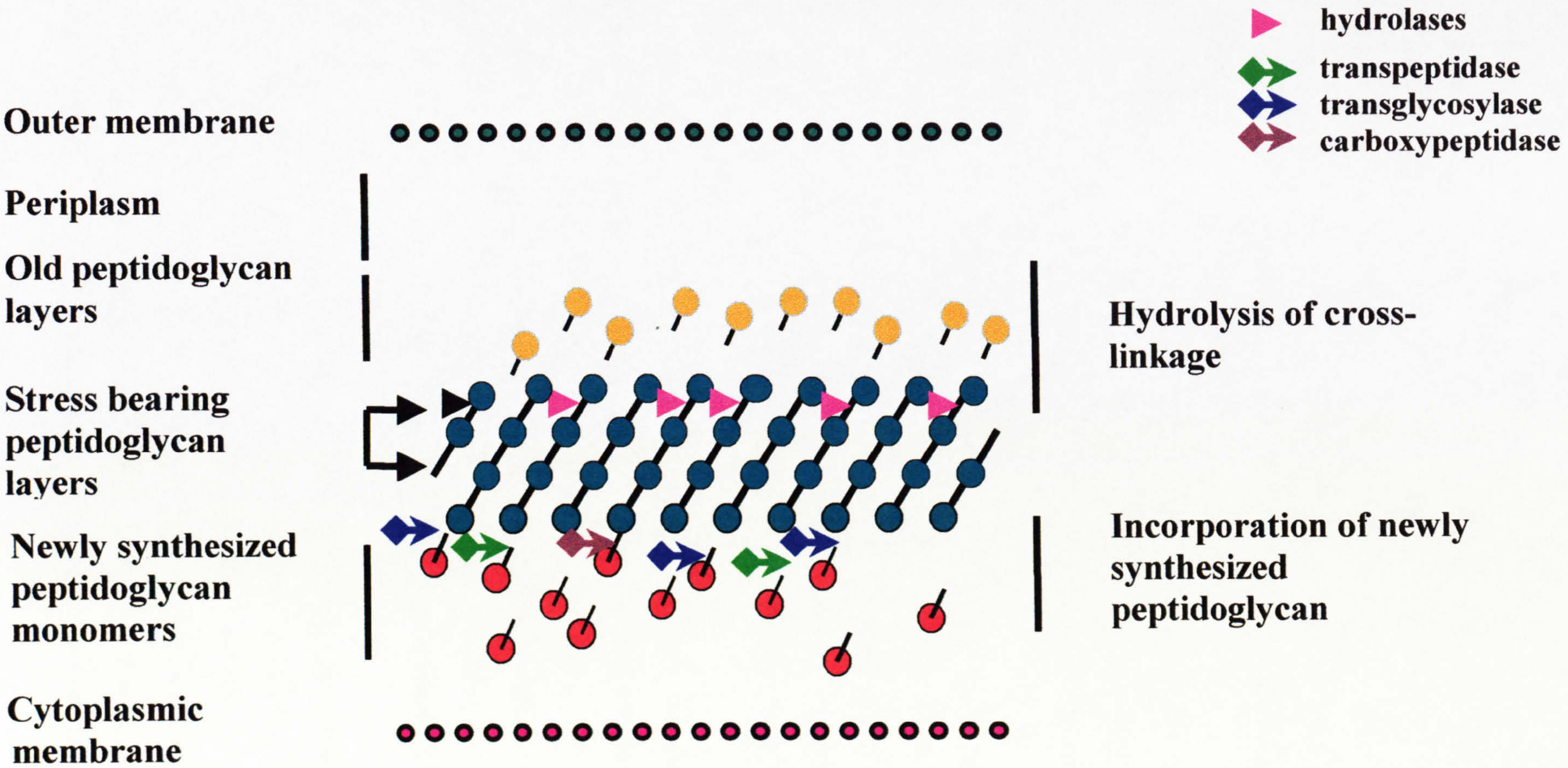



Fig. 5.11 Model for the turnover of the prokaryotic peptidoglycan layer. For details see text.

molecules, that are synthesized during the high-temperature pulse, or the newly synthesized peptidoglycan molecules may be inserted into the existing wall layer in a different way, ie the cross-linking of the new molecules to the existing peptidoglycan layer is altered. If such a scenario takes place, the time period of the short-term incubation to the increased temperature determines the amount of altered peptidoglycan that is added to the cell wall. This hypothesis is supported by observation of Glauner et al. (1988) who found a (then) novel type of cross-linkage between single peptidoglycan mono-layers in the cell wall of *Escherichia coli*, which was influenced by environmental factors, including temperature.

It is suggested that helix reversal takes place if the following two conditions are fulfilled.

1. The amount of newly synthesized peptidoglycan has to be sufficient for the reversal of helix orientation and the newly synthesized and incorporated peptidoglycan layers reach a certain position within the peptidoglycan that is critical for the helix orientation, ie the stress bearing layers within the peptidoglycan sacculus of the cell wall (Höltje, 1998).
2. The newly synthesized peptidoglycan has to be incorporated in sufficient cells of a trichome for the reversal to take place. The latter is thought to be necessary to overcome the steric integrity of a helical trichome in order to reverse the orientation. Aspects similar to those discussed for the formation of helical trichomes from straight ones (Section 5.4.2.1), apply here.

The suggested model would explain both the reversal of helix orientation during permanent growth at elevated temperature as well as the change in helix orientation induced by the short-term incubation at increased temperatures. Further evidence in support of this model is provided by the fact that cultures of strain D0923 that grew in the presence of 40 μM chloramphenicol did not reverse their helix orientation with permanent exposure to 32 °C, suggesting that protein synthesis is involved in reversal of helix orientation.

There were always a few (< 2%) trichomes with the opposite helix orientation in cultures of strains showing this phenomenon. As a consequence, one could argue, that the increased percentage of trichomes with reversed helix in a culture is due to better growth of this type of trichome at the higher temperature, thus not due to the reversal of helix orientation. However, this would also agree with the model, as it confirms that a structurally altered type of peptidoglycan is favoured under increased growth temperature.

The cyanobacterial cell wall contains an outer membrane like the gram-negative bacterial cell wall. However, the thickness of the cyanobacterial peptidoglycan layer and the high degree of cross-linkage between the peptidoglycan monolayers (56% in *Synechocystis* sp. PCC 6714; Jürgens et al., 1983) correspond well with the peptidoglycan of gram-positive bacteria (Jürgens et al., 1983). Furthermore, cross-linked peptidoglycan of the γ1-type has been found in *Synechocystis* strains PCC 6714 (Jürgens et al., 1983) and PCC 6803 (Kegg Encyclopedia, 1996) as well as in *Bacillus subtilis* (Archibald et al., 1993). Based on these similarities between the gram-positive and cyanobacterial peptidoglycan structures it is not surprising, that the shape determining cell wall layer of members of both prokaryotic groups respond similarly to environmental changes despite the different phylogenetic lines (Fox et al., 1980). The similarity in the concept of helical growth between both classes of prokaryotes has also been suggested by Hernández-Muniz and Stevens (1988). Hernández-Muniz and Stevens (1988) describe similarities in the formation of braided, helical trichomes in *Mastigocladus laminosus* and the filamentous, helical mutants of *Bacillus subtilis* (Mendelson, 1976).

*Reversal of helix orientation through mechanical force*

Further experiments on changes in helical conformation concern the observation that filaments of D0925 grown at 30 °C using continuous shaking change their helix orientation. Following this environmental change, some filaments show both orientations: clockwise and anti-clockwise with a snag at the point of the helix, where the orientation is reversed. No such state has been found in cultures grown without continuous shaking at 32 °C or higher. Explanation for this phenomenon may be aided by recent theoretical, biomechanical studies, which have tried to explain the changes in orientation of helical filaments like the tendrils of climbing plants (Goriely & Tabor, 1998). In fact, most of the trichomes with reversed helix orientation were found within aggregates of approximately 10-100 trichomes. Aggregation of trichomes seems to be favoured by the helical trichome conformation and the length of the trichomes, which provide sufficient anchoring for each other. Within those aggregates tension and strain may originate due to the continuous shaking of the culture, thus leading to helix reversal similar to that of climbing plants.

This model, however, is not in agreement with the explanation provided by Tilby (1977), who suggested that fibrils wound in helical manner around the cell may be

responsible for reversal of the helical shape of *Bacillus subtilis* through snag formation. According to Tilby (1977), reversal of helix orientation by formation of a snag could then occur at the point of the trichome where the orientation of the fibrils changes or where the equality of clockwise and anti-clockwise fibrils around the trichome is disturbed. Fibrils helically wound around trichomes of filamentous cyanobacteria have been described either on top of the outer membrane (Hoiczyk & Baumeister, 1995) or between the peptidoglycan layer and the outer membrane (Adams et al., 1999). However, both types of fibrils have been found at the cell surface of cyanobacteria with straight trichome morphology (ie *Oscillatoria princeps, Phormidium uncinatum*). Therefore, it seems unlikely that surface fibrils are involved in helical shape of cyanobacteria, supporting the hypothesis of the peptidoglycan layer as the part of the cell wall, that determines the trichome morphology.

Trichomes showing both helix orientations with a snag at the point of helix reversal has only been found for strain D0925 but not D0918/H or D0923. This may be due to differences in helix parameters. The helix diameter of trichomes of strain D0925 ranges from 50 to 80 µm at the mid of the trichome and most of the trichomes of a culture show barrel-shaped helix morphology. In contrast, trichomes of strains D0918/H (at 32 °C) and D0923 have a trichome diameter of 37 to 50 µm and 47 to 55 µm, respectively, and show fusiform or dumbbell-shaped helices. The wider helix diameter and the barrel shape of strain D0925 may facilitate the formation of helix reversal. In fact, the occurrence of reversal of helix orientation by mechanical forces has been shown to be dependent on the helix diameter (Goriely & Tabor, 1998; Seife, 1998).

### 5.4.3 Motility

#### 5.4.3.1 Correlation between ability to glide and trichome morphology

The ability of *Arthrospira* strains to glide on solid substrate can be deduced from the macroscopic appearance of the growth morphology on agar plates. Colony forming strains lack the ability to glide, while strains that grow as a feathery spread are highly motile. As judged by the speed of spreading out on the agar plate, lawn forming strains are less motile than those forming feathery spread (usual observations). The macroscopic appearance of the growth morphology on agar plates was reproducible and, thus, seems to be

characteristic for a strain. This, however, is not the case for the straight morphotypes of *Arthrospira* strains.

Characters of the helical trichome morphology were found to be responsible for the type of growth pattern to be formed by the strain, and, thus, the degree of motility. Looser coiled trichomes, such as many of the strains showing regular trichome helices, proved to be highly motile, while closely coiled trichomes, such as fusiform and dumbbell-shaped trichomes, are less or not motile.

A possible explanation for the correlation between the degree of motility and the helical trichome morphology may be provided by the mechanism of gliding of a helical filament. Straight trichomes of several species of different genera (eg *Oscillatoria princeps*: Halfen & Castenholz, 1970; *Phormidium uncinatum*: Hoyczyk & Baumeister, 1998) were found to glide while rotating within the slime, thus, facilitating the "pushing forward" of the trichome. In contrast, strains that glide by lateral movement to the long axis of the trichome, must overcome a higher mechanical resistance. This type of gliding has been observed for *Anabaena variabilis* by excretion of slime perpendicularly to the long axis of the filament (Hoyczyk & Baumeister, 1998). Furthermore, the aid of the surface fibrils helically wound along the trichomes of oscillatoriacean species including *Arthrospira* strains (Adams et al., unpubl. results) does not apply for lateral movement. Therefore, lateral gliding may well result in slower movement and thus be responsible for formation of a lawn. Reasons for the lack of motility are unknown, but may be similar to those found for non-motile cultures of motile strains of other oscillatoriacean species. Hoyczyk and Baumeister (1997) showed that the non-motile filaments differed from the motile filaments by lack of slime production and surface ("oscillin") fibrils.

### 5.4.3.2 Motility in liquid medium

A necessary pre-requisite for gliding movement is contact to a solid substrate serving as a structural basis on which the gliding mechanism of the cyanobacterium can act (Jarosch, 1962). However, observations on the growth pattern of cultures in liquid medium suggest, that at least those strains that show aggregation to clumps (D0904, D0905) under any growth environment tested, may be motile also in liquid medium. The aggregation, of course, may be the result of random encounters driven by diffusion, and subsequent anchoring together due to the helical trichome morphology. Active motility, however,

may also be involved in the formation of aggregates. Castenholz (1967) showed that a culture of *Oscillatoria terebriformis* dispersed in a petri dish containing liquid medium aggregates within less than four min to a single clump at the centre of the dish. The motility was suggested to be based on flexional and contractional or torsional movements, ie gliding of trichomes against each other upon contact.

A similar mechanism to that suggested for the motility of *O. terebriformis* in liquid medium may be responsible for *Arthrospira* strains. However, the precise mechanism is still unclear. Adding 10 μM cAMP to the liquid culture medium Ohmori et al. (1992) observed aggregation of *Spirulina* (*Arthrospira*) *platensis* trichomes with a speed of 189 (± 22) μm $min^{-1}$. It seems unlikely that such a high motility, which is even higher than the 120-300 μm $min^{-1}$ observed for straight oscillatoriacean trichomes on solid substrate (Fritsch, 1945), can be achieved in liquid medium by gliding through excretion of slime. An alternative explanation for the high degree of motility may be provided by a motility mechanism in liquid medium that uses fimbriae at the cell surface as mechanical tools. Travelling surface waves of surface fimbriae were also discussed for the swimming motility of the marine *Synechococcus* isolates (Ehlers et al., 1996). Using an improved fixation protocol for the sample preparation for TEM led to the discovery of up to now unknown fimbriae (glycocalyx) at the cell surface of *Arthrospira* cells (Section 6.3; Fig. 6.2C).

## 5.5 Summary

i) 17 morphological characters of the set of 35 *Arthrospira* strains, five duplicate strains and eight different morphotypes as well as seven *Spirulina* strains were examined. Morphological data of 17 *Arthrospira* species description were also collected.

ii) There is a high correlation between the three characters that describe the trichome helix, thus allowing to divide the helical strains into two clusters.

iii) The loss of helical trichome morphology was observed in four *Arthrospira* strains during the course of the project and is thought to be caused by mutation. Under laboratory conditions the straight trichomes were found to grow faster than the helical ones, thus

outcompeting the helical morphotype. Straightening of helical trichomes through increased salinity was reversibel.

iv) One of the nine strains with straight trichomes showed some reverse mutants. However, the straight morphotype was stable as a culture.

v) Some *Arthrospira* strains were found to reverse their helix orientation by either genetic drift, or temperature or mechanical forces. Protein synthesis proved to be a pre-requisite for temperature-induced helix reversal.

vi) The macroscopic appearance of growth morphology on agar was found to occur in three major types and, thus, can be used to identify helical *Arthrospira* strains, but not straight ones. There is also a high correlation between the helical trichome morphology of *Arthrospira* strains and the macroscopic appearance of growth morphology on agar.

vii) Two *Arthrospira* strains are highly motile also in liquid medium by, presumably, flexigonal gliding, possibly aided by surface fimbriae.

# CHAPTER 6 ULTRASTRUCTURE

## 6.1 Introduction

In contrast to previous studies, which have investigated ultrastructural characters at the generic level, this work was undertaken to screen a large set of *Arthrospira* strains for intrageneric characters for taxonomic purposes. Six *Spirulina* strains were also included in the screening program to identify ultrastructural differences between the genera *Arthrospira* and *Spirulina*.

## 6.2 *Arthrospira* versus *Spirulina*

The results of the ultrastructural studies on 35 *Arthrospira* strains, the five duplicate strains and six morphological variants, as well as six *Spirulina* strains (D0868, D0869, D0870, D0871, D0877, D0878) showed the following characters. *Spirulina* strains show six rows of pores at the concave side of the coil next to cross-walls, whereas *Arthrospira* strains show only a single row around the filament at each site of a cross-wall (Fig. 6.1A, B). In general, *Arthrospira* strains showed by far more inclusions than *Spirulina* strains. Cylindrical bodies (Fig. 6.1C) were found only in *Arthrospira*, but not in *Spirulina* strains. Furthermore, gas vacuoles containing gas cylinders were found in all *Arthrospira* strains, but in none of the *Spirulina* strains.

## 6.3 Ultrastructure of *Arthrospira* strains

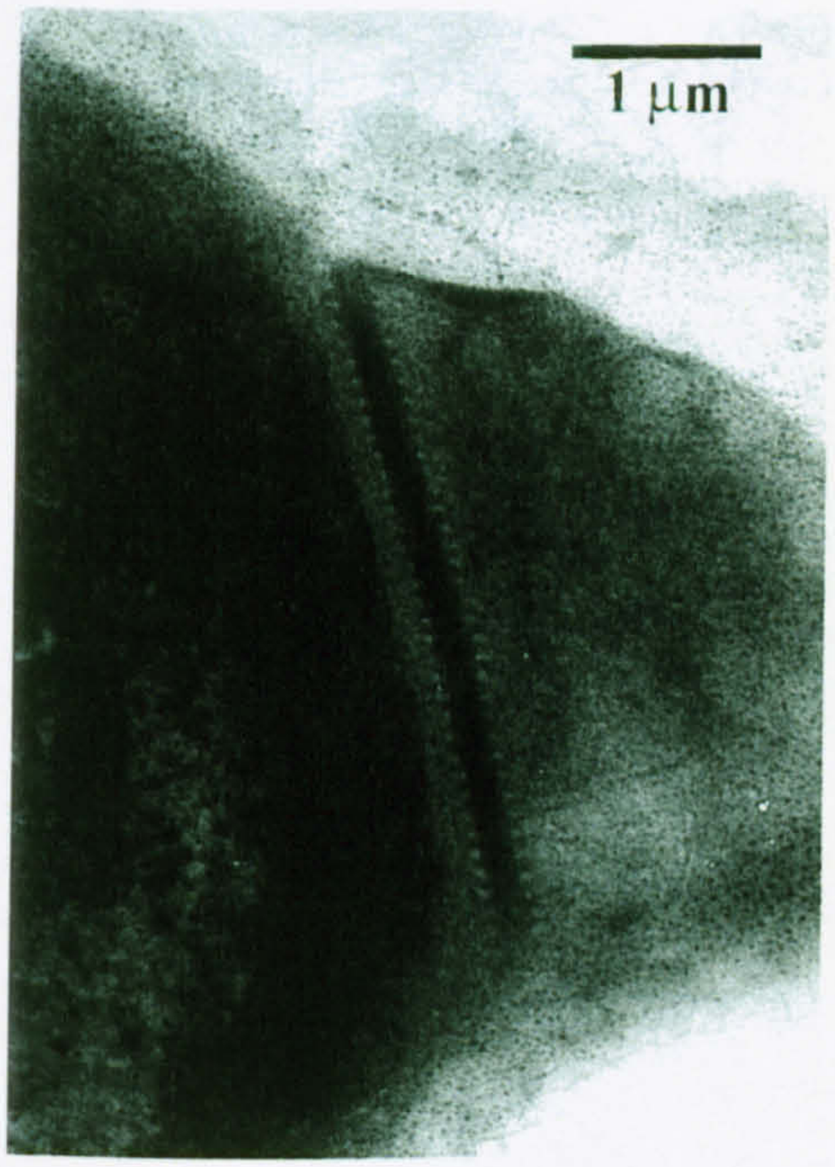
Intensive screening of the ultrastructure of 20 *Arthrospira* strains showed that a variety of cell inclusions (eg mesosomes, carboxysomes, membranous spheres; Fig. 6.2A, B) are present in all *Arthrospira* strains. Furthermore, fimbriae were identified at the cell surface of *Arthrospira* strains indicating the presence of a glycocalyx (Fig. 6.2C). Screening of the rest of strains did not concentrate on these characters as they occurred in all strains and, therefore, did not allow any differentiation between strains.

Fig. 6.1 Genus-specific ultrastructural features of *Arthrospira* and *Spirulina*.

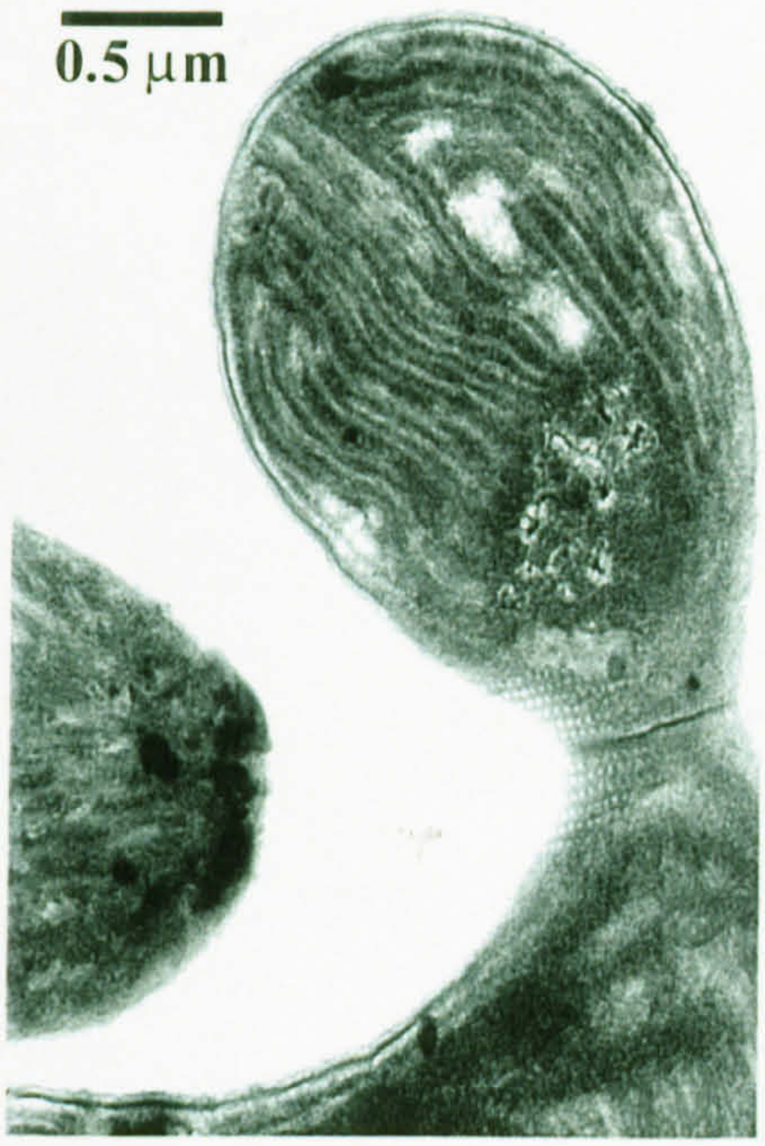
All 35 *Arthrospira* strains, their duplicates and different morphotypes showed one row of pores at either site of a cross wall (A), while six rows of pores have been found at the concave site of the cross walls of all six *Spirulina* strains (B). Electron micrographs C and D show ultrastructural features typically found in cross sections of *Arthrospira* and *Spirulina* strains, respectively. (A = D0884, B = D0870, C = strain D0876, D = D0871)

# *Arthrospira* versus *Spirulina*

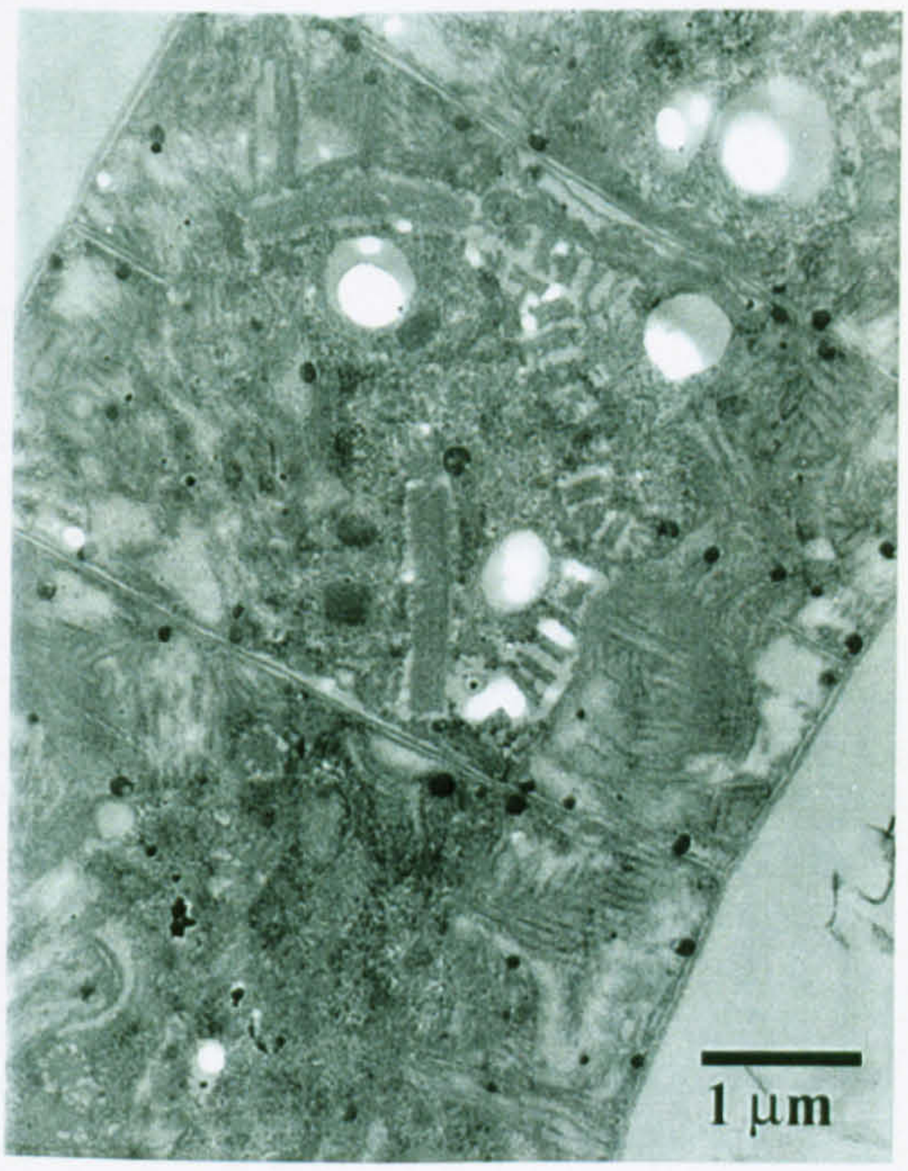
**A)**




**B)**




**C)**



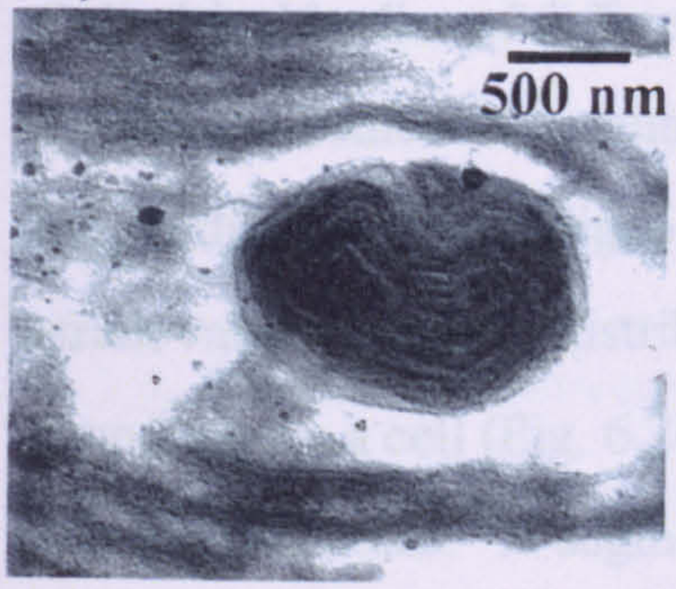

**D)**



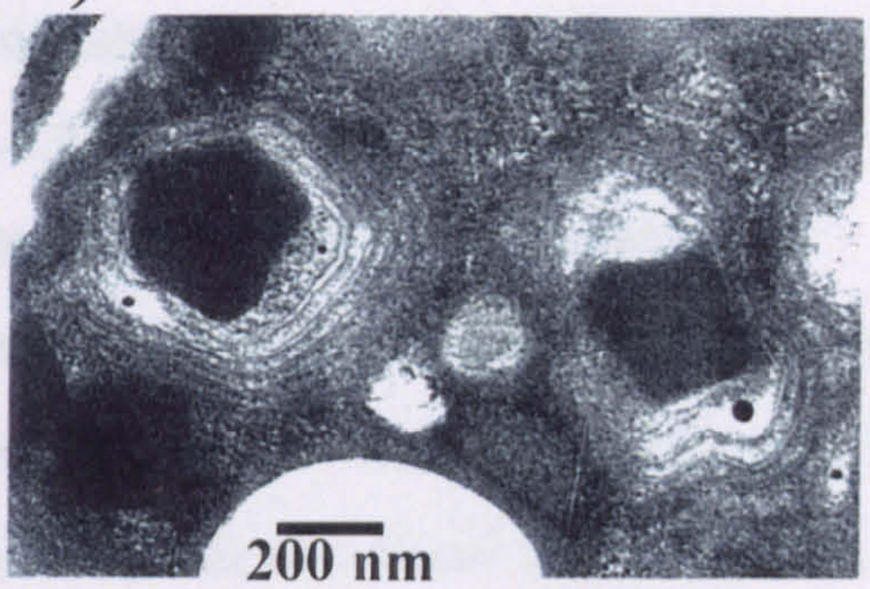
Fig. 6.2 Ultrastructure of *Arthrospira* strains.

A. Mesosome in strain D0885/H1. B. Carboxysomes surrounded by spherical membranous structures (strain D0891). C. Surface structures of *Arthrospira* strain D0900 indicating the presence of a glycocalyx. D - H. Cylindrical bodies. Continuous type (D, strain D0897) and segmented type (F, H, strain D0895) of cylindrical bodies in longitudinal sections. E, G. Cross sections of cylindrical bodies. Cross and longitudinal sections of cylindrical bodies in strain D0880 (E). Eight cylindrical bodies were found in this cross section of filaments of strain D0897 (G). I. Negative stained electron micrograph of a diacetylenic phospholipid after self assembly to a multilayered tubule from a 1 mg $mL^{-1}$ sample in methanol/water (8:2). Taken from Spector et al. (1998).

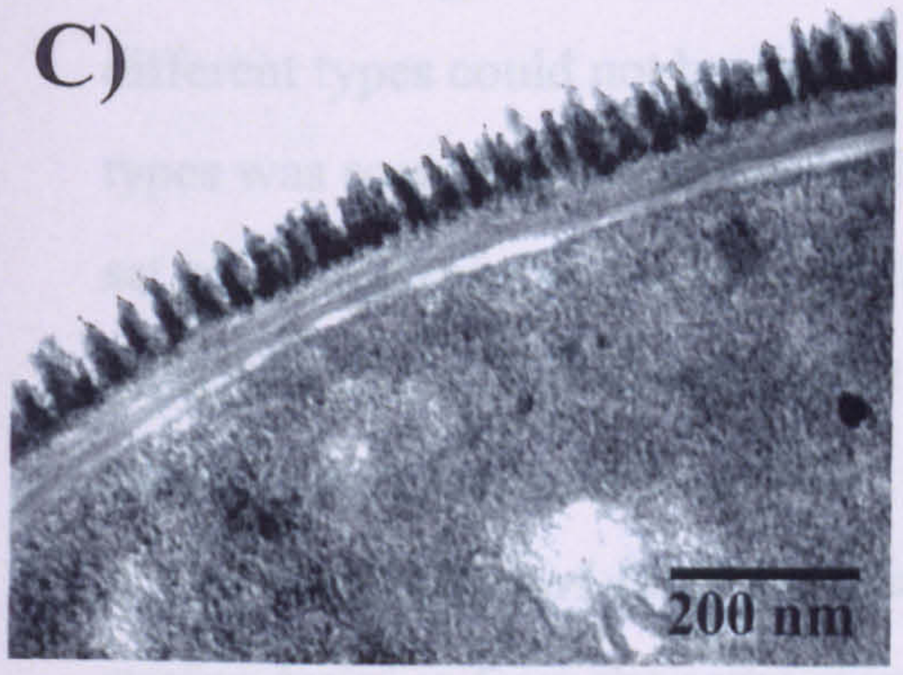
A)



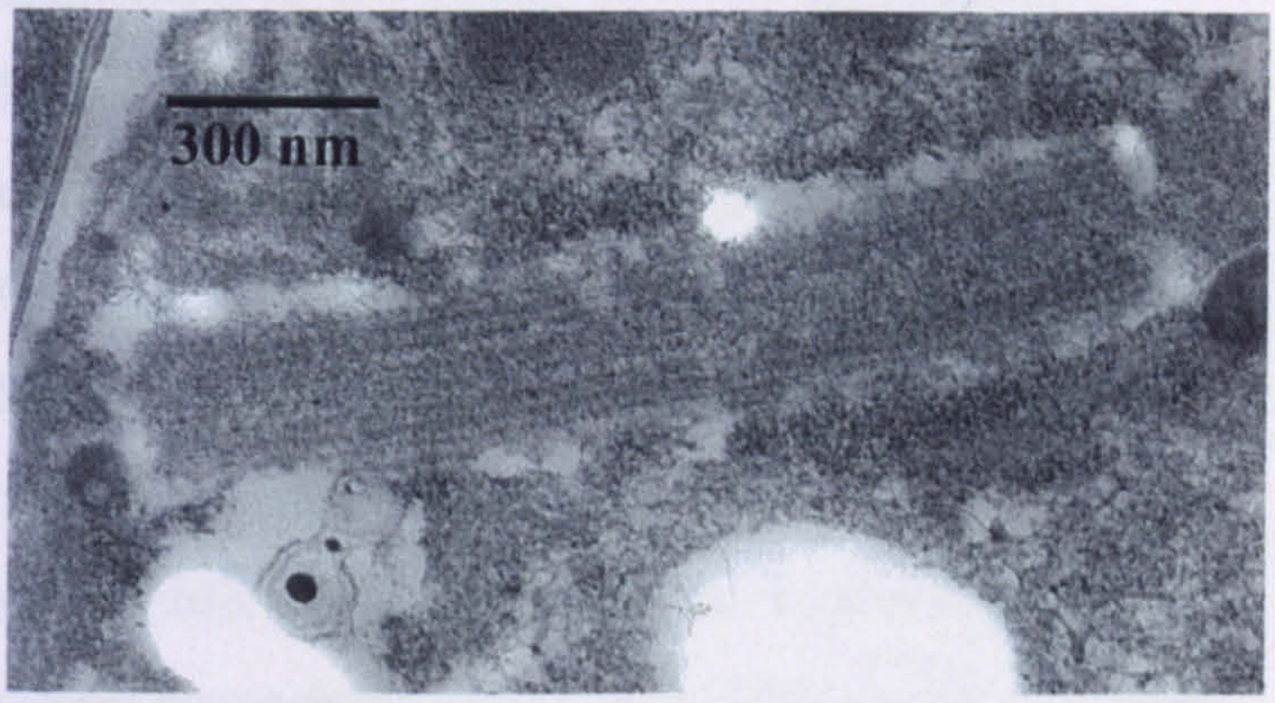

B)



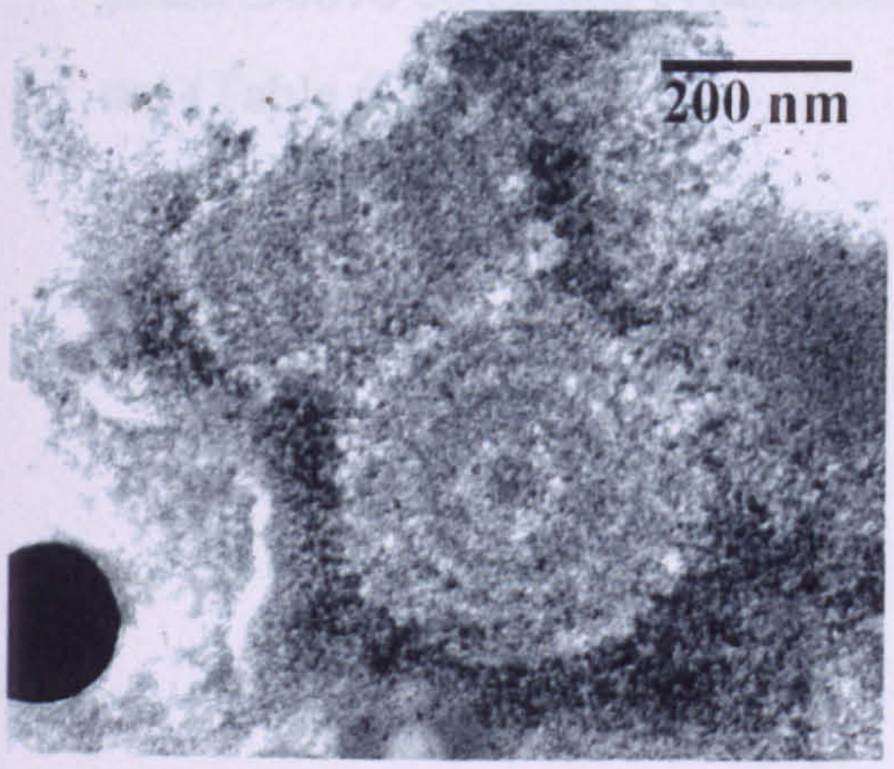

C)



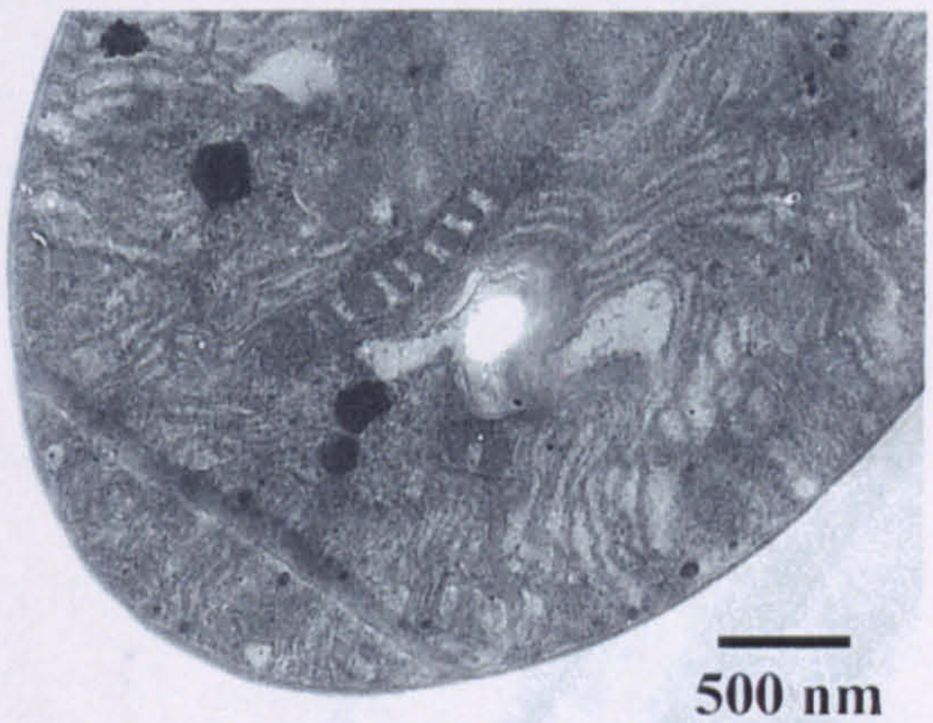

D)



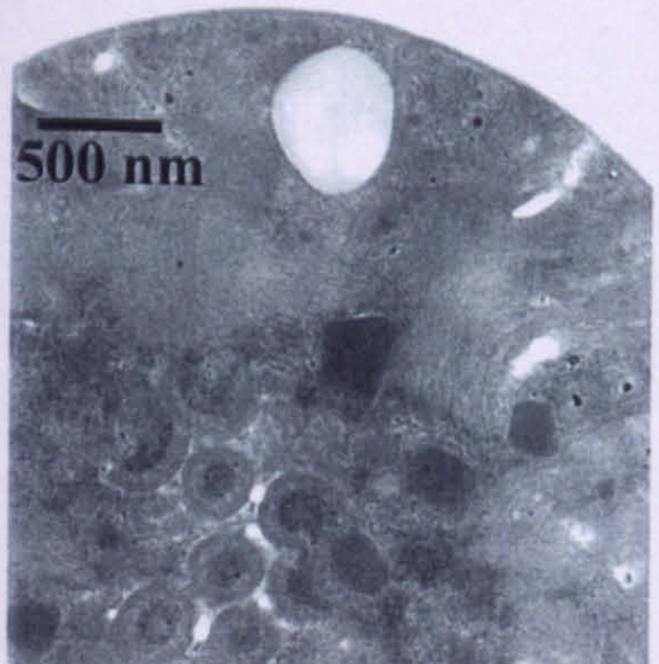

E)



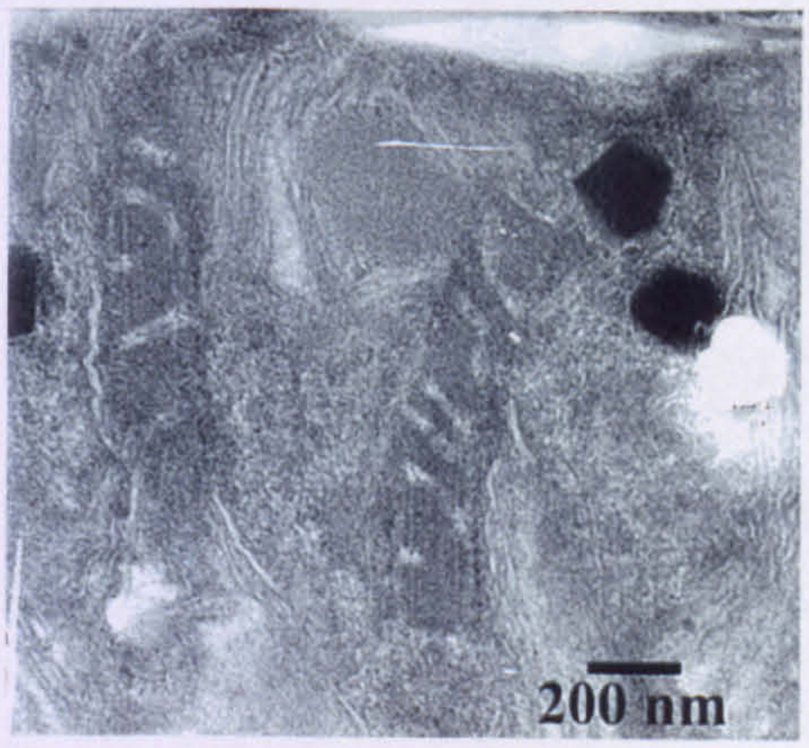

F)



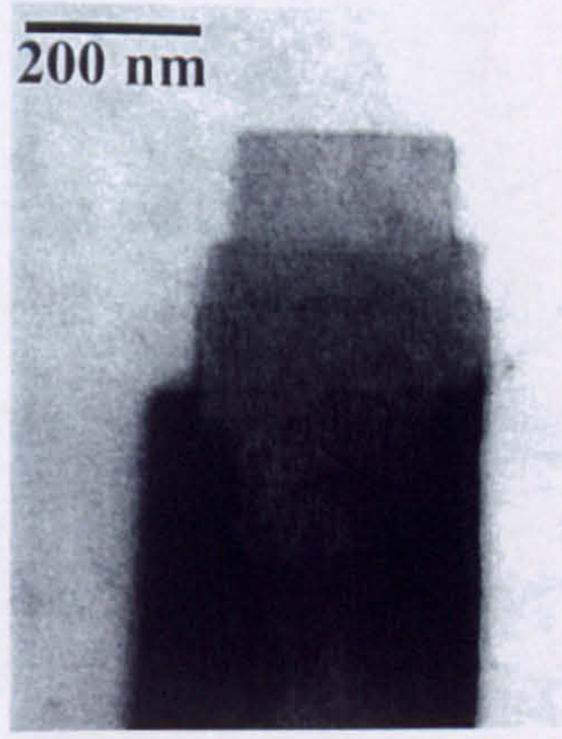

G)




H)




I)



Cylindrical bodies were found in most (14), but not all of the 20 *Arthrospira* strains tested in the initial analysis of cell inclusions. Their diameter ranged from 200 to 400 nm. Cylindrical bodies of different sizes were observed within the same cell. Generally, cylindrical bodies were found in cross-section (Fig. 6.2G), but sometimes also in longitudinal sections (Fig. 6.2D, F, H). The amount of cylindrical bodies found for a strain varied, but their distribution was irregular: sometimes several cylindrical bodies were found within a cell (Fig. 6.2G), while many other cells of the same sample did not contain cylindrical bodies. A segmented type of cylindrical body was also found in some strains, but only in longitudinal sections of filaments (Fig. 6.2 F, H). In cross-sections the different types could not be distinguished. Therefore, the occurrence of either of the two types was scored as cylindrical bodies being present. The screening was extended to the set of 35 *Arthrospira* strains (Table 6.1A), the five duplicate strains (Table 6.1B) and the eight different morphotypes (Table 6.1C). In total, under the environmental conditions tested, more strains contained (25) cylindrical bodies than did not (10).

The comparison of duplicate strains and different morphotypes shows that most of the strains deriving from the same clone show the same character state (Table 6.1B, C). Only strain D0876 contained cylindrical bodies, while its duplicate strains (D0875, D0911) did not (Table 6.1B).

Table 6.1 Presence of cylindrical bodies in *Arthrospira* strains. 100 cross or longitudinal sections of filaments were examined for each of the 35 strains, five duplicate strains and four different morphotype for the presence of cylindrical bodies. The presence of either of either of the two types of cylindrical bodies ("normal" and segmented) is scored as 'cylindrical bodies present'.

6.1.A Comparison between strains.

| | Cylindrical bodies | | | Cylindrical bodies | |
|---|---|---|---|---|---|
| Strains | present | absent | Strains | present | absent |
| D0867 | + | - | D0909 | + | - |
| D0872/H | - | + | D0910/H | + | - |
| D0873 | - | + | D0911 | - | + |
| D0880 | + | - | D0913 | - | + |
| D0881 | + | - | D0914 | + | - |
| D0882 | + | - | D0915 | + | - |
| D0884 | + | - | D0916 | + | - |
| D0885/H1 | + | - | D0918/H | + | - |
| D0890 | - | + | D0919 | + | - |
| D0891 | + | - | D0920 | + | - |
| D0895 | + | - | D0921 | + | - |
| D0896 | - | + | D0922 | + | - |
| D0897 | + | - | D0923 | + | - |
| D0899 | + | - | D0925 | - | + |
| D0900 | - | + | D0929 | + | - |
| D0904 | - | + | D0930 | - | + |
| D0905 | + | - | D0933 | + | - |
| D0907 | + | - | | | |

6.1B Comparison of duplicate strains.

| | Cylindrical bodies | |
|---|---|---|
| Strains | present | absent |
| **D0873** | - | + |
| D0879 | - | + |
| **D0880** | + | - |
| D0887 | + | - |
| D0906/H | + | - |
| D0875 | - | + |
| D0876 | + | - |
| **D0911** | - | + |

6.1C Comparison of morphological variants of the same strains.

| | Cylindrical bodies | |
|---|---|---|
| Strains | Present | absent |
| D0872/H | + | - |
| D0872/S | + | - |
| D0910/H | + | - |
| D0910/S1 | + | - |
| D0918/H | + | - |
| D0918/S | + | - |
| D0906/H | + | - |
| D0906/S | + | - |

## 6.4 Discussion

### 6.4.1 Genus specific characters of *Arthrospira* and *Spirulina* strains

The results of the comparison of the ultrastructure of *Arthrospira* and *Spirulina* strains confirmed the type of cell wall pores as a genus-specific character of *Arthrospira* and *Spirulina*, as suggested by Guglielmi and Cohen-Bazire (1982) and most recently by Palinska and Krumbein (2000).

Besides the differences of the cell wall pore system, the ultrastructure of *Spirulina* spp. show fewer inclusions. For example, cylindrical bodies were diagnostic (Tomaselli et al., 1996), being found only in *Arthrospira*, but not in *Spirulina* strains.

Polyphosphate bodies were abundant in almost all cultures of either of the two genera. This is due to the high phosphate concentration in either of the two media used (Section 4.2).

Due to the absence of a variety of inclusions as potential markers the analysis of the ultrastructure of *Spirulina* strains does not provide a successful approach for taxonomic characterization at the intrageneric level. In contrast, the variety of cell inclusions found in *Arthrospira* strains suggests that there are differences between strains of *Arthrospira* that might be used for taxonomic purposes.

### 6.4.2 Ultrastructure of *Arthrospira*

The initial intensive analysis of ultrastructural characters of 20 *Arthrospira* strains was carried out to evaluate their potential as characters for taxonomic purposes. Characters were selected on the basis of the work of Jensen (1984, 1985) and/or the easy identification due to obvious structural features. However, despite the variety of cell inclusions most of them proved inadequate for taxonomic purposes. This was mainly due to the fact that most of the inclusions were present in all strains and only differed slightly in size and shape (eg carboxysomes, polyphosphate bodies). Other cell inclusions of "irregular occurrence" (Jensen, 1985), such as tubular structures (Jensen, 1985) are very small and very irregularly distributed and, therefore, difficult to detect. Other structures (eg mesosomes) may represent artefacts as a result of the preparation of the sample for TEM.

Fimbriae at the cell surface of prokaryotes are formed by anionic polysaccharides (Costerton et al., 1981). The addition of lysine in the fixation solution (Section 3.4.3) proved to stabilize the bacterial glycocalyx (Fassel et al., 1993). In addition, the acidic nature of the surface polysaccharides make the glycocalyx also amenable to polyanion-specific stains, such as ruthenium red (Luft, 1971), thus leading to better visualization.

Employing a modified fixation protocol (Section 3.4.3), it was possible to obtain better contrast of the cell wall as well as to visualize surface structures such as the glycocalyx of *Arthrospira* strains. Analysis of 20 strains, however, showed that very similar glycocalyx structures were present at the cell surface of all strains tested, allowing no differentiation between strains.

*Cylindrical bodies*

After intensive screening of 20 *Arthrospira* strains the presence (or absence) of cylindrical bodies was identified as taxonomic character of practicable use for differentiating between *Arthrospira* strains. Approximately 70% of the *Arthrospira* strains tested contained cylindrical bodies. Furthermore, the length of the cylindrical bodies (up to 1.5 μm; eg observed in strain D0887; Fig.6.3D) make them very likely to be detected. In addition the clear, distinguished structure of two concentric cylinders with an electro-dense core, which is strongly stained by osmium tetroxide, and the constant diameter of cylindrical bodies (200-400 nm) also facilitate the detection of this inclusion during a screening programme. As environmental changes influenced the formation and structure of cylindrical bodies (Van Eykelenburg, 1980), it is necessary to compare cultures grown under standardized conditions.

Testing 100 cross- or longitudinal sections of filaments of each strain showed that 25 strains of the 35 strains examined contain cylindrical bodies under the growth conditions tested (Table 6.1A). Generally, it was not possible to distinguish between the "normal" and the segmented type of a cylindrical body when the inclusion was identified in a cross-section. As most of the cylindrical bodies were detected in cross-sections, no differentiation has been made between the two types of cylindrical bodies, but detection of either of the two forms was scored as cylindrical bodies being present. There may well be environmental conditions that trigger the formation or degradation of cylindrical bodies in strains that proved to lack or possess, respectively, cylindrical bodies. It was, therefore, important that growth of cultures of the strains took place under precisely controlled

conditions. Furthermore, care was taken to use cultures of strains that reached the same phase of growth, in this study the phase of fast growth.

Cylindrical bodies occurred in some strains more frequently than in others, suggesting a further character for taxonomic purposes. The irregular presence of cylindrical bodies, however, causes great problems for the statistical analysis of the average amount of inclusions occurring in a strain, and, therefore, was not considered as taxonomic marker.

*What are cylindrical bodies?*

Cylindrical bodies have been first described in *Symploca muscorum* (Pankratz and Bowen, 1963). Later on, cylindrical bodies were also found in three *Oscillatoria* and two *Lyngbya* isolates (Jensen, 1985), *Trichodesmium erythraeum* (Van Baalen and Brown, 1969) and *Spirulina (Arthrospira) platensis* (Van Eykelenburg, 1979). Jensen (1985) reports that cylindrical bodies are common in those species that possess them. Observations presented in this work (Fig. 6.5A) confirm these results.

The structure of cylindrical bodies shows that cylindrical bodies consist of two concentric cylinders formed by double membranes which are very similar to lipid membranes (eg compare Fig. 6.3D, E). This is especially obvious when observed directly under the electron microscope at very high magnification.

Lipid-based tubular structures (Fig. 6.3I) have been known for some time and their potential as molecularly engineered microstructures (Schnur, 1993) and tool for helical cristalization of proteins has been discussed (Ringler et al., 1997). Lipid tubules are generally formed by self assembly in solutions due to their inherent nature, but many parameters can influence their structure (Schnur, 1993). For example, the concentration and structure of the lipids and the mixture of the solvents determine the number of bilayers in the tubule (Spector et al., 1998), while the diameter of the tubule may be related to the chirality of the lipid molecules (Schnur, 1993). In fact, the diameter of the tubules can range from 27 nm (Wilson-Kubalek, 1998) to 400 nm (Schnur, 1993). Although the self assembly of lipids into tubules has so far only been shown for synthetic phospholipids with diacetylenes in the acyl chain (Spector et al., 1998), self assembly to tubular membrane structures may also be possible in nature. Furthermore, the assembly of lipids to membranous tubules within the cytoplasm of *Arthrospira* cells may be aided by unknown cellular mechanisms, thus allowing tubule formation of naturally occurring lipids.

If cylindrical bodies consist of lipids, their function may be that of a reservoir or intermediate storage form of lipids. Several different explanations are provided in the literature for the origin and function of cylindrical bodies in cyanobacteria. Pankratz and Bowen (1963) considered the possibility of cylindrical bodies as intracellular parasites or symbionts. Van Baalen and Brown (1969) suggest that they are involved in thylakoid formation. This explanation was also supported by Jensen (1985). The occurrence of the segmented type of cylindrical bodies may confirm this scenario. The segmented type of cylindrical body may represent the state of degradation and utilization of the lipids or membrane structures of a cylindrical body for other purposes, such as the formation of thylakoids, as suggested by Van Baalen and Brown (1969). The fact that the segmented type was found in cultures grown at the lower and upper end (19 and 37 °C, respectively) of tolerated growth temperature of *Spirulina* (*Arthrospira*) *platensis* (Van Eykelenburg, 1980) indicates that the transition to the segmented type takes place as a response to an environmental stress. Furthermore, the light intensity the cultures were grown at also influenced the occurrence of cylindrical bodies (Van Eykelenburg, 1980). In fact, changes in growth temperature (Cohen et al., 1987; Section 8.2) and light intensity (Section 8.2) were found to influence the fatty acid content and composition of *Arthrospira* strains. Although the cultures tested in this work were grown at 30 °C, the segmented type of cylindrical bodies was found, suggests that the turnover of cylindrical bodies is the 'standard pathway' of lipid utilization from the storage form, or that there are further, still unknown factors that trigger the transition of cylindrical bodies.

In conclusion, it appears very likely that cylindrical bodies are formed by lipids. Attempts to identify main components of the cylindrical bodies using the microanalysis system attached to the transmission electron microscope (Section 3.4.3) were, however, unsuccessful. Characterization by lipid specific staining, lipid specific cell probes or by isolation seem to be the only means to clarify unequivocally the consistency of cylindrical bodies.

## 6.5 Summary

i) The separation of *Arthrospira* and *Spirulina* strains into two genera was confirmed based on differences in cell wall pores and presence (*Arthrospira*) or absence (*Spirulina*) of cylindrical bodies.

ii) Fimbriae like surface structures have been identified at the cell surface of *Arthrospira* strains indicating the presence of a glycocalyx.

iii) Cylindrical bodies provide an ultrastructural character useful for taxonomic purposes, due to their easy detection and differences between strains.

# CHAPTER 7 PHYSIOLOGY

## 7.1 Introduction

Screening of cyanobacteria for their ability to grow heterotrophically has revealed great variation at the inter- and intrageneric level (Section 1.5.1) and may also provide taxonomic characters in the case of *Arthrospira* strains. Other physiological characters of potential value for taxonomic purposes are the ability to utilize alternative N- and P-sources. In the case of N, it has already been shown that alternative compounds are being utilized by *Arthrospira* strains (Section 1.5.2), but no information is available on variation between strains. In the case of P, no information is available on *Arthrospira* strains, but variation in the utilization of organic P-sources of other cyanobacteria have been found to be useful for the taxonomic classification (Section 1.5.2).

## 7.2. Heterotrophy

### 7.2.1 Dark heterotrophy

#### 7.2.1.1 Variation in the ability to grow in the dark on sugars

The ability to grow in the dark on sugars was tested for all 35 *Arthrospira* strains, the five duplicate strains and the different morphological subclones of four of the strains. During an initial screening program (no replicates) glucose, fructose, sucrose, galactose, maltose and lactose (each 20 mM) and starch (0.05% (w/v)) were tested as organic carbon source. The screening was carried out in 100-mL flasks containing 30 mL Zarrouk's medium and one of the carbon sources. The flasks were incubated at 30 °C in a cardboard box, which was aerated each other day for 20 min while being shaken irregularly. Of the seven carbohydrates tested only glucose and fructose could be utilized by some of the strains (data not shown). Therefore, more detailed experiments were carried out on those carbon sources, but sucrose was also included as it has been shown to be utilized by many cyanobacteria in the past and represents.

The data from the analysis of four replicates of each strain, confirmed the original results in respect to sucrose. None of the strains grew on sucrose in the dark, only glucose and

fructose were utilized for growth by some of the strains, glucose by more strains than fructose (Table 7.1). Of the 35 strains tested 34 grew on glucose, but only 24 grew on fructose. The pH of the medium of the dark heterotrophic cultures as well as of the medium of those replicates, which did not grow to cultures, was determined at the end of the experiment. In all cases the pH remained within the range 9.5-9.8.

Table 7.1 Results of assays for dark heterotrophic growth of *Arthrospira* strains. Four replicates have been investigated for each sugar (20 mM). (None grew on sucrose in the dark.)

| Strain | **Glucose** | | **Fructose** | |
|---|---|---|---|---|
| | No. of flasks showing | | No. of flasks showing | |
| | Growth | no growth | growth | no growth |
| D0867 | 4 | 0 | 4 | 0 |
| D0872/H | 4 | 0 | 0 | 4 |
| D0873 | 4 | 0 | 0 | 4 |
| D0880 | 1 | 3 | 0 | 4 |
| D0881 | 1 | 3 | 0 | 4 |
| D0882 | 4 | 0 | 2 | 2 |
| D0884 | 4 | 0 | 2 | 2 |
| D0885/H1 | 4 | 0 | 1 | 3 |
| D0890 | 4 | 0 | 4 | 0 |
| D0891 | 4 | 0 | 2 | 2 |
| D0895 | 4 | 0 | 3 | 1 |
| D0896 | 4 | 0 | 4 | 0 |
| D0897 | 4 | 0 | 2 | 2 |
| D0899 | 2 | 2 | 0 | 4 |
| D0900 | 2 | 2 | 1 | 3 |
| D0904 | 4 | 0 | 3 | 1 |
| D0905 | 4 | 0 | 4 | 0 |
| D0907 | 4 | 0 | 0 | 4 |
| D0909 | 4 | 0 | 0 | 4 |
| D0910/H | 4 | 0 | 1 | 3 |
| D0911 | 4 | 0 | 2 | 2 |
| D0913 | 4 | 0 | 2 | 2 |
| D0914 | 1 | 3 | 0 | 4 |
| D0915 | 0 | 4 | 1 | 3 |
| D0916 | 4 | 0 | 2 | 2 |
| D0918/H | 4 | 0 | 3 | 1 |
| D0919 | 2 | 2 | 3 | 1 |
| D0920 | 4 | 0 | 2 | 2 |
| D0921 | 4 | 0 | 4 | 0 |
| D0922 | 4 | 0 | 0 | 4 |
| D0923 | 4 | 0 | 4 | 0 |
| D0925 | 4 | 0 | 2 | 2 |
| D0929 | 4 | 0 | 4 | 0 |
| D0930 | 4 | 0 | 0 | 4 |
| D0933 | 4 | 0 | 0 | 4 |

However, some of the 34 strains, that grew in the dark on glucose, showed growth only in one, two or three replicates out of four (Table 7.2). This phenomenon was even more expressed when fructose was used as carbon source.

Table 7.2 Summary of results for replicates in assays for heterotrophic growth of *Arthrospira* strains. 35 strains were tested for heterotrophic growth in the dark using 20 mM glucose or fructose.

| | Glucose | Fructose | Glucose and fructose |
|---|---|---|---|
| All grow | 28 | 8 | 8 |
| Some grew | 6 | 16 | - |
| None grew | 1 | 11 | 0 |

Generally, those strains, which did not grow at all or which grew only in some of the replicates on glucose, failed also to grow on fructose in any or at least some of the replicates (Table 7.3). Only one strain (D0915) grew in some replicates on fructose, but failed to grow on glucose. The eight strains, that showed growth on fructose in all of the four replicates tested, grew also on glucose in all of the replicates.

Table 7.3 Summary of abilities (including details for replicates) of particular strains to grow in the dark using a particular substrate if they can use the other substrate.

| | Total | All on glucose | Some on glucose | None on glucose | All on fructose | Some on fructose | None on fructose |
|---|---|---|---|---|---|---|---|
| All grew on glucose | 28 | | | | 8 | 13 | 7 |
| Some grew on glucose | 6 | | | | 0 | 2 | 4 |
| None grew on glucose | 1 | | | | 0 | 1 | 0 |
| All grew on fructose | 8 | 8 | 0 | 0 | | | |
| Some grew on fructose | 16 | 13 | 2 | 1 | | | |
| None grew on fructose | 11 | 7 | 4 | 0 | | | |

As none of the 35 strains tested grew on sucrose in the dark, the influence of the sucrose concentration on the ability to utilize it for growth was investigated of those eight strains, that showed growth in all replicates on both glucose and fructose. Carbohydrate concentrations tested were 5mM, 10mM and 20mM. Four replicates were tested for each strain and sugar, but only two for each strain as control (no sucrose added). Although the incubation time was extended to 60 days none of the strains grew on sucrose independent of the sucrose concentration.

*Dark heterotrophy of duplicate strains*

The three sets of duplicate strains were also tested for their ability to grow in the dark on glucose, fructose or sucrose. The results indicate that duplicate strains show broadly a similar ability to grow in the dark on glucose (Table 7.4). More variation between duplicate strains was found when fructose was used as carbon source. Only one of the three sets of duplicate strains behaved the same. None of the cultures of the three sets of duplicate strains grew on sucrose.

Table 7.4 Dark heterotrophic growth on glucose and fructose of duplicate strains deriving from the same clone. The clone of each set of duplicate strains, that is included in the set of 35 strains (Table 7.1), is indicated in bold letters. Four replicates have been investigated for each sugar.

| Strain | **Glucose** | | **Fructose** | |
|---|---|---|---|---|
| | No. of flasks showing | | No. of flasks showing | |
| | growth | no growth | Growth | no growth |
| **D0873** | **4** | **0** | **0** | **4** |
| D0879 | 4 | 0 | 4 | 0 |
| **D0880** | **1** | **3** | **0** | **4** |
| D0887 | 2 | 2 | 0 | 4 |
| D0906/H | 0 | 4 | 0 | 4 |
| D0875 | 4 | 0 | 4 | 0 |
| D0876 | 2 | 2 | 0 | 4 |
| **D0911** | **4** | **0** | **2** | **2** |

*Dark heterotrophy of different morphotypes of a strain*

The different morphotypes of four strains (D0872, D0885, D0906, D0910) were also tested for their ability to grow in the dark on glucose, fructose or sucrose. Again, there was more variation between the morphotypes of the same strain when fructose was used as carbon source, compared to glucose, and none of the different morphotypes grew on sucrose (Table 7.5).

Table 7.5 Dark heterotrophic growth on glucose and fructose of different morphotypes of *Arthrospira* strains. Four replicates have been investigated for each sugar.

| Strain | **Glucose** No. of flasks showing | | **Fructose** No. of flasks showing | |
|---|---|---|---|---|
| | growth | no growth | growth | no growth |
| D0872/H | 4 | 0 | 0 | 4 |
| D0872/S | 4 | 0 | 2 | 2 |
| D0885/H1 | 4 | 0 | 1 | 3 |
| D0885/H2 | 4 | 0 | 2 | 2 |
| D0906/H | 0 | 4 | 0 | 4 |
| D0906/S | 4 | 0 | 1 | 3 |
| D0910/H | 0 | 4 | 0 | 4 |
| D0910/S1 | 4 | 0 | 1 | 3 |
| D0910/S2 | 4 | 0 | 1 | 3 |

7.2.1.2 Reproducibility

Studies were also carried out to test the reproducibility of the results on dark heterotrophic growth. A subset of 10 strains (four replicates each) was tested again for dark heterotrophic growth on glucose and fructose as before. The results are compared with the originally obtained data in Table 7.6. None of the strains that failed to grow in all replicates in the original experiment showed growth in any of the replicates of the repeat experiment. Generally, less strains and less replicate cultures of those strains, which showed growth only in some of the replicates, grew in the repeat experiment. This was more markedly when fructose was used as a carbon source compared to glucose. However, compared to the first screening more replicates of strains D0880 and D0914 grew in the repeat on glucose. Similarly, more replicates of strains D0915 and D0918/H grew on fructose in the repeat compared to the original experiment.

Table 7.6 Results of the independent repeat experiment testing the reproducibility of the ability to grow in the dark on glucose or fructose. Reproducibility was tested on ten *Arthrospira* strains. Numbers in parentheses indicate the number of replicates (out of four) that showed growth in the original experiment.

| Strain | **Glucose** | | **Fructose** | |
|---|---|---|---|---|
| | No. of flasks showing | | No. of flasks showing | |
| | growth | no growth | growth | no growth |
| D0867 | 4 (4) | 0 (0) | 4 (4) | 0 (0) |
| D0880 | 3 (1) | 1 (3) | 0 (0) | 4 (4) |
| D0885/H1 | 4 (4) | 0 (0) | 0 (1) | 4 (3) |
| D0905 | 4 (4) | 0 (0) | 4 (4) | 0 (0) |
| D0914 | 3 (1) | 1 (3) | 0 (0) | 4 (4) |
| D0915 | 0 (0) | 4 (4) | 2 (1) | 2 (3) |
| D0916 | 4 (4) | 0 (0) | 1 (2) | 3 (2) |
| D0918/H | 4 (4) | 0 (0) | 4 (3) | 0 (1) |
| D0922 | 4 (4) | 0 (0) | 0 (0) | 4 (4) |
| D0923 | 4 (4) | 0 (0) | 4 (4) | 0 (0) |

Further tests of the reproducibility of the results were carried out on a larger number of replicates (10 replicates). Two strains (D0867, D0905), where in both independent experiments all four replicates used both sugars (Table 7.6), were compared versus two strains (D0906/H (Table 7.4), D0915/D0922 (Table 7.6)), where no growth was observed in any of the replicates on either of the two sugars. (D0915 did not grow on glucose, but in some replicates on fructose; therefore, D0922 was added to this set as test strain for 'no growth on fructose'.) In addition, for each of the two sugars one strain was investigated that grew in some of the four replicates (D0914/H and D0916 for glucose and fructose, respectively). Ten replicates were used in this repeat experiment for each sugar and strain, together with controls lacking sugar: four replicates in most cases, but only one set of controls for those strains that were tested for more than one sugar. The cultures were incubated in the dark (cardboard boxes) and aerated each day for 30 min while being shaken frequently.

The repeat experiments again showed differences between replicates (Fig. 7.1), although the only difference in the treatment this time was that the cultures were aerated more frequently (every day instead of every other day) and the aeration time was extended (from 20 min to 30 min). For instance, strain D0867, growing originally in all of the four replicates, showed growth on glucose in only seven out of ten replicates. In contrast, all cultures of strain D0914 grew on glucose, while in the original experiment growth was observed only in one out of four replicates.

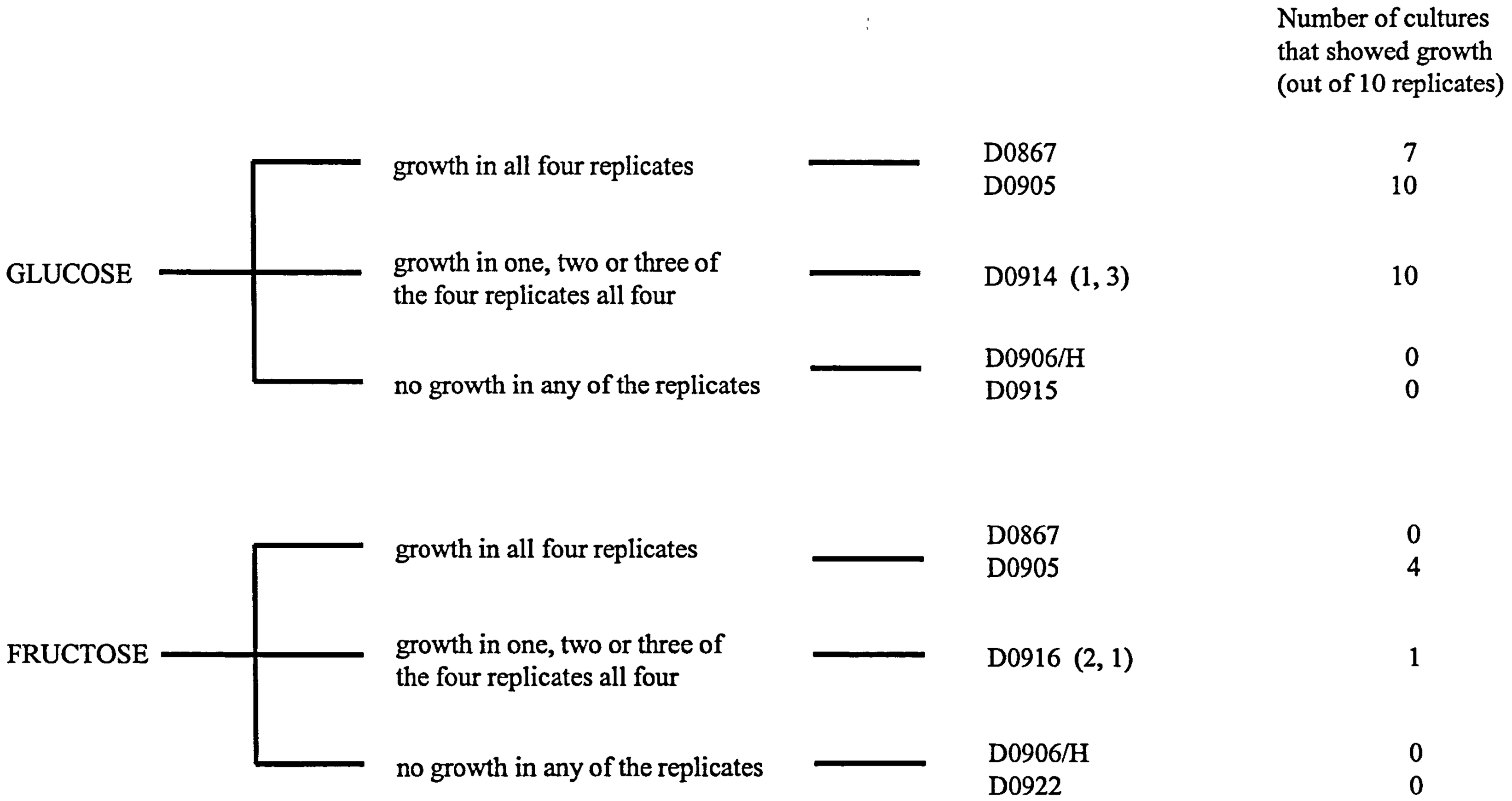



Fig. 7.1 Results of second repeat experiment testing the ability to grow dark heterotrophically.

Ten replicates were tested in the repeat experiment. Strains chosen as test strains for 'growth in all four replicates' or 'no growth in any of the replicates' fulfilled the criteria in both first experiment and first repeat. Numbers in parentheses indicate the number of replicates (out of four) grown in the first experiment and the first repeat.

Again, the differences to the previous experiments were greater when fructose was used as carbon source. While all replicates of D0867 and D0905 had previously grown on fructose, none of the replicates of D0867 grew in this case and only four of the replicates of D0905. D0916 showed growth in one of the ten replicates. None of the strains that did not show growth in any of the four replicates in the original experiment showed growth in the repeat experiment.

Further experiments were carried out to test whether improvements of environmental conditions would lead to growth of all replicates. The same set of strains was tested in this experiment. Changes of the environmental conditions involved improvement of the aeration system by continuous bubbling filter-sterilised air into the dark heterotrophic culture (Section 3.6.1); this also insured a consistent physical mixing of the culture. Due to the set-up of the system only eight replicates were analyzed for each strain.

Despite these improvements more of the replicates tested grew only in one of the cases tested (D0905 on fructose; Table 7.7) compared to the previous test which did not involve continuous aeration and mixing. Although grown in the first screenings on both sugars, strain D0867 failed in all replicates to grow in this experiment as well as in all subsequent attempts to obtain dark heterotrophically grown biomass (ie for analysis of fatty acid composition; Section 8.2.2).

Table 7.7 Results (including details for replicates) on the ability to grow in the dark on glucose or fructose under continuous aeration and mixing. Eight replicates were assayed. Numbers in parentheses indicate the number of replicates (out of 10 or 4) that showed growth in the previous three tests of the same strain. (nd = not determined.)

| Strain | **Glucose** | | **Fructose** | |
|---|---|---|---|---|
| | No. of flasks showing growth | no growth | No. of flasks showing growth | no growth |
| D0867 | 1 (4, 4, 7) | 7 (0, 0, 3) | 0 (4, 4, 0) | 8 (0, 0, 10) |
| D0905 | 8 (4, 4, 10) | 0 (0, 0, 0) | 8 (4, 4, 4) | 0 (0, 0, 6) |
| D0906/H | 0 (0, 0, 0) | 8 (4, 4, 10) | 0 (0, 0, 0) | 8 (4, 4, 10) |
| D0914 | 8 (1, 3, 10) | 0 (3, 1, 0) | nd | nd |
| D0916 | nd | nd | 0 (2, 1, 1) | 8 (2, 3, 9) |

### 7.2.2 Photoheterotrophy

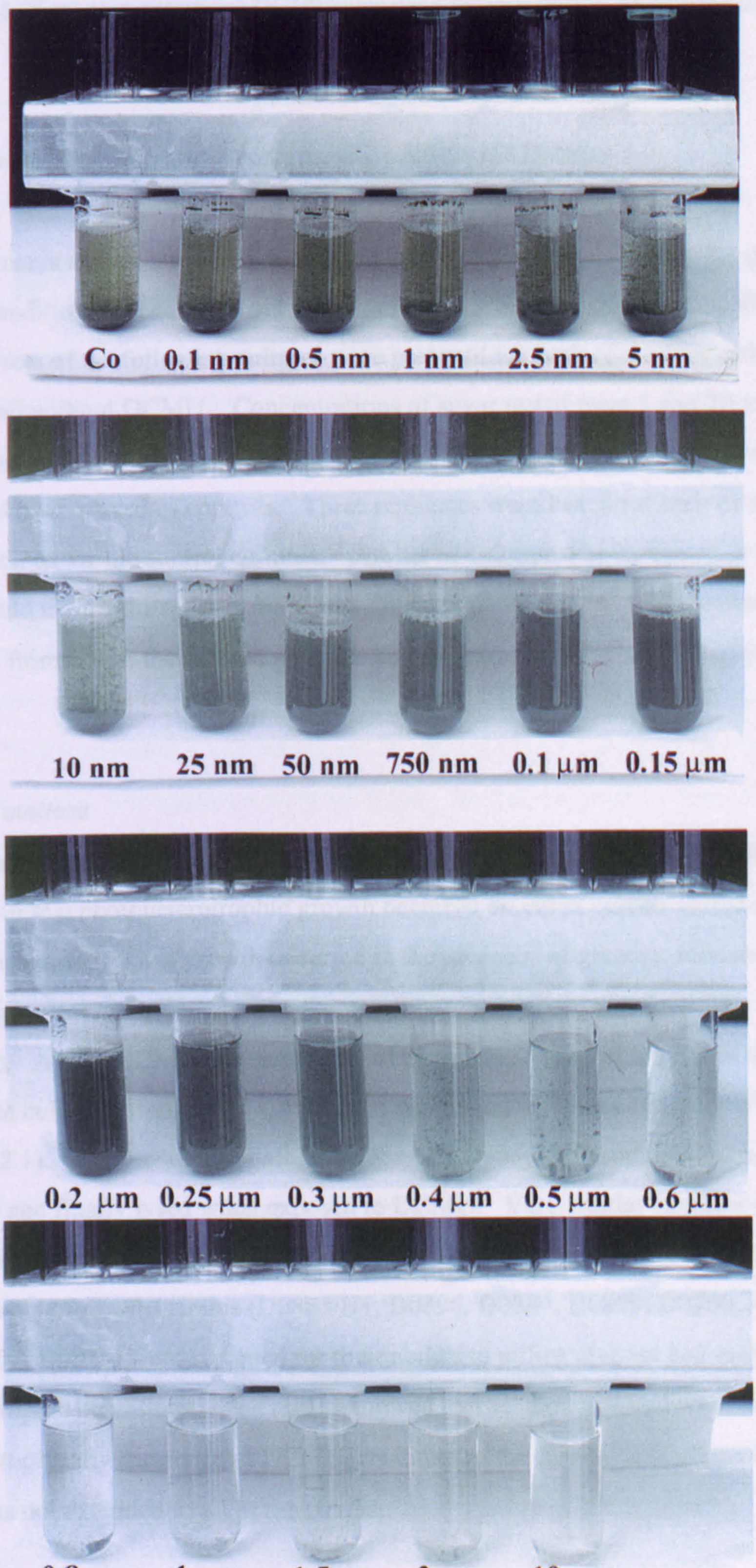
#### 7.2.2.1 Influence of DCMU

DCMU was employed to inhibit photosynthesis, thus allowing to test whether *Arthrospira* strains utilize sugars for growth under photoheterotrophic conditions. The concentration of DCMU necessary to inhibit photosynthesis was investigated using strain D0923 as test strain, as it grew in all replicates on glucose and fructose in the dark, thus representing a good candidate for tests for ability to grow photoheterotrophically. DCMU was added to 16 mL medium in 50-mL boiling tubes and inoculated with 2-mL aliquots of a stock culture of *Arthrospira* strain D0923 grown at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$. The concentrations of DCMU tested were: 0, 0.05, 0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 75, 100, 150, 200, 250, 300, 400, 500, 600, 800 nM, 1, 1.5, 2, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25, 30, 35, 40, 45, 50, 55, 60 µM. Based on the literature on photoheterotrophic growth of *Arthrospira* strains, the light intensity chosen for the growth of the cultures was 70 µmol photon $m^{-2}$ $s^{-1}$. However, the light intensity, the cultures were exposed to, varied due to the large number of tubes (three replicates for each concentration). One set of one replicate of each of the concentrations of DCMU was incubated at 60 (± 2) µmol photon $m^{-2}$ $s^{-1}$, the second at 70 (± 4) µmol photon $m^{-2}$ $s^{-1}$ and the third at 80 (± 5) µmol photon $m^{-2}$ $s^{-1}$.

Despite the exposure to different light intensities, broadly the same results were obtained for each of the three replicates. Within the first 48 h of incubation the inocula of the photoautotrophic cultures and those cultures containing less than 10 nM DCMU showed surprisingly loss of pigmentation and clumping of the biomass. Inocula in the presence of >10 nM DCMU, remained, however, dark blue-green. After further incubation growth occurred in all cultures containing less than 1 µM DCMU. After 10 to 30 days it became apparent that photoautotrophic cultures looked healthiest in the presence of 75 to 150 nM DCMU, the biomass being dark blue-green and buoyant (Figure 7.2). In contrast, parts of the cultures containing less than 10 nM DCMU showed still less pigmentation resulting from the initial photodamage. Growth did not take place in cultures containing DCMU in concentrations higher than 800 nM and the inocula lysed in those cases after 20 to 30 days of incubation (Figure 7.2).

Fig. 7.2 Test for the inhibitory concentration of the herbicide DCMU.

Photographs of autotrophic cultures of strain D0923 grown in the presence of DCMU. The photographs were taken after 30 days of incubation at 30 °C and 70 μmol photon $m^{-2}$ $s^{-1}$. (Co = control)


Co
0.1 nm
0.5 nm
1 nm
2.5 nm
5 nm
10 nm
25 nm
50 nm
750 nm
0.1 μm
0.15 μm
0.2 μm
0.25 μm
0.3 μm
0.4 μm
0.5 μm
0.6 μm
0.8 μm
1 μm
1.5 μm
2 μm
10 μm

To ensure that the inhibition of photosynthesis was always achieved for all cells of a culture 10 µM (final concentration) DCMU was used for any further photoheterotrophic experiments.

7.2.2.2 Photoheterotrophic growth on glucose, maltose and fructose

All experiments testing the ability to utilize glucose, maltose and fructose for growth under light conditions were carried out in the same way. Strain D0923 was again the test strain. Two sets of the following cultures were prepared for each of the four carbohydrates tested with and without DCMU. Concentrations of sugar tested were 5 and 20 mM and in addition in the case of glucose 111 mM (2% (w/v)). Photoautotrophic cultures with or without DCMU were used as controls. Three replicates were tested for each of the cultures as well as for the control cultures. One set of cultures was incubated at 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$, the other at 30 °C and 70 µmol photon $m^{-2}$ $s^{-1}$. The culture of strain D0923 from which the inocula were taken, was grown at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$.

*Glucose and maltose*

The results obtained for the cultures grown at either of the two light intensities tested were similar in that photoheterotrophic growth occurred on either carbon source under both light intensities tested. Best growth occurred in the presence of glucose, followed by the photoautotrophic control cultures and cultures containing glucose plus DCMU (observation). Again, DCMU prevented loss of pigmentation and clumping of the biomass when cultures containing DCMU were grown under 70 µmol photon $m^{-2}$ $s^{-1}$ (Section 7.2.2.1). The inoculum of all replicates of the photoautotrophic (control) cultures did not grow and finally lysed when exposed to DCMU. Very similar results were obtained when maltose was used as carbon source.

Further nine *Arthrospira* strains (D0885/H1, D0896, D0897, D0899, D0900, D0904, D0905, D0907, D0915) were screened for their ability to utilize glucose and maltose under photoheterotrophic conditions. The results showed that all strains tested were able to grow photoheterotrophically (presence of DCMU) on either of the two sugars. Therefore, the screening was not extended to all of the strains.

*Fructose*

The results obtained for strain D0923 and fructose as carbon source showed that slow growth takes place in the presence of this sugar. In contrast to photoheterotrophic growth on glucose and maltose, fructose can not be utilized by *Arthrospira* strains for growth in the presence of DCMU under either of the light conditions provided. Moreover, extended incubation (50 days) led to cell lysis of the complete biomass in all of those replicates that contained 20 mM fructose (with or without DCMU).

Further nine strains (same as for glucose and maltose; Section 7.2.2.2) were screened for their ability to utilize fructose under photoheterotrophic conditions. Again, the results showed that all strains tested behaved the same. Therefore, the screening was not extended to all of the strains.

### 7.2.2.3 Photoheterotrophic growth on sucrose

All *Arthrospira* strains failed to grow in the dark on sucrose. However, experiments were also carried out to test, whether sucrose can be utilized under photoheterotrophic conditions. The experiments were carried out in 100-mL flasks containing 30 mL (final volume) medium and four replicates were analyzed. Inocula were taken from cultures of the strains grown at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$. Although the large number of flasks meant that it was impossible to provide all of the 20 strains tested with the same irradiance in the growth room, all replicates of the same strain were exposed to the same irradiance (Section 3.6.1). The overall range of light intensity used for this study was 30 to 70 µmol photon $m^{-2}$ $s^{-1}$.

The 20 *Arthrospira* strains tested (as for glucose and fructose plus D0881, D0884, D0890, D0895, D0910/H, D0916, D0918/H, D0919, D0922, D0925) responded similarly to the sucrose up-shock. Within the first four to six days after inoculation seemingly all cells of the inoculum of all four replicate cultures containing sucrose (20 mM; with or without DCMU) lysed as judged by light-microscopic analysis of an aliquot of the culture. However, after six to seven days after inoculation, growth was observed in some replicates of cultures containing sucrose only. This was the case for all 20 strains tested. After another three to six days these replicates had grown to healthy cultures. Generally, the higher the irradiance was within the range provided (30 to 70 µmol photon $m^{-2}$ $s^{-1}$), the sooner was growth observed and the more replicates of the four tested grew. All cultures

exposed to a light intensity in the range 60 to 70 µmol photon $m^{-2}$ $s^{-1}$ recovered. None of the replicates of any of the 20 strains tested recovered when DCMU was added.

The photoheterotrophic growth experiment was repeated to investigate further the loss of pigmentation and subsequent cell lysis during the first days of incubation in sucrose and growth after approximately seven days. Zarrouk's medium without (control) and with added sucrose (20 mM) or sucrose (20 mM) plus DCMU (10 µM) was inoculated with aliquots of photoautotrophic cultures of strain D0923 followed by incubation at 10 or 70 µmol photon $m^{-2}$ $s^{-1}$. Ten subcultures containing sucrose were tested for each of the two different light environments and three subcultures were used for the controls (no sucrose).

The observations are summarized in Table 7.8. All replicates of the subcultures grown in the presence of sucrose at 70 µmol photon $m^{-2}$ $s^{-1}$ behaved exactly the same: loss of pigmentation followed by cell lysis and subsequent growth to dense cultures. Again, no recovery was observed in cultures that contained DCMU. The inoculum of subcultures grown at 10 µmol photon $m^{-2}$ $s^{-1}$ bleached too, but more slowly and recovery occurred in only three of the ten replicates after eight days of incubation. Light-microscopic analysis of small "green patches" in the flask observed on day seven in cultures grown at 10 µmol photon $m^{-2}$ $s^{-1}$ showed that only very few cells had survived the sucrose stress, all the others having lysed (Fig. 7.3). The cells that survived were larger than normal cells (10-11 µm wide and up to 12 µm long instead of 9-10 µm wide and 3-5µm long) and had large spherical inclusions (Fig. 7.3). Similar granules were not observed in cultures grown under 70 µmol photon $m^{-2}$ $s^{-1}$ light intensity.

Fig. 7.3 Occurrence of interthylakoidal granules in *Arthrospira* cells upon sucrose up-shock.

The micrographs were taken from samples of cultures after incubation for seven days in the presence of 20 mM sucrose at 30 °C and 10 μmol photon $m^{-2}$ $s^{-1}$.

A)




B)




C)

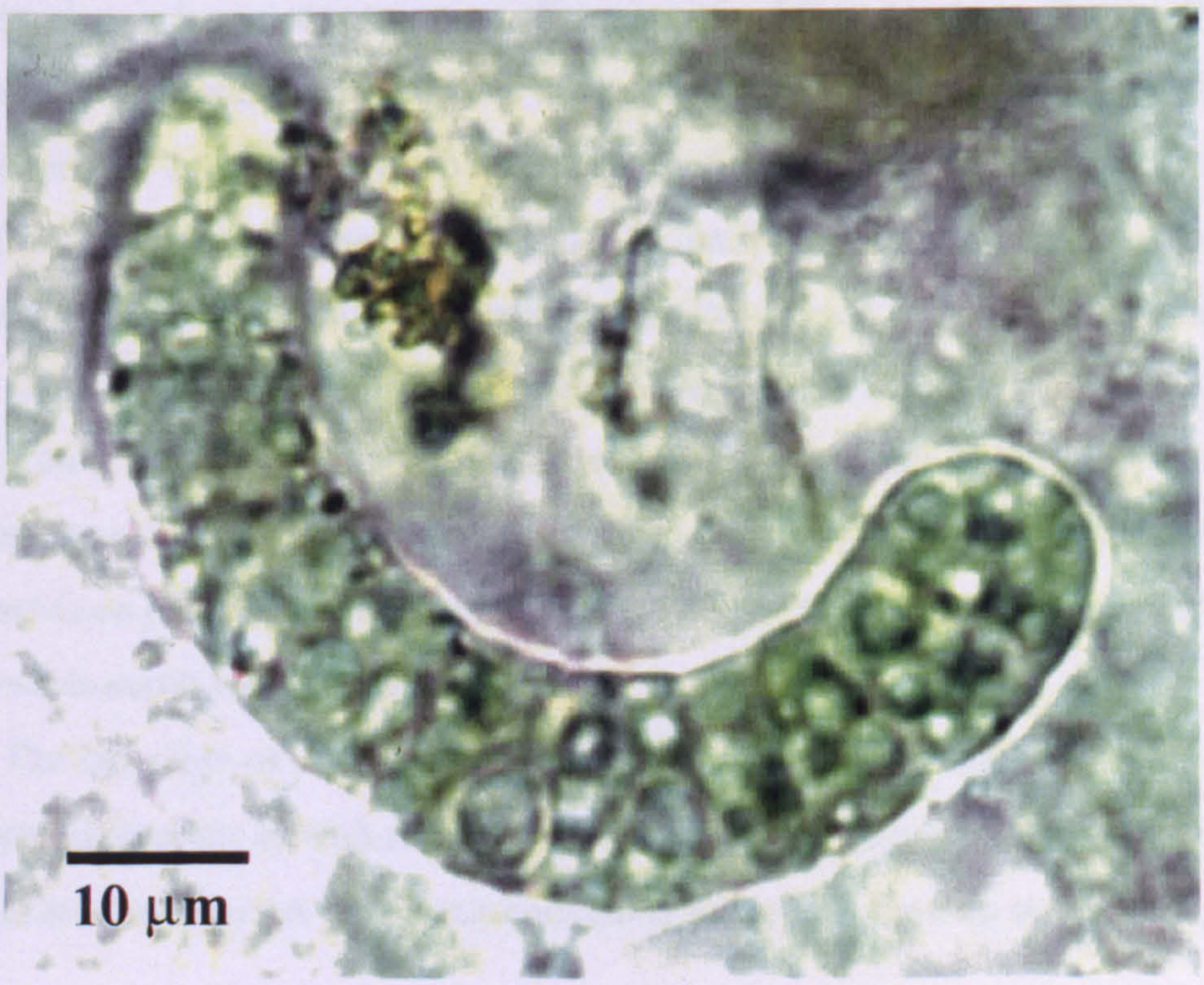


Table 7.8 Time course of adaptation of *Arthrospira* strain D0923 to growth in the presence of sucrose (20 mM). None of the replicates containing sucrose plus DCMU grew (not included in the Table).

| Days after inoculation | 10 μmol photon $m^{-2}$ $s^{-1}$ – control | 10 μmol photon $m^{-2}$ $s^{-1}$ + 20 mM sucrose | 70 μmol photon $m^{-2}$ $s^{-1}$ – control | 70 μmol photon $m^{-2}$ $s^{-1}$ + 20 mM sucrose |
|---|---|---|---|---|
| 2 | Inoculum dark blue-green; growth | Inoculum loosing pigmentation (yellow to green); cell lysis | Loss of pigmentation; biomass of inoculum aggregated in a clump, but growth taking place | Inoculum loosing pigmentation (yellow to green) ; cell lysis |
| 3 | Inoculum dark blue-green; growth | Inoculum loosing pigmentation (yellow to green) ; cell lysis | Biomass of inoculum clumped; growth occurring | Inoculum loosing pigmentation (yellow to green) ; cell lysis |
| 4 | Dark blue-green culture; growth | Inoculum loosing pigmentation (yellow to green) ; cell lysis | Recovery of all replicates and growth; biomass blue-green; good growth | Inoculum colourless: all cells seem to be lysed |
| 6 | Dark blue-green culture; growth | Inoculum loosing pigmentation (yellow or colourless) ; cell lysis | Culture blue-green; good growth | Inoculum colourless, but initiation of growth taking place |
| 7 | Dark blue-green culture; growth | Inoculum colourless; in three cultures little green patches of biomass visible | Culture blue-green; growth | More green filaments visible; growth in all ten replicates |
| 8 | Dark blue-green culture; growth | Initiation of slow growth in three replicates | Culture blue-green; reaching stationary phase | All ten replicates growing |

Obviously, the sucrose concentration of 20 mM had an effect on the growth behaviour. Therefore, a lower concentration of sucrose (5 mM) was tested. Essentially the same results were obtained, ie cell lysis of the majority of cells of the inoculum followed by recovery of a few cells, which then grew continuously, but no recovery in the presence of DCMU. However, when grown under 10 μmol photon $m^{-2}$ $s^{-1}$, more (six) replicates of the ten cultures recovered in the presence of 5 mM sucrose compared to 20 mM sucrose.

Aliquots of the ten subcultures that recovered from the sucrose up-shock at 70 μmol photon $m^{-2}$ $s^{-1}$ were used to inoculate an equivalent number (ten replicates) of 100 mL-flasks containing 30 mL Zarrouk's medium with or without 20 mM sucrose for both light conditions (10 and 70 μmol photon $m^{-2}$ $s^{-1}$). Four subcultures of strain D0923 were used as controls under each light condition.

The inoculum of neither set of subcultures grown at 70 μmol photon $m^{-2}$ $s^{-1}$, photoautotrophic or with sucrose added, showed cell lysis or underwent a lag phase. Similarly, neither set of cultures containing sucrose and grown at 10 μmol photon $m^{-2}$ $s^{-1}$

underwent bleaching or showed a lag phase.

To test whether sucrose adapted cultures are able to grow heterotrophically on sucrose, further experiments were conducted using the ten cultures adapted to sucrose at 70 µmol photon $m^{-2}$ $s^{-1}$ as inoculum.

*i) Under dark-heterotrophic conditions*

Aliquots of the ten replicates of those cultures of strain D0923, which were adapted to sucrose, were used to inoculate Zarrouk's medium containing 5 mM or 20 mM sucrose (ten replicates each). Controls existed of Zarrouk's medium without added sucrose inoculated with aliquots of either sucrose adapted cultures or photoautotrophic cultures (three replicates each). None of the sets of subcultures led to growth despite the extended period of incubation in the dark (100 days).

*ii) Under photoheterotrophic conditions*

To test the ability of sucrose adapted cultures to grow photoheterotrophically on sucrose, a comparison was drawn between the use of inoculum taken from a sucrose adapted subculture and from a photoautotrophic stock of strain D0923. 18 mL medium in 50-mL boiling tubes containing either 5 mM or 20 mM sucrose with or without DCMU added were inoculated with aliquots from a stock culture or a sucrose adapted subculture of strain D0923 and incubated at 30 °C and 70 µmol photon $m^{-2}$ $s^{-1}$. Ten replicates were tested for each of the combinations. The results showed that all replicate cultures that contained sucrose and DCMU failed to grow and lysed after approximately 20 days of incubation. This response to photoheterotrophic growth conditions was independent of the concentration of sucrose (within the range tested). However, the inocula of the cultures containing only sucrose did not show again the initial cell lysis and lag phase of growth.

## 7.3 Use of alternative nitrogen sources

The analysis of the ability of *Arthrospira* strains to utilize alternative N-sources revealed that all strains behaved qualitatively similar, ie all were able to utilize nitrate as well as ammonium and urea. The yield of biomass was in any of the N-containing media bigger than in the control cultures (Zarrouk's medium minus N). To adjust for possible differences in the inoculum between strains and potential differences in the internal N-

reserves of the strains, the resulting biomass of those N-free cultures that survived the incubation period, was deduced from the biomass of the cultures containing one of the three N-sources. The results are summarized in Table 7.9.

Table 7.9 Results of the screening for the ability of *Arthrospira* strains to utilize alternative N-sources. An N-source is regarded as having produced more biomass, if its final biomass is two mg higher than that of another N-source. N-sources separated by a hyphen indicate equal growth. (nd = not determined.)

| | Biomass yield [mg] of cultures containing combined N in form of | | | |
|---|---|---|---|---|
| Strain | Nitrate | Ammonium | Urea | "Best" N-source |
| D0867 | 11.2 | 11.9 | 11.2 | A – N – U |
| D0872/H | 19.2 | 9.4 | 8.5 | N > A – U |
| D0873 | 5.5 | 9.8 | 4.6 | A > N – U |
| D0880 | 12.9 | 10.4 | 16.8 | U > N > A |
| D0881 | 12.1 | 15.7 | 10.3 | A > N – U |
| D0882 | 11.9 | 11.2 | 9.7 | N – A – U; N > U |
| D0884 | 12.0 | 15.2 | 11.7 | A > N – U |
| D0885/H1 | 3.2 | 9.4 | 1.0 | A > N > U |
| D0890 | 11.4 | 7.1 | 7.0 | N > A – U |
| D0891 | 9.6 | 8.3 | 3.2 | N – A > U |
| D0895 | 11.3 | 14.1 | 12.5 | A > U – N |
| D0896 | 8.9 | 4.4 | 5.2 | N > U – A |
| D0897 | 12.8 | 13.3 | 6.5 | A – N > U |
| D0899 | 7.6 | 7.2 | 6.6 | N – A – U |
| D0900 | 9.5 | 11.3 | 18.8 | U > A > N |
| D0904 | 7.2 | 25.7 | 5.7 | A > N – U |
| D0905 | 17.1 | 13.9 | 4.7 | N > A > U |
| D0907 | 8.5 | 8.4 | 5.3 | N – A > U |
| D0909 | 10.5 | 12.2 | 9.9 | A – N – U; A > N |
| D0910/H | 7.6 | 7.9 | 3.8 | N – A > U |
| D0911 | 10.2 | 7.7 | 6.5 | N > A – U |
| D0913 | 8.6 | 5.6 | 7.8 | N – U > A |
| D0914 | 13.2 | 13.3 | 6.2 | A – N > U |
| D0915 | 12.3 | 8.4 | 2.8 | N > A > U |
| D0916 | 15.6 | 16.5 | 11.7 | A – N > U |
| D0918/H | 4.2 | 7.6 | 3.6 | A > N – U |
| D0919 | 6.0 | 9.7 | 4.8 | A > N – U |
| D0920 | 17.0 | 15.1 | 12.3 | N – A > U |
| D0921 | 10.3 | 10.0 | 3.6 | N – A > U |
| D0922 | 11.9 | 4.8 | 8.0 | N > U > A |
| D0923 | 1.1 | 6.8 | 0.1 | A > U – N |
| D0925 | 4.2 | 9.7 | 11.1 | U – A > N |
| D0929 | nd | nd | nd | nd |
| D0930 | nd | nd | nd | nd |
| D0933 | nd | nd | nd | nd |

Analysis of the strains for their preferred N-source shows that nitrate and ammonium are approximately equally well utilized (Table 7.10). Although urea was the substrate best utilized by least of the strains, 20 strains grew on urea better or at least as good as on either nitrate or ammonium.

Table 7.10 Summary of results of screening for the ability to utilize alternative N-sources. 32 strains were tested in total.

| | |
|---|---|
| Best growth on nitrate | 7 |
| Best growth on ammonium | 9 |
| Best growth on urea | 2 |
| Equivalent growth on nitrate and ammonium | 12 |
| Growth on urea better or as good as either on nitrate or on ammonium | 20 |

## 7.4 Growth on organic phosphorus sources and surface phosphatase activity

### 7.4.1 Growth on organic phosphorus sources

The ability to utilize organic P sources was observed qualitatively on 10 strains (Table 7.11). All 10 strains show similar responses. Growth on pNPP and Na-β-GP was similar to that on inorganic phosphate.

The four strains (D0887, D0918, D0922, D0925) which showed some growth in Zarrouk's medium with phosphodiesters and phytate also showed some growth in the absence of any phosphate source.

A control was carried out parallel to the experiments to test whether light and/or medium can cause degradation of the organic P substrate. pNPP proved to be stable in Zarrouk's medium (without inoculum) at 30 °C and 30 μmol photon $m^{-2}$ $s^{-1}$ (M. Scott, pers. comm.).

Table 7.11 Results of screening *Arthrospira* strains for their ability to utilizeorganic P-sources. + to +++++ = relative biomass after growth for 3 weeks.

| Strain | $K_2HPO_4$ | Na-β-GP | pNPP | bis-pNPP | DNA | Phytate | Control |
|---|---|---|---|---|---|---|---|
| D0867 | +++ | ++ | ++ | - | - | - | - |
| D0887 | ++++ | ++++ | ++++ | + | + | + | + |
| D0895 | +++ | ++ | +++ | - | - | - | - |
| D0896 | +++ | ++ | +++ | - | - | - | - |
| D0914 | +++++ | +++++ | ++++ | - | - | - | - |
| D0916 | ++++ | +++ | ++++ | - | - | - | - |
| D0918 | +++ | +++ | +++ | + | + | + | + |
| D0920 | +++ | ++ | +++ | - | - | - | - |
| D0922 | +++ | +++ | +++ | + | + | + | + |
| D0925 | +++ | ++ | +++ | + | + | + | + |

## 7.4.2 Surface phosphatase activity

Measurements of surface PMEase of four P-limited strains (D0885/H1, D0911, D0913, D0916) using pNPP as a substrate showed that all had detectable activity. However, despite the ability of the strains to grow on phosphomonoesters, their PMEase activity was low in all cases (in the region of 4-8 nmol pNPP μg chl $a^{-1}$ $h^{-1}$) and no obvious differences in activity existed.

Table 7.12 Results of experiments investigating surface phosphatase activity of *Arthrospira* strains.

| Strain | Phosphatase activity (nmol PNPP $h^{-1}$ $\mu g^{-1}$ chl *a*) | Standard deviation |
|---|---|---|
| D0887 | 4.11 | 0.57 |
| D0914 | 7.58 | 2.91 |
| D0916 | 8.26 | 0.61 |
| D0920 | 5.74 | 1.62 |

To investigate whether modification in experimental conditions lead to increasing phosphatase activity further, more detailed studies were undertaken on the same four strains. (These experiments were carried out by M. Scott.) The studies involved investigation of a series of experimental and environmental factors (pH, substrate concentration, Zn-concentration of growth and assay medium, light intensity, time course of P-limitation, induction of enzyme activity by growth in organic P-substrate).

The results obtained from those experiments confirmed the initial data (Table 7.12), showing that under any of the conditions tested *Arthrospira* strains possess detectable, but very low (in the range of 2-8 nmol pNP μg chl $a^{-1}$ $h^{-1}$) surface PMEase activity and lack PDEase activity.

## 7.5 Discussion

### 7.5.1 Heterotrophy

#### 7.5.1.1 Dark heterotrophy

*Arthrospira strains are capable of dark heterotrophic growth on glucose and fructose*

The screening program showed that *Arthrospira* strains can utilize in the dark only the monosaccharides glucose and fructose, but none of the disaccharides. This is the first report on the ability of *Arthrospira* strains to grow in the dark on fructose. The data agree with those from others who reported that cyanobacteria utilize glucose in the dark more frequently than fructose and sucrose (eg Rippka & Stanier, 1973).

The results of the screening program for the ability to grow in the dark on sugars were surprising in that in some cases the four replicates did not behave the same, a result differing from that of previous studies on dark heterotrophy of other cyanobacteria (eg Rippka & Stanier, 1973; Khoja & Whitton, 1975).

Two reasons are put forward to explain the differences in results and variability between replicates within a single experiment. Firstly, some minor difference in environmental conditions (such as oxygen supply) may have influenced the growth under dark heterotrophic conditions. Secondly, there may be a relatively high mutation frequency resulting in genetic drift towards the phenotype with the more favourable characters under a given environment.

*i) Strain-specific requirements for dark heterotrophy*

Strain-specific requirements for dark heterotrophic growth can be concluded from the comparison of the data for strain D0914/H from the repeat experiments. While only one or two out of four replicates of strain D0914/H grew in the original and first repeat experiment, respectively, the more frequent and intensive aeration procedure during the incubation period in the repeat experiment may be responsible for growth of all ten replicates (Fig. 7.1). Further optimization of the growth conditions by consistent bubbling air into the dark heterotrophic cultures led again to growth in all ten replicates suggesting that in the original experiment the aeration was the growth limiting factor for strain D0914/H. This suggests that there may be potential for at least those strains, that showed partial growth on glucose and/or fructose, to grow in the dark on glucose and fructose in all replicates. However, it would be beyond the scope of this project to study the specific environmental parameters necessary for dark heterotrophic growth of such a large set of *Arthrospira* strains. Furthermore, these experiments would require bioreactors that allow control of environmental parameters, such as oxygen, pH, mixing speed, as well as continuous sugar supply.

*ii) Ability to dark heterotrophy may be affected by genetic drift*

Evidence for genetic drift being responsible for the loss of the ability to grow in the dark on sugar may be provided by the report of Hihara and Ikeuchi (1997), who observed two cell types within a photoautotrophic culture of *Synechocystis* PCC 6803 showing distinct growth properties. While one type grew better under photoautotrophic conditions, the other produced larger colonies under photoheterotrophic conditions (glucose as carbon source). The authors identified a single base mutation in an up to then unknown gene (designated *pmgA*) that was responsible for the different growth characters (Hihara & Ikeuchi, 1997). In this case the loss of a functional gene essential for photoheterotrophic growth led to better growth under photoautotrophic conditions. Comparison of the laboratory cultures with a cryo-preserved sample showed that during subsequent culturing of the strain under photoautotrophic conditions the cell type capable of photoheterotrophic growth had been expelled from the photoautotrophic culture of *Synechocystis* PCC 6803, which had lost the ability to grow under photoheterotrophic conditions (Hihara & Ikeuchi, 1997).

Although the work of Hihara and Ikeuchi (1997) demonstrates that genetic drift can affect the ability to grow photoheterotrophically, a similar scenario may be responsible for the loss of the ability of *Arthrospira* strains to grow in the dark on sugars.

Due to the absence of a successful method for long-term storage of *Arthrospira* strains (Section 4.4) genetic drift affecting the ability to grow heterotrophically can only be prevented by maintenance of subcultures under both, autotrophic and heterotrophic conditions.

An alternative explanation for the occurrence of dark heterotrophic growth in some of the replicates tested may be provided by back mutation. In the case of a "simple" mutation like a point mutation, the likelihood of a potential back mutation from inability to ability to grow in the dark on sugars may be relatively high. This scenario could also explain the fact that strain D0914/H showed more frequent growth in the repeat than in the original experiment.

The hypothesis of genetic drift being responsible for the subsequent loss of the ability to utilize sugars for growth in the dark seems, however, unlikely due to the short time scale of the project.

*Lack of ability to grow in the dark on sucrose*

There is no information in the literature available on the ability of *Arthrospira* strains to grow in the dark on sucrose. The studies undertaken here show that *Arthrospira* strains can not utilize sucrose in the dark. Studies on photoheterotrophic growth of *Arthrospira* strains proved that a light-dependent adaptation process is necessary to gain the ability to grow in the presence of sucrose (Section 7.2.2.3). Using aliquots of the sucrose adapted cultures as inoculum for dark heterotrophic growth, however, did also not result in dark heterotrophic growth on sucrose.

There are some reports in the literature on how to achieve dark heterotrophic growth of cyanobacteria, which originally failed to grow on an organic carbon source in the dark (eg *Synechocystis* sp. PCC 6803: Anderson & McIntosh, 1991; *Cyanothece* sp. ATCC 51142: Schneegurt et al., 1997). However, in all those cases the carbon source was utilized by the cyanobacterium under photoheterotrophic conditions, while sucrose does not support photoheterotrophic growth of *Arthrospira* strains. Therefore, the application of similar approaches to those successful for *Synechocystis* sp. PCC 6803 and *Cyanothece* sp. ATCC 51142 are not promising for *Arthrospira* strains.

7.5.1.2 Photoheterotrophic growth

*Influence of DCMU*

To avoid partial inhibition of photosynthesis a screening program was carried out to determine the inhibitory concentration of DCMU. One µM DCMU was found to inhibit completely growth in photoautotrophic cultures of strain D0923, and, therefore, presumably, all *Arthrospra* strains in general. However, to have comparable experimental conditions to those in the literature (eg Rippka & Stanier, 1973), DCMU was used in a (final) concentration of 10µM in any of the experiments investigating photoheterotrophic growth. This was also to ensure that photosynthesis of all cells of a culture, including those, that may be surrounded by a thicker sheath or excreted slime, would be inhibited.

The decrease of pigmentation upon exposure of photoautotrophic cultures to light intensities of 60 to 80 µmol photon $m^{-2}$ $s^{-1}$ indicates photodamage through high light stress (Jensen & Knutsen, 1993). This was surprising, as most of the research on physiological and biochemical aspects of *Arthrospira* strains has been testing even bigger shifts in light intensity (eg 75/150/300 µmol $m^{-2}$ $s^{-1}$; Cohen et al., 1987) without cultures being adapted to the environment, under which they were tested. However, in such cases cultures of larger volume (eg 500 mL; Cohen et al., 1987), often in combination with continuous mixing, were used for the experiments. A large culture volume leads to shading effects of cells, especially if the cell density of the culture is high. Furthermore, continuous mixing ensures that cells of a culture grown under high a light environment are not permanently exposed to the high light flux (Richmond, 1999). Due to the large set of strains tested in this work, however, the only practicable way to achieve standardized conditions for all cultures of an experiment was to use stationary 30-mL cultures. The permanent exposure to light flux of the cells at the surface of a stationary culture seemingly causes photoinhibition at lower light intensity compared to mixed cultures.

However, the initial failure to do so led to the observation that DCMU in concentrations >10nM in both photoautotrophic and photoheterotrophic cultures leads to protection of the culture against photodamage caused by a high irradiance. Although much work has been done on the ability to photoheterotrophic growth of *Arthrospira* strains including the use of DCMU (Marquez et al., 1993; Chen & Zhang, 1997) such an effect has not been reported previously for this genus. Similar observations of a photosystem-stabilizing effect of DCMU have only been described recently for *Synechocystis* PCC 6803 and *Synechococcus*

PCC 7942 (Komenda & Masojídek, 1998). The authors report that DCMU cause a stabilization of photosystem II by slowing down the D1 turnover.

*Photoheterotrophic growth on glucose and maltose*

The ability to grow photoheterotrophically on glucose and maltose was analyzed for 10 *Arthrospira* strains. All of the strains tested proved to be capable to grow on these sugars in the presence of DCMU, but better growth was obtained without the inhibitor. This agrees with the results of Marquez et al. (1993), who observed that the sum of the yield of photoautotrophic and photoheterotrophic growth on glucose equals the yield of growth in the presence of glucose. As the ten strains analyzed included strains that grew heterotrophically in the dark in none, some or all of the four replicates on glucose, it can be assumed that the results are representative for all *Arthrospira* strains. This is confirmed by several reports of photoheterotrophic growth of *Arthrospira* strains which have not been analyzed in this project (eg Ogawa & Terui, 1972). Therefore, the screening was not extended to all strains.

*Lack of ability to grow photoheterotrophically on fructose and sucrose*

None of the ten strains tested utilized fructose for growth under photoheterotrophic conditions. This is somewhat surprising, as the same strains, that grew on fructose in the dark, failed to support photoheterotrophic cultures. The lack of photoheterotrophic growth does not seem to be due to lack of an fructose uptake mechanism, as addition of 20 mM fructose to the medium caused cell death. An explanation for this phenomenon may be provided by a toxic effect of fructose. Fructose toxicity under photoheterotrophic conditions has also been observed for *Synechocystis* spp. (Joset et al., 1988) and *Cyanothece sp.* (Schneegurt et al., 1997). In the latter case, the toxicity was also dependent on the concentration of fructose added to the medium.

Similarly to fructose, sucrose did not support growth of any of the 20 *Arthrospira* strains tested in the presence of DCMU and is, presumably, characteristic for all *Arthrospira* strains. A further similarity to fructose concerns the toxic effect of sucrose on *Arthrospira* strains. This was dependent on the concentration of sucrose when grown under 10 µmol photon $m^{-2}$ $s^{-1}$, as the number of replicates recovering from the sucrose up-shock was higher when 5 mM sucrose were added compared to 20 mM. The time period needed for cell lysis and subsequent growth of a culture from very few cells as well as the number of replicates

recovering proved to be dependent on the light intensity the cultures were exposed to. The cells of the inocula were not adapted to high light intensity. The additional stress may be responsible for the fact that cell lysis occurred the faster, the higher the light intensity was, which the cells were exposed to. However, the combination of the two stress factors is not the reason for the cell lysis taking place, as the cell lysis occurred under any of the light intensities tested (within the range provided: 10 µmol photon $m^{-2}$ $s^{-1}$ to 70 µmol photon $m^{-2}$ $s^{-1}$). The fact that more replicates recovered at the higher light flux may indicate that the adaptation process is energy dependent.

The data obtained in this study agree with the results of Tomaselli et al. (1978) (Section 1.5.1). The strain of *Arthrospira platensis* used by Tomaselli et al. (1978) is also included here (strain "C1" = D0918). Both studies show that strain C1 (D0918/H) is able to grow in the presence of sucrose but not able to utilize it for growth. Unfortunately, the strain of *Arthrospira maxima* of the study of Tomaselli et al. (1978) was not available for this work.

*Formation of intra-thylakoidal granules*

The analysis of the "colourless" inoculum of *Arthrospira* strain D0923 upon sucrose up-shock shows that the process is not bleaching followed by regaining of the pigmentation, but that a few cells survive the sucrose up-shock by formation of intra-thylakoidal vacuoles. All other cells are lysed and cell walls are broken. Therefore, the very few survivors must be the "seeds" for the growth to a new culture. However, "bleaching" of photoheterotrophic cultures of cyanobacteria has been reported previously for *Spirulina* (*Arthrospira*) *platensis* in medium containing 111 mM glucose (Ogawa & Terui, 1970, 1972). The authors defined this phenomenon as "mixotrophic lysis". To test the occurrence of "mixotrophic lysis" of *Arthrospira* strains caused by photoheterotrophic growth on glucose, the experiment of Ogawa and Terui (1972) was repeated. The results of the experiment show that photoheterotrophic growth of strain D0923 on 2% (w/v) glucose (111mM) does not lead to bleaching or lysis of the inoculum. The reasons for the different result in this study are unknown, but may be explained by the fact that different strains have been used in both studies.

While trying to obtain a photoheterotrophic culture of *Cyanothece* sp. growing on 50mM glycerol, Schneegurt et al. (1997) also report that after an initial "period of acclimation, and accompanying bleaching and slowed growth, a stable line was obtained that grew well under these conditions". None of the researchers, however, analysed the "bleaching" cells

of the inoculum for morphological changes. Schneegurt et al. (1997) assumed that the "bleached" inoculum was "regaining its pigmentation".

Once a cell has been adapted to the presence of sucrose in the growth medium, no further lysis of the inoculum occurs upon transfer into fresh sucrose containing medium, indicating that the mechanism necessary for growth in the presence of sucrose has been established in these cells. The mechanism that is responsible for degradation and/or excretion of the intra-thylakoidal granules or their breakdown products is, however, unknown.

Only a very few cells seem to be capable of this adaptation process while the majority of cells of the inoculum lyse. The surviving cells seem to be either mutants able to grow in the presence of sucrose or cells that are in a very 'healthy' state with sufficiently large ATP reserves, which seems to be necessary for the active deposition of sucrose or sucrose breakdown products into intra-thylakoidal vacuoles. Furthermore, the availability of intra-thylakoidal vacuoles at the time of the sucrose up-shock for the immediate deposition of sucrose or its breakdown products may provide a further advantage those survivors have over other cells.

Sucrose added to Zarrouk's medium may have been deposited directly as sucrose into the intra-thylakoidal vacuoles observed in *Arthrospira* strains, or, after initial degradation, in form of a break down product, thus avoiding cell death by osmotic pressure. However, detailed analysis of the composition and ultrastructure of the intra-thylakoidal granules induced by sucrose up-shock will prove difficult. Main reason for this is the fact that only very few cells survive the sucrose up-shock. Furthermore, once these cells start to grow they also lose the intra-thylakoidal granules. This and the fact that less cells seem to survive the sucrose up-shock under 70 µmol photon $m^{-2}$ $s^{-1}$ may be the reasons for the failure to find cells containing the large intra-thylakoidal granules in the cultures exposed to 70 µmol photon $m^{-2}$ $s^{-1}$.

### 7.5.1.3 Heterotrophy as taxonomic marker

Variation in the ability to heterophic growth on organic carbon sources has often been used in the past to characterize cyanobacterial isolates (eg Rippka et al., 1979). However, this study shows that differences in the ability to utilize the organic carbon source may occur between replicates as well as lack in reproducibility. This must be considered if the

ability to heterotrophic growth is being used to characterize *Arthrospira* strains, or, possibly, cyanobacteria in general.

### 7.5.2 Growth on alternative nitrogen sources

The different N-sources tested were utilized by all *Arthrospira* strains, but none of the strains grew in N-free medium, indicating absence of nitrogenase activity (Rippka et al., 1979). The addition of urea, however, proved to necessitate fed-batch culture, as urea added to a final N-concentration equivalent to that of Zarrouk's medium (411 mg N $L^{-1}$) led to cell death. Sakamoto et al. (1998) showed that growth on urea triggers cell death of *Synechococcus* sp. strain PCC 7002. The precise mechanisms for this phenomenon, however, are still unknown, but fatty acid peroxidation has been found to represent an initial step in the biochemical cascade leading to cell death (Sakamoto et al., 1998). To overcome urea-induced cell death the concentration of N in Zarrouk's medium was lowered to 5 mg N $L^{-1}$ under fed-batch conditions.

The analysis of the growth observed for the different N-sources shows that there are quantitative differences between strains in their abilities to utilize the different N-sources. Although standardized conditions were ensured for all strains, care must be taken when using the data for taxonomic purposes. The final biomass obtained by cultures is much dependent on the physiological state of the inoculum. Although the stock cultures were treated similarly minor differences (Section 3.6.2) in the state of the inoculum may have affected the yield of the culture.

### 7.5.3 Growth on organic phosphorus sources

Analysis of ability to grow in the presence of phosphomonoesters as the sole P-source indicates that all of the *Arthrospira* strains tested grew approximately as well as the control cultures in medium containing inorganic P. However, assays for surface PMEase activity showed that *Arthrospira* strains only possess a very low surface phosphatase activity. It seems that the substrate and/or product is either retained inside the cell or at the cell surface. After extended culture the product is released as judged by the yellow colour (pNP) of the medium.

The four strains (D0887, D0918, D0922, D0925) which appeared to show some growth

in Zarrouk's medium with phosphodiesters and phytate also did so in the absence of any phosphate source, suggesting that the original inocula in these cases were less P starved than that of the other strains.

None of the diesters or phytic acid were utilized by any of the *Arthrospira* strains tested. Similarly, Whitton et al. (1991) reported that phosphomonoesters are being utilized by all cyanobacteria tested (50), while some lack the ability to utilize diesters or phytic acid.

The survey of the surface phosphatase activity of 50 cyanobacterial isolates from 10 genera showed that this physiological character can be used for taxonomic grouping of strains at both genus and strain level (Whitton et al., 1991). In contrast to that survey, *Arthrospira* strains do not show great variation between the growth on phosphomonoesters and that on the inorganic P-source. Furthermore, the lack of utilization of a wide range of organic P-substrates limits the availability of characters for taxonomic purposes. Therefore, growth on alternative P-sources does not represent a useful marker for taxonomic purposes within the genus *Arthrospira*, and the experiments were not extended to the rest of the strains.

## 7.6 Summary

i) Glucose and fructose were the only carbohydrates that supported dark heterotrophic growth. Glucose was utilized by more strains than fructose. For several strains, however, the results proved to be not reproducible. This was more significant when fructose was used as carbon source.

ii) The inconsistency of results observed for the ability of *Arthrospira* strains to dark heterotrophic growth may be due to the requirement for strain specific environmental conditions or genetic drift. This has to be considered when dark heterotrophy is being used for taxonomic purposes.

iii) All *Arthrospira* strains tested were able to grow photoheterotrophically on glucose and maltose.

iv) *Arthrospira* strains grew in the presence of 5 mM fructose, but fructose did not seem to support photoheterotrophic growth. This was the case for all strains tested. 20 mM fructose was toxic to *Arthrospira* strains.

v) Growth of *Arthrospira* spp. in the presence of sucrose occurs via an adaptation process. Most of the cells of the inoculum lyse after inoculation of Zarrouk's medium containing sucrose. Subsequently, the few surviving cells grow to a culture.

vi) The occurrence of interthylakoidal granules in those cells, which survived the sucrose upshock, suggests that sucrose or its breakdown products are actively deposited in granules. Sucrose induced cell death may be caused by either toxicity of sucrose or its breakdown products or osmotic pressure within the cell after uptake of sucrose.

vii) The survival rate of the inoculum and start of growth after recovery depended on the light intensity the cultures are exposed to: the higher the light intensity (within the range tested) the faster the adaptation process and yield.

viii) *Arthrospira* strains showed quantitative differences in their abilities to grow on alternative N-sources, but nitrate is the N-source that best supports generally growth.

ix) *Arthrospira* strains can utilize organic phosphomonoesters, but not diesters or phytic acid. However, the quantitative differences in the enzyme activity between strains seem to be insufficient for taxonomic purposes. Measurement of PMEase activity indicates that the product is immobilized at the cell surface.

# CHAPTER 8 BIOCHEMICAL CHARACTERS

## 8.1 Introduction

As fatty acids have been shown in the past to be useful for the classification of a large number of cyanbacterial taxa, including the genera *Arthrospira* and *Spirulina* (Section 1.6.1), it was planned to analyze the fatty acid composition of the set of *Arthrospira* strains. To test whether γ-linolenic acid can be used as a chemotaxonomic marker to differentiate between both genera, the fatty acid composition of five *Spirulina* strains was also analysed.

Information on the impact of environmental factors on the fatty acid composition of *Arthrospira* strains is still very limited regarding both, number of strains and different environments tested. Therefore, it was planned to investigate the impact of five different environments on the fatty acid desaturation of 10 *Arthrospira* strains.

Because *Arthrospira* strains were found to possess a glycocalyx (Section 6.3) which may show appropriate binding sites for plant lectins, it was planned to test this feature for its potential as a taxonomic character.

## 8.2 Fatty acids

### 8.2.1 Fatty acid composition and content

The fatty acid composition of the *Arthrospira* strains was formed by a range of fatty acids. The most abundant fatty acids in all strains tested were palmitic acid (hexadecanoic acid), γ-linolenic acid and linoleic acid. In contrast, none of the six *Spirulina* strains tested contained γ-linolenic acid. As a precise determination of the content of a fatty acid is only possible for those fatty acids, which are present in high concentrations, the following studies focus on the three most abundant ones (Fig. 8.1).

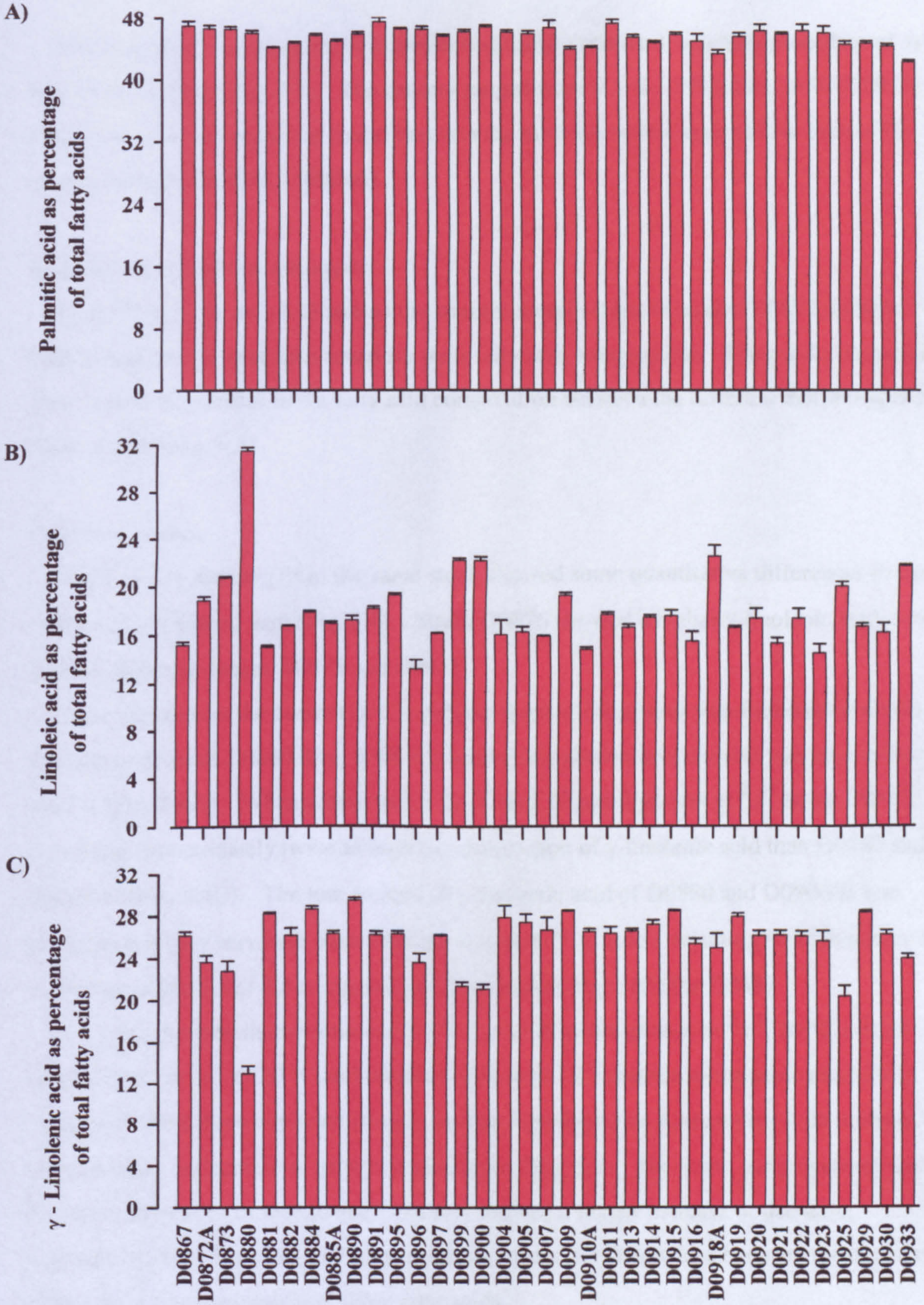



Fig. 8.1 Comparison of the fatty acid composition of the 35 *Arthrospira* strains.

Cultures were grown for ten days at 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$. Data for the three most abundant fatty acids are shown. The error bars represent the standard deviation of three replicates.

The γ-linolenic acid content was determined using a standard weighing procedure of ash-free dried biomass (Fig. 8.2). The content ranged from 0.3 % (D0920) to 2.6 % (D0910). However, great variation was found between the three replicates analyzed for each of the strains (ie high standard deviation).

*Helical and straight morphotypes*

In addition to the set of 35 strains the straight morphotypes of strains D0872, D0906, D0910 and D0918 were also tested for their fatty acid composition. The results showed that there are no differences in the fatty acid composition between the different morphotypes of those strains (Fig. 8.3).

*Duplicate strains*

Other strains deriving from the same stock showed some quantitative differences in the content of γ-linolenic acid (Fig. 8.4). Strain D0876 showed a higher γ-linolenic acid content than its duplicate strains D0875 and D0911.

Great variation in the content of $C_{18}$ polyunsaturated fatty acids was found between the duplicate strains of D0880 (Fig. 8.4B, C), while the palmitic acid content was approximately similar (Fig. 8.4A). When grown under 30 °C and 10 μmol photon $m^{-2}$ $s^{-1}$ strain D0887 contained approximately twice as high a concentration of γ-linolenic acid than D0880 and D0906/H (Fig. 8.4C). The low content of γ-linolenic acid of D0880 and D0906/H was accompanied by a very high content of linoleic acid (Fig. 8.4B), resulting in a γ-linolenic acid to linoleic acid ratio of 0.4 compared to a four-fold as high ratio for D0887.

Changing the growth environment to 30 °C and 70 μmol photon $m^{-2}$ $s^{-1}$, 20 °C and 10 μmol photon $m^{-2}$ $s^{-1}$ or 20 °C and 60 μmol photon $m^{-2}$ $s^{-1}$ resulted in the same qualitative changes of these three duplicate strains: increasing γ-linolenic acid content with increase of light intensity or decrease of growth temperature (Fig. 8.5). However, the γ-linolenic and linoleic acid content of D0880 and D0906/H showed a bigger increase or decrease, respectively, than that of D0887, thus resulting in smaller differences between the duplicate strains regarding the content of these fatty acids.

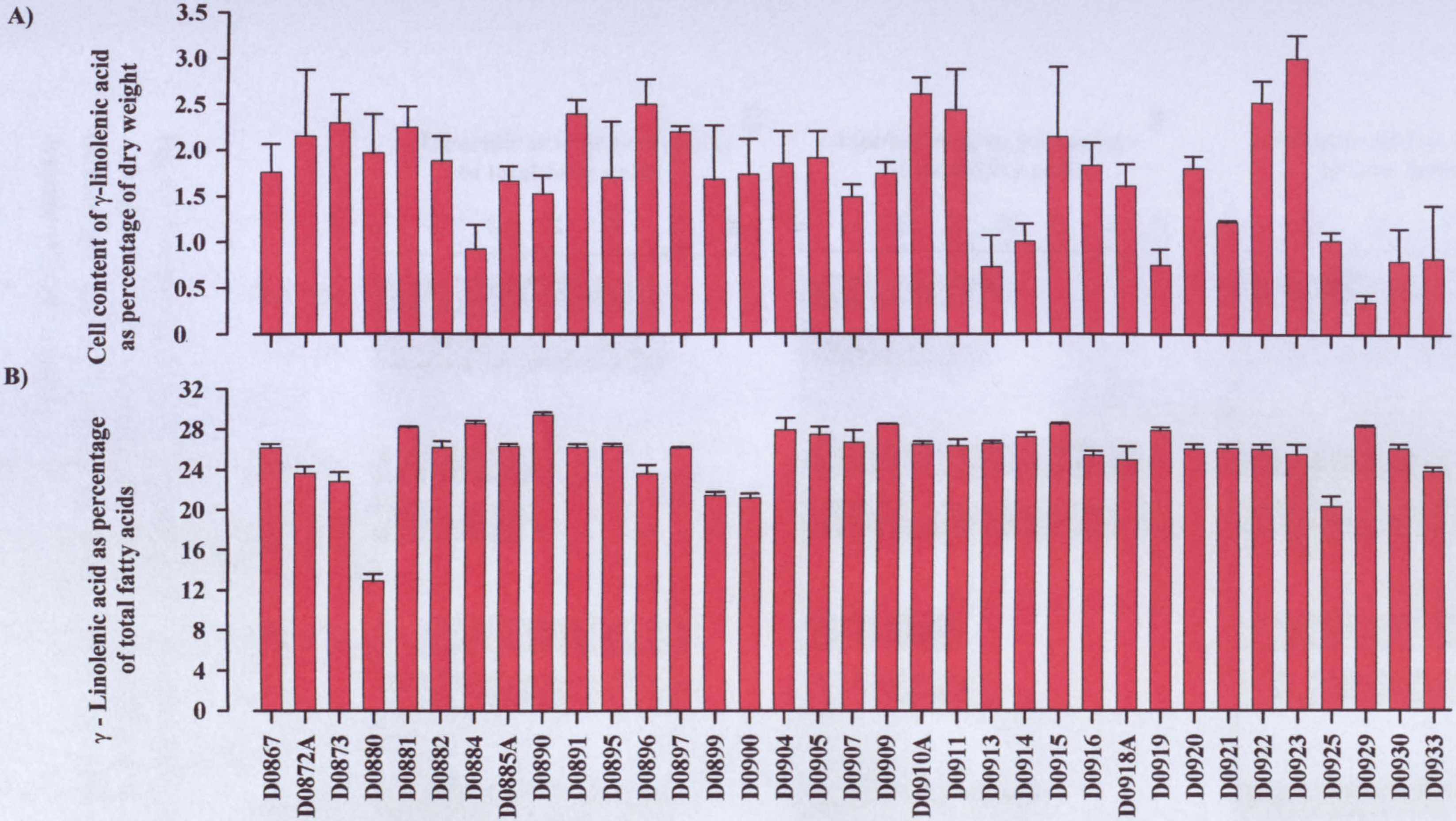



Fig. 8.2 Cellular content of γ-linolenic acid of the 35 *Arthrospira* strains.

Cultures were grown for ten days at 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$. The error bars represent the standard deviation of three replicates.

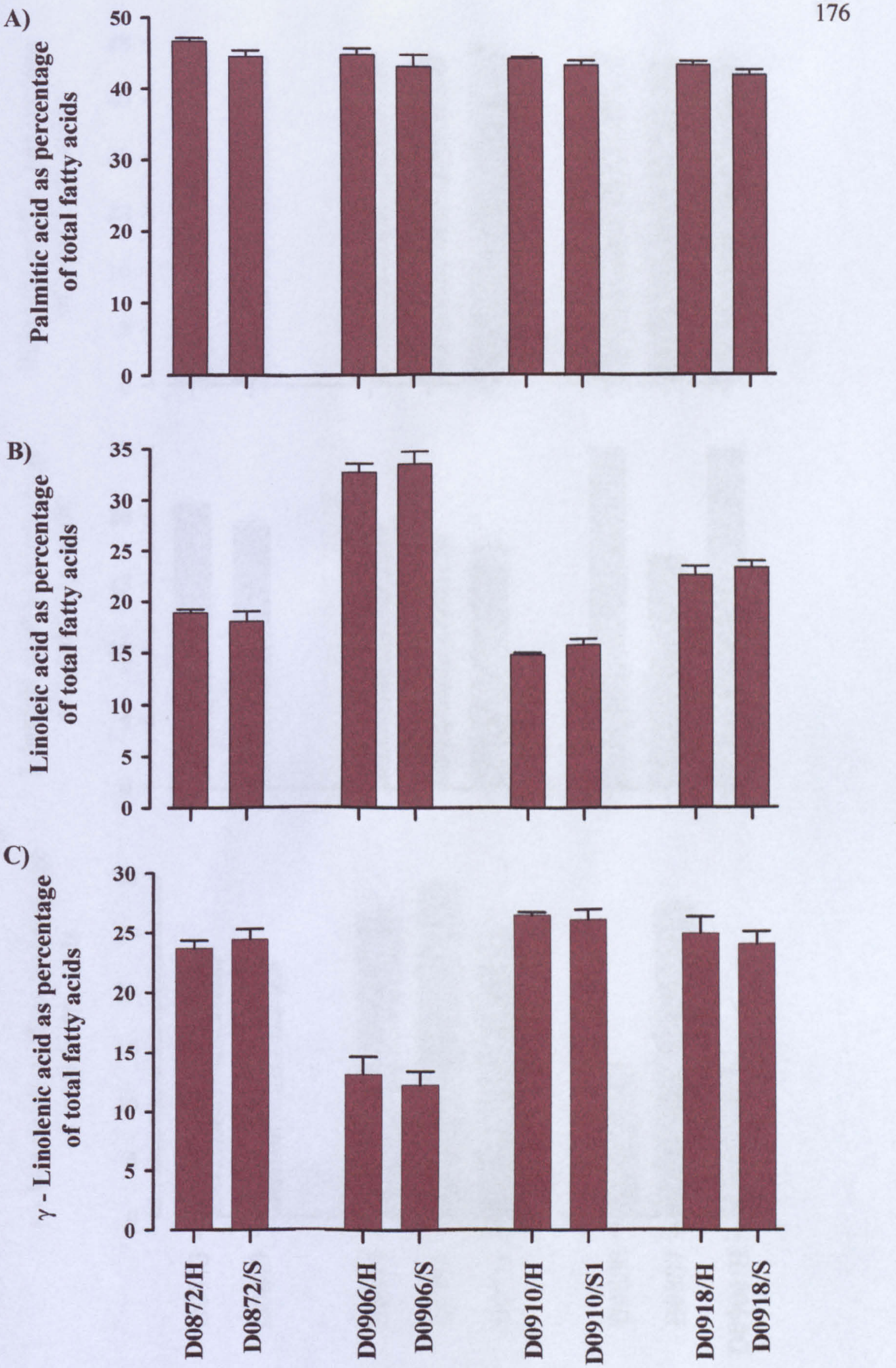



Fig. 8.3 Comparison of the fatty acid composition of helical and straight morphotypes.

Cultures were grown for ten days at 30 °C and 10 μmol photon $m^{-2}$ $s^{-1}$. Data for the three most abundant fatty acids are shown. The error bars represent the standard deviation of three replicates.

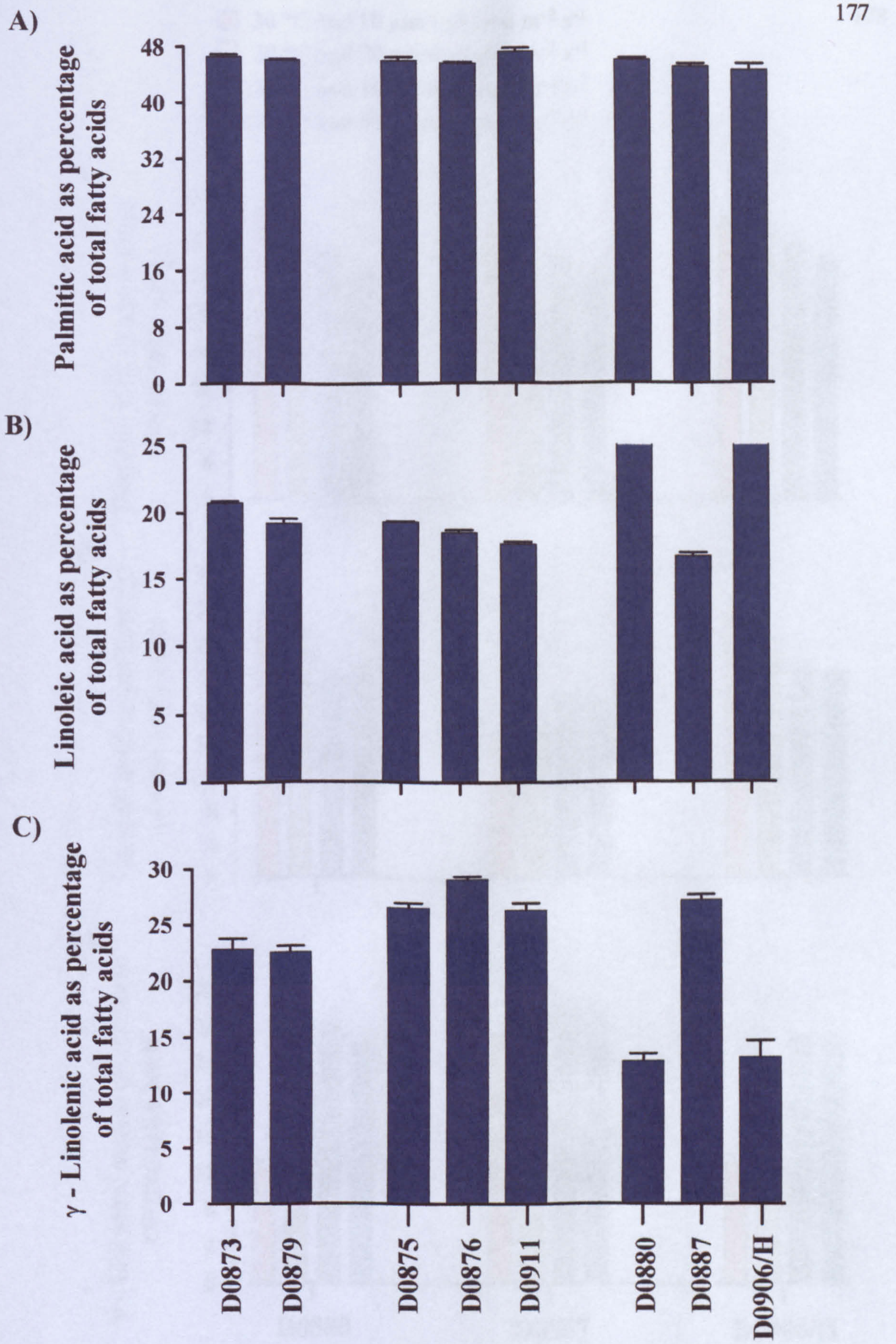



Fig. 8.4 Comparison of the fatty acid composition of duplicate strains.

Cultures were grown for ten days at 30 ºC and 10 µmol photon $m^{-2}$ $s^{-1}$. Data for the three most abundant fatty acids are shown. The error bars represent the standard deviation of three replicates.

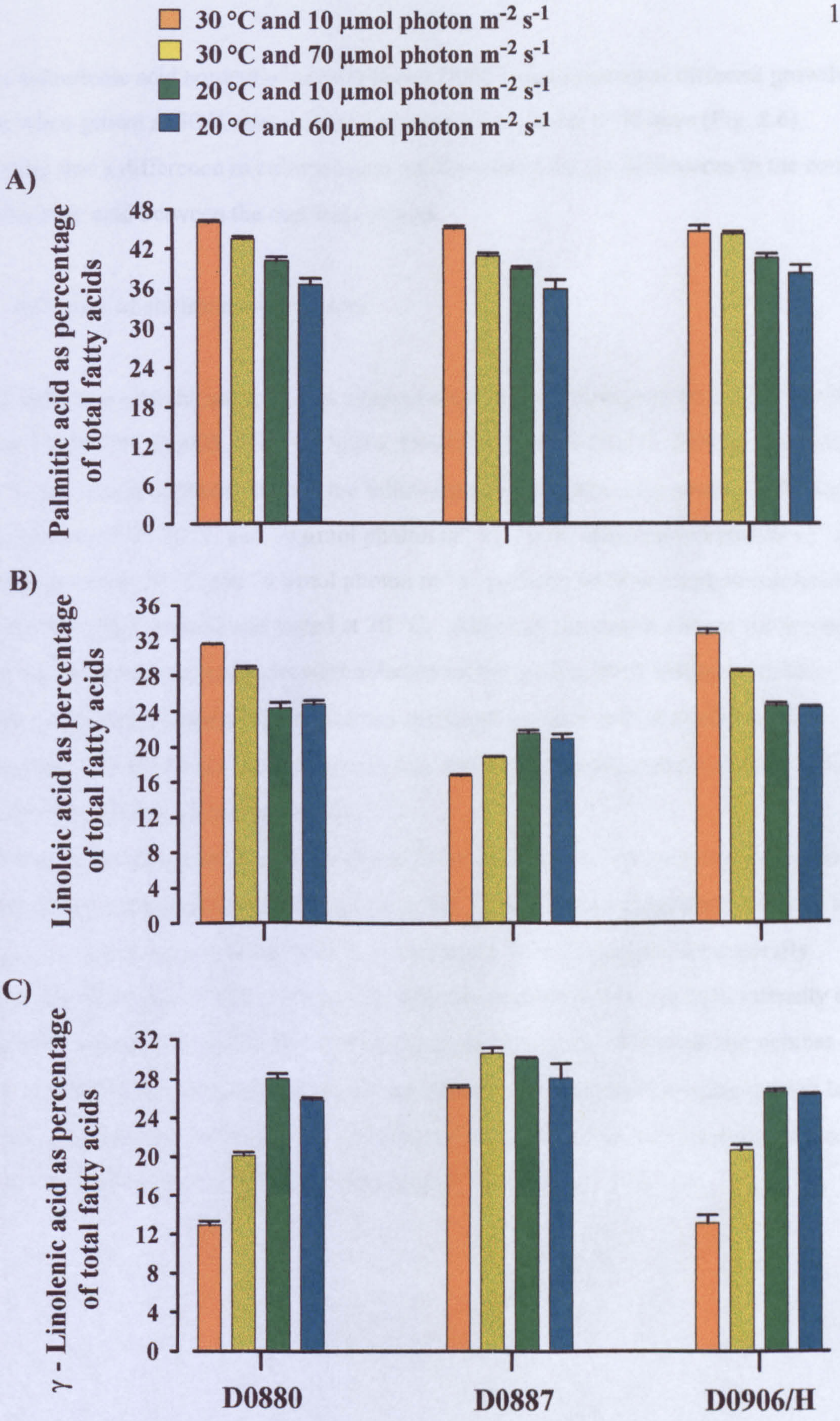



Fig. 8.5 The fatty acid composition of the duplicate strains D0880, D0887 and D0906 under four different environments.

Data for the three most abundant fatty acids are shown. The error bars represent the standard deviation of three replicates.

The γ-linolenic acid content of D0906/H and D0887 was constant at different growth stages when grown at 30 °C and 70 μmol photon $m^{-2}$ $s^{-1}$ for up to 40 days (Fig. 8.6), indicating that a difference in culture age is not the reason for the differences in the content of γ-linolenic acid between the duplicate strains.

### 8.2.2 Influence of environmental factors

The influence of light intensity and temperature was investigated on 10 strains: D0867, D0880, D0885/H1, D0905, D0911, D0916, D0918/H, D0920, D0923, D0925. In addition to 30 °C and 10 μmol photon $m^{-2}$ $s^{-1}$, the following environments were tested: 30°C and 70 μmol photon $m^{-2}$ $s^{-1}$, 20 °C and 10 μmol photon $m^{-2}$ $s^{-1}$, 20 °C and 60 μmol photon $m^{-2}$ $s^{-1}$ (cultures grown at 20 °C and 70 μmol photon $m^{-2}$ $s^{-1}$ partially underwent photoinhibition). Dark-heterotrophic growth was tested at 30 °C. Although the strains chosen for the dark-heterotrophic growth experiments were selected on the basis of their ability to utilize glucose in the dark (Section 7.2.1), biomass sufficient for fatty acid analysis was only obtained for 7 of the 10 strains tested (only one dark-heterotrophic culture of strains D0920 and D0925 was obtained and analyzed).

The strains tested responded differently to the environmental changes concerning the content of the polyunsaturated $C_{18}$ fatty acids (Fig. 8.7), though a general trend can be seen (Table 8.1). Increasing light intensity and decreasing growth temperature generally favoured the desaturation of $C_{18}$ fatty acids, while a combination of low light intensity and high growth temperature led to elevated levels of palmitic acid. Although the number of strains tested was limited to seven, the results indicate that dark heterotrophic growth led to increasing desaturation of linoleic to γ-linolenic acid compared to cultures grown under low light intensity at the same temperature (Fig. 8.7B, C; Table 8.1).

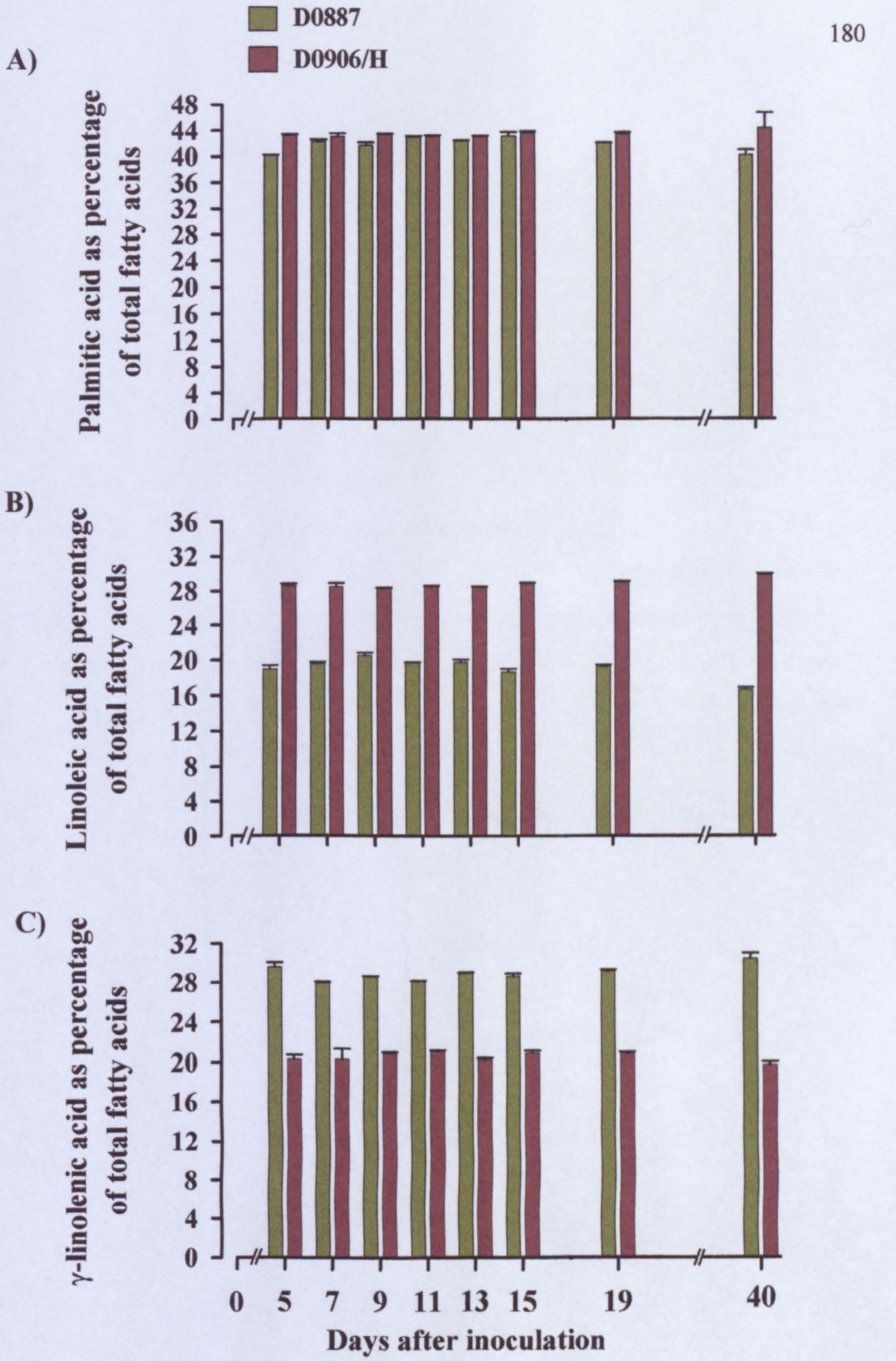



Fig. 8.6 Fatty acid composition of D0887 and D0906/H during a culture period of forty days.

Cultures were grown for ten days at 30 ºC and 70 µmol photon $m^{-2}$ $s^{-1}$. The error bars represent the standard deviation of three replicates.

Fig. 8.7 Comparison of the fatty acid composition of ten strains grown under five different environments.

The growth period was dependent on the growth environment (see text for details). Data for the three most abundant fatty acids are shown. The error bars represent the standard deviation of three replicates. The colours represent the following environmental conditions under which the cultures were grown:

- Dark-heterotrophic growth
- 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$
- 30 °C and 70 µmol photon $m^{-2}$ $s^{-1}$
- 20 °C and 10 µmol photon $m^{-2}$ $s^{-1}$
- 20 °C and 60 µmol photon $m^{-2}$ $s^{-1}$

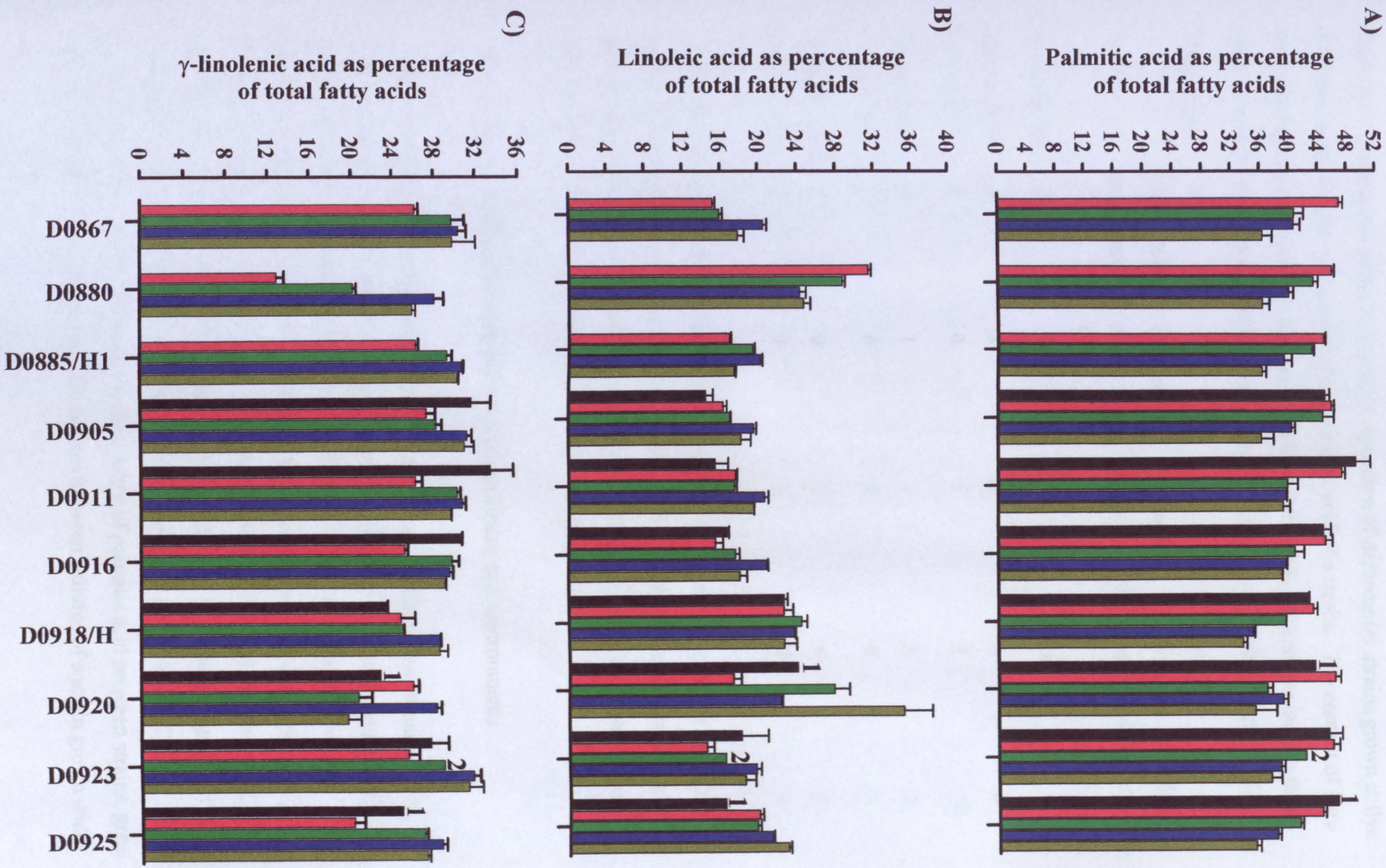

A)
Palmitic acid as percentage of total fatty acids
0 4 8 12 16 20 24 28 32 36 40 44 48 52
B)
Linoleic acid as percentage of total fatty acids
0 4 8 12 16 20 24 28 32 36 40
C)
γ-linolenic acid as percentage of total fatty acids
0 4 8 12 16 20 24 28 32 36
D0867
D0880
D0885/H1
D0905
D0911
D0916
D0918/H
D0920
D0923
D0925
1
2

Table 8.1 Comparison of the fatty acid composition of *Arthrospira* strains grown at five different environments - The most abundant fatty acid of a strain. The content of fatty acids is assessed in form of the percentage of total fatty acids. (Numbers include those cases in which two or more strains showed a similar content of a particular fatty acid.)

| Temperature | 30 °C | | | 20 °C | |
|---|---|---|---|---|---|
| | Dark hetero-trophic growth | 10 µmol photon $m^{-2}$ $s^{-1}$ | 70 µmol photon $m^{-2}$ $s^{-1}$ | 10 µmol photon $m^{-2}$ $s^{-1}$ | 60 µmol photon $m^{-2}$ $s^{-1}$ |
| Strains tested | 7 | 10 | 10 | 10 | 10 |
| Highest 16:0 | 4 | 8 | 0 | 0 | 0 |
| Lowest 16:0 | 0 | 0 | 1 | 1 | 10 |
| Highest 18:2 | 0 | 1 | 1 | 5 | 2 |
| Lowest 18:2 | 4 | 6 | 0 | 0 | 0 |
| Highest γ-18:3 | 3 | 0 | 0 | 6 | 3 |
| Lowest γ-18:3 | 1 | 9 | 1 | 0 | 0 |

The overall content of γ-linolenic acid of the cultures grown under any of the four further environments tested was also determined. The results obtained, however, showed very high variations between the replicates analyzed for each strain, thus making any statement about the overall content of the fatty acids impossible.

### 8.2.3 Variation in fatty acid composition between strains and environments

Analysis of the differences in the fatty acid content of the three most abundant fatty acids shows that greatest variation between strains is found in the polyunsaturated $C_{18}$ fatty acids compared to palmitic acid (Fig. 8.1; Table 8.2A). Under any of the five environments tested linoleic acid varied more between strains than γ-linolenic acid. Greatest variation between strains was found in the content of the polyunsaturated $C_{18}$ fatty acids of cultures grown under 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$. Cultures grown at 20 °C showed generally less variation between strains than cultures grown at 30 °C.

In contrast to the small differences in the content of palmitic acid between strains, great variation was found in the palmitic acid content between cultures of a strain grown under

different environments (Fig. 8.7A; Table 8.2B). The polyunsaturated $C_{18}$ fatty acids showed generally less variation between the different environments (Table 8.2B) than between strains (Table 8.2A). However, the overall variation was greatest in γ-linolenic acid followed by linoleic acid, while palmitic acid showed the least variation (Table 8.2C).

Table 8.2 Variation in fatty acid composition between strains or induced by changes in the growth environment. The variation is expressed as the percentage of fatty acids of the lowest content (as percentage of total fatty acids) of a particular fatty acid to the highest content of the same fatty acid. The data is based on ten strains studied at four different environments and seven strains studied under dark heterotrophic conditions. Only data for the three most abundant fatty acids is shown.

8.2A Variation in the fatty acid content between strains.

| | 16:0 | 18:2 | γ-18:3 |
|---|---|---|---|
| 30°C and dark-heterotrophic growth [1)] | 86.5 | 60.4 | 68.6 |
| 30°C and 10 µmol photon $m^{-2}$ $s^{-1}$ | 91.5 | 45.9 | 47.1 |
| 30°C and 70 µmol photon $m^{-2}$ $s^{-1}$ | 88.2 | 49.5 | 67.5 |
| 20°C and 10 µmol photon $m^{-2}$ $s^{-1}$ | 86.0 | 80.3 | 88.4 |
| 20°C and 60 µmol photon $m^{-2}$ $s^{-1}$ | 86.0 | 49.4 | 66.7 |

[1)] Based on data of only seven of the ten strains.

8.2B Variation in the fatty acid content of a strain grown under different environments.

| | 16:0 | 18:2 | γ-18:3 |
|---|---|---|---|
| D0867 [1)] | 77.6 | 74.1 | 88.0 |
| D0880 [1)] | 79.2 | 77.2 | 46.1 |
| D0885/H1 [1)] | 80.9 | 84.2 | 85.7 |
| D0905 | 78.7 | 79.5 | 86.6 |
| D0911 | 77.7 | 78.9 | 78.9 |
| D0916 | 84.9 | 74.3 | 82.8 |
| D0918/H | 77.8 | 92.7 | 81.8 |
| D0920 | 76.1 | 49.1 | 69.9 |
| D0923 | 81.5 | 73.4 | 80.7 |
| D0925 | 74.0 | 68.7 | 70.6 |

[1)] Based on data of only four of the five environments tested (not dark heterotrophic growth).

Table 8.2C Analysis of variation between strains and environments. The variation is expressed as the percentage of fatty acids of the lowest environmentally induced variation to the highest environmentally induced variation (see Table 8.2B).

| Fatty acid | 16:0 | 18:2 | γ-18:3 |
|---|---|---|---|
| Variation | 87.2 | 74.1 | 52.4 |

### 8.2.4 Reproducibility of the data

High variation in the fatty acid content was found between strains when the cultures of the strains were grown under either 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$ or 30 °C and 70 µmol photon $m^{-2}$ $s^{-1}$ (Table 8.2A). To test the reproducibility of the data from the analysis of the fatty acid composition of cultures grown under these environments, the corresponding experiments were repeated using several randomly chosen strains and the two environmental conditions: eight strains (D0867, D0872/H, D0873, D0904, D0905, D0918/H, D0920, D0923) were grown again at 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$ and six strains (D0880, D0911, D0916, D0920, D0923, D0925) under 30 °C and 70 µmol photon $m^{-2}$ $s^{-1}$.

The data of the repeat experiments demonstrate high reproducibility of the fatty acid composition of *Arthrospira* strains (Fig. 8.8), which was higher for the cultures grown at 30 °C and 70 µmol photon $m^{-2}$ $s^{-1}$ (Fig. 8.8B) than those grown at 30 °C and 10 µmol photon $m^{-2}$ $s^{-1}$ (Fig. 8.8A).

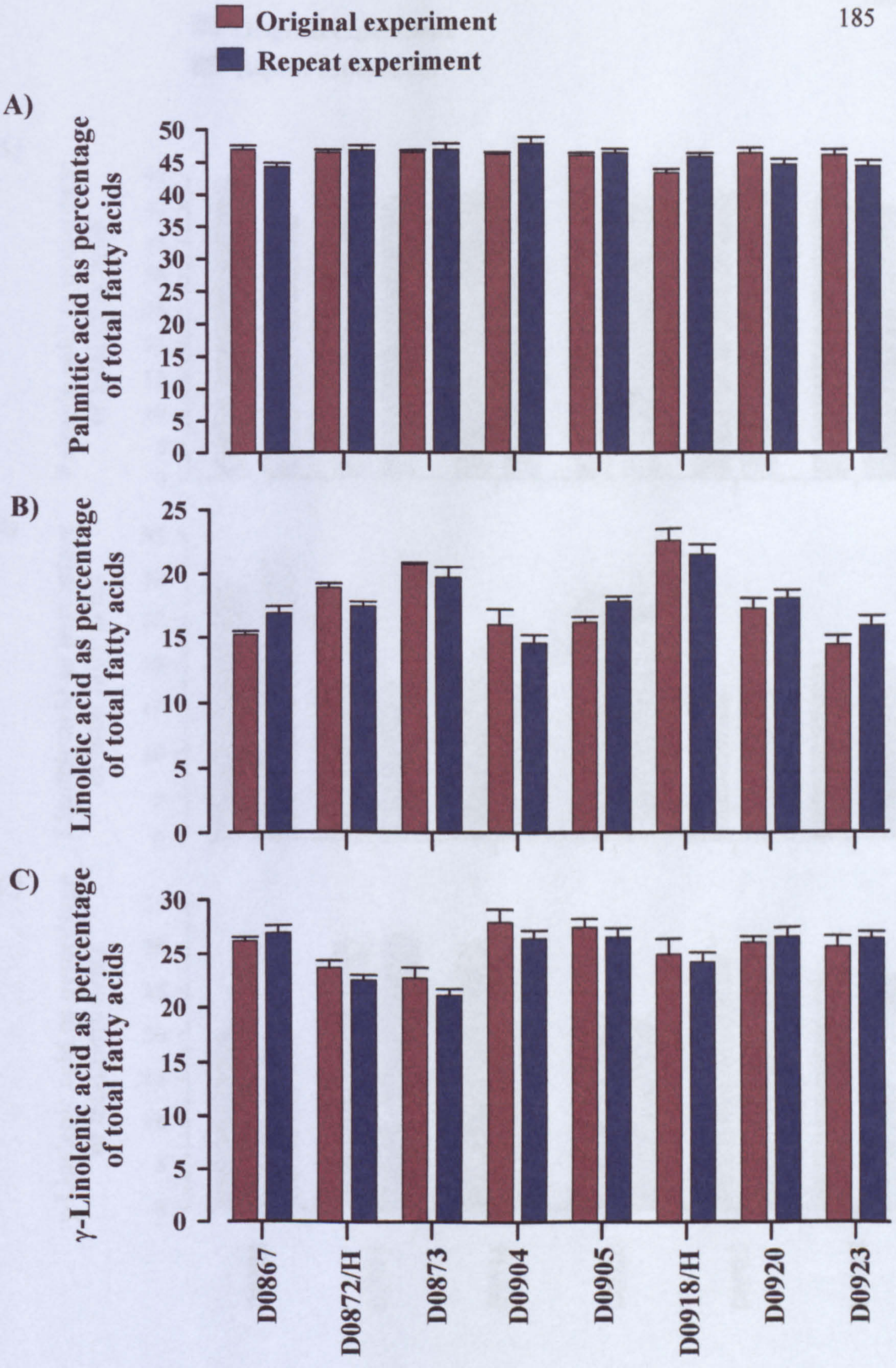



Fig. 8.8 Reproducibility of the results for the fatty acid composition of *Arthrospira* strains from two independent experiments.

8.8A Comparison of data from the original and repeat experiment using cultures grown under 30 °C and 10 μmol photon $m^{-2}$ $s^{-1}$. Data for the three most abundant fatty acids are shown. The error bars represent the standard deviation of three replicates.

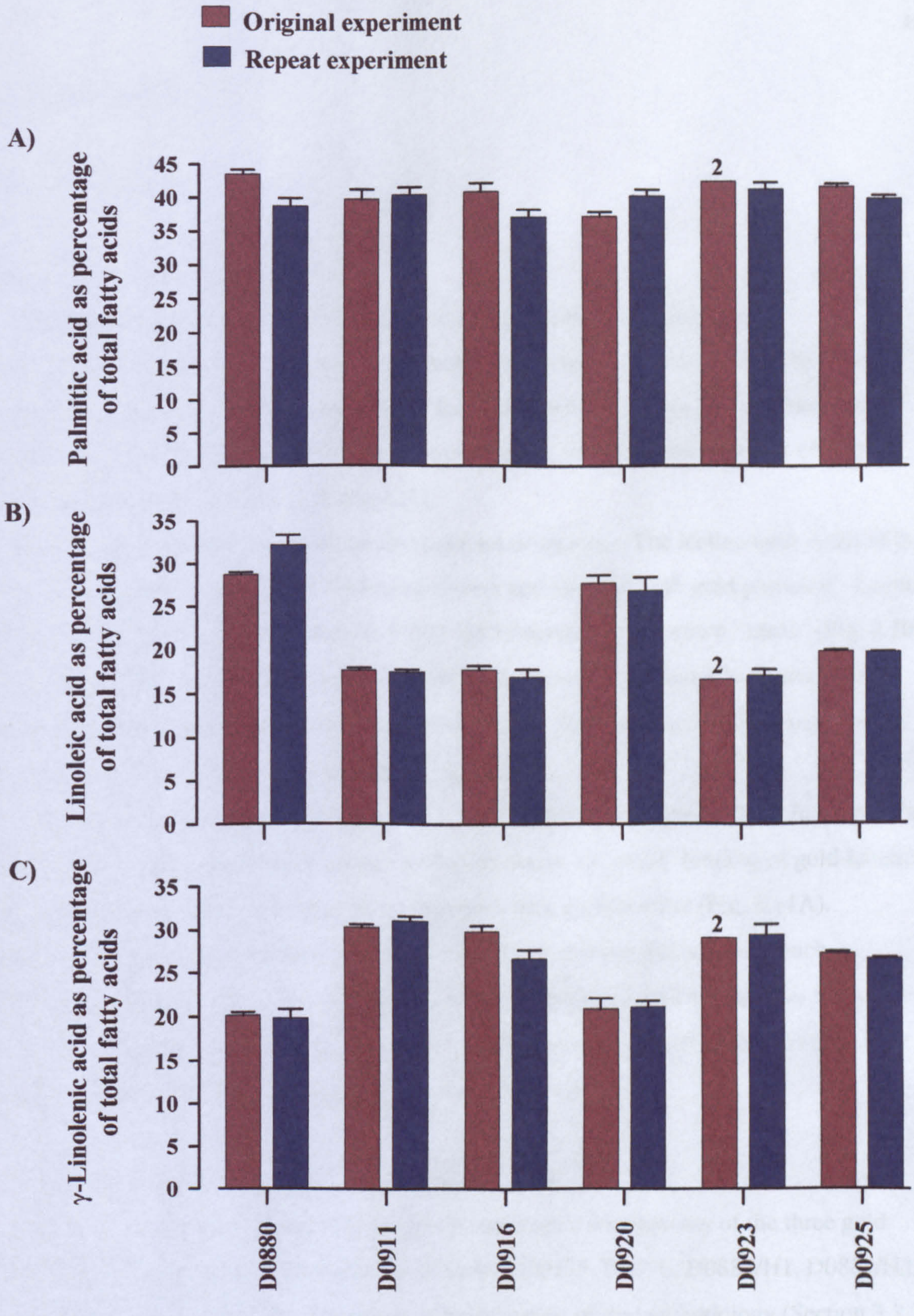



Fig. 8.8 Reproducibility of the results for the fatty acid composition of *Arthrospira* strains from two independent experiments.

8.8B Comparison of data from the original and repeat experiment using cultures grown under 30 °C and 70 µmol photon $m^{-2}$ $s^{-1}$. Data for the three most abundant fatty acids are shown. Data are shown for the three most abundant fatty acids. (n = 3, except where number of replicates are above the bar).

## 8.3 Lectin-binding

### 8.3.1 Methodology

*Use of FITC- and gold-labelled lectins*

Fluorescein isothiocyanate (FITC)-labelled lectins from *Lens culinaris* and *Tetragonolobus purpureas* were used to investigate whether these lectins bind to the cell surface of *Arthrospira* strains. Analysis of the lectin-binding ability revealed that none of the 35 strains and five duplicate strains or any of the six different morphotypes of a strain bind to either of the two lectins (Table 8.3).

Three further lectins were tested on the same set of strains. The lectins were isolated from *Glycine max*, *Helix pomatia* and *Triticum vulgaris* and labelled with gold particles. Lectin-binding can thus be observed either by bright-field microscopy as brown "spots" (Fig. 8.10B) or by the use of UV-light in combination with an IGS-excitation filter which produces polarised light. Polarised light leads to a gold-yellow 'fluorescence' of the gold particles which the lectin molecule is labelled with (Fig. 8.10A).

Control experiments elucidated that cells taken from an *Arthrospira* culture in Zarrouk's medium show very similar fluorescence to that produced by 'weak' binding of gold-labelled lectins when observed by UV-light in combination with an IGS-filter (Fig. 8.11A). However, trichomes of *Arthrospira* washed with distilled water did not show such fluorescence (Fig. 8.11B). This was also the case when the washed sample was treated with silver enhancement solution only. Therefore, in all subsequent experiments samples were washed with $H_2O$ prior to incubation with gold- labelled lectins.

*Improvement of the visualization of gold-labelled lectins*

In an initial experiment it was not possible to determine whether any of the three gold-labelled lectins were binding to trichomes of strains D0875, D0876, D0885/H1, D0885/H2, D0899, D0906/H and D0933. Therefore, a modification of the methodology (Section 3.7.2) used for the detection of the gold-labelled lectins was tested. The modification tested concerned the exposure time to the silver enhancement solution which was extended from 10-12 min to 15-20min.

Fig. 8.9 Binding of lectins to cell surface structures of *Arthrospira* strains.

Lectin-binding of strain D0923. Micrographs of a filament were taken after treatment with lectins using UV-light in combination with an IGS-excitation filter (A) or by 'standard' light microscopy (B).

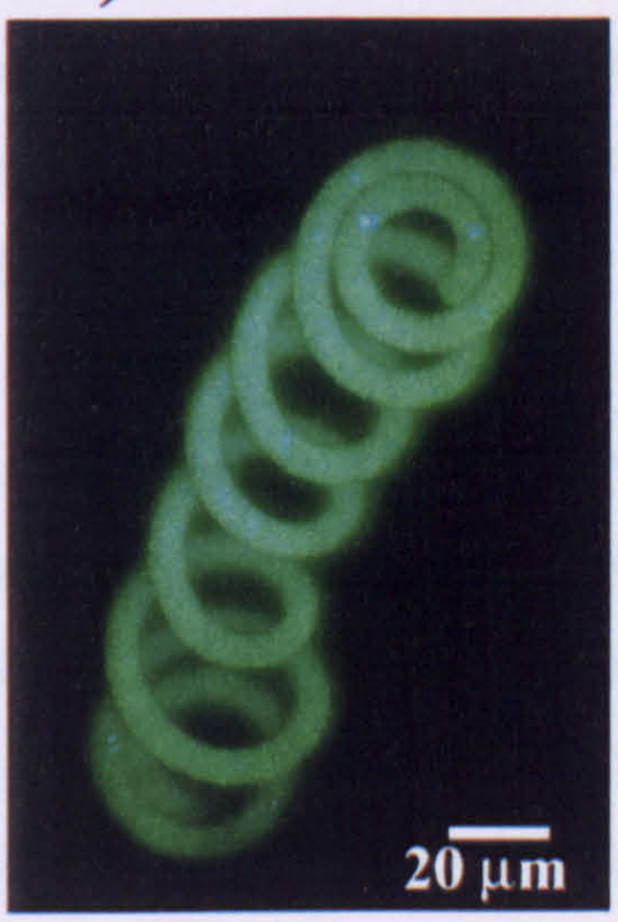
Fig. 8.10 Filament of *Arthrospira* observed under UV-light in combination with an IGS-excitation filter.

Samples of *Arthrospira* strain D0923 studied were taken either directly from cultures in Zarrouk's medium (A) or after washing with distilled water (B).

Fig. 8.11 Examples of lectin-binding to surface layers other than the glycocalyx.

Binding of lectins near the constrictions at the sites of cross walls D0904 (A). Binding of lectins to the slime excreted by cells (B, C). Micrographs were taken using either a combination of UV-light, IGS filter and Damarski interference prism (A), or 'standard' light microscopy (B) or UV-light in combination with an IGS filter (C).

A)




B)



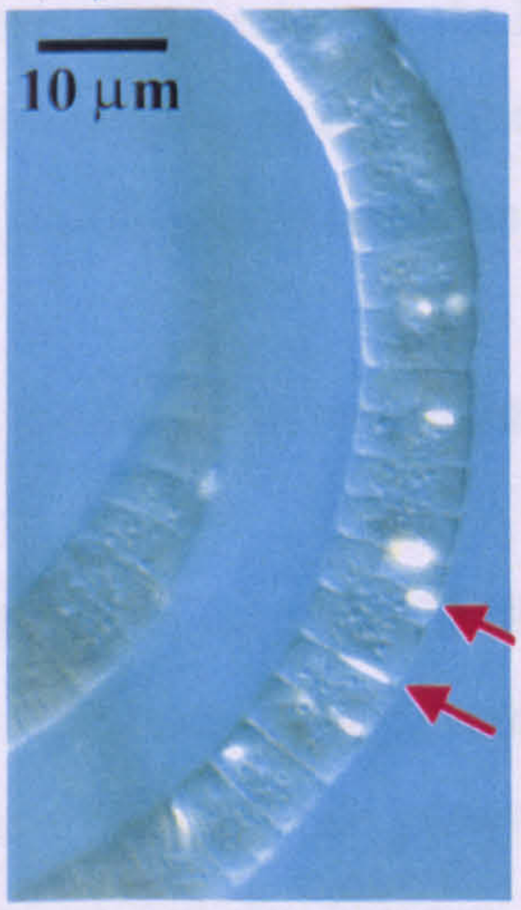

A)



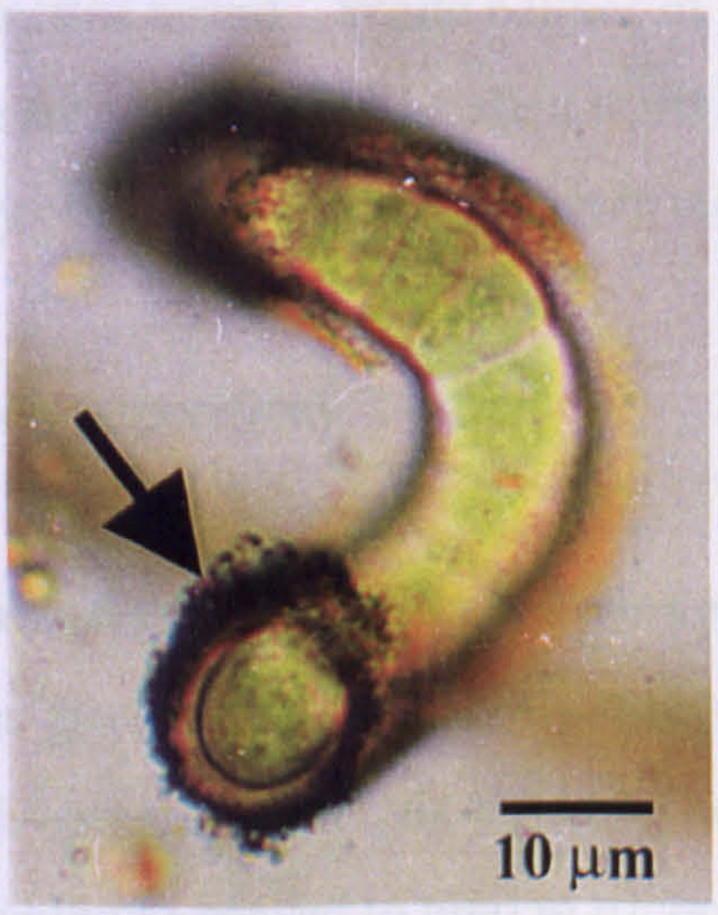

B)



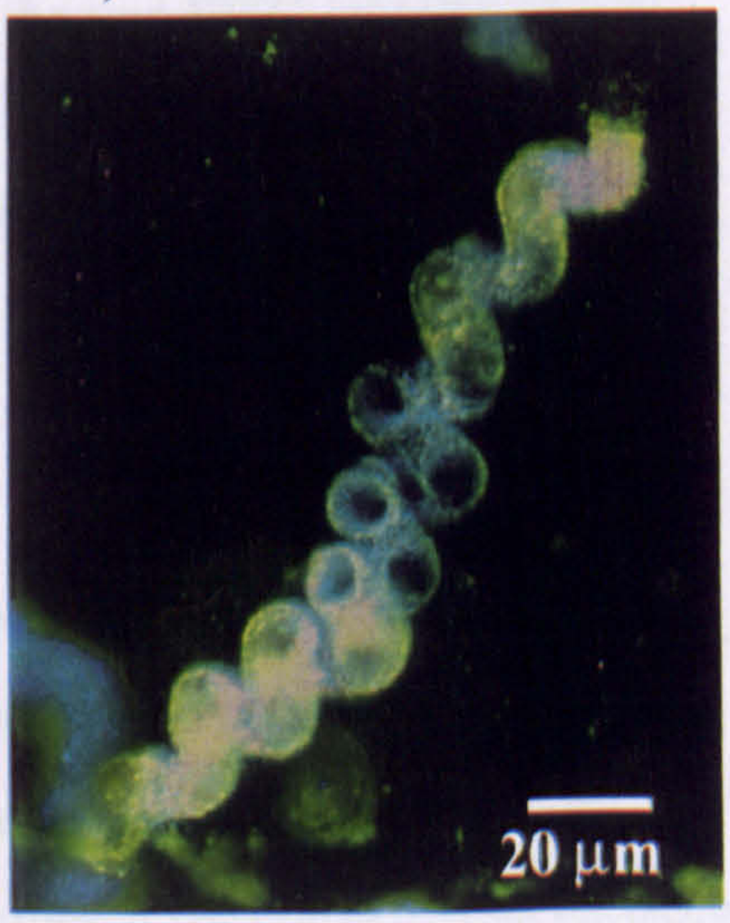

A)




B)




C)



The modified method proved that lectins from *Glycine max* bind strongly to surface structures of trichomes of most of those strains, for which binding of lectins to cell surface structures was uncertain in the initial experiment. Exceptions were strains D0875, D0876 and D0933, for which the ability to bind to lectins could again not be determined beyond doubt (Table 8.3.).

*Dilution of lectins and growth conditions used*

The dilution of the lectins leading to best binding was tested on three strains (D0918/H, D0923, D0925). The tests were carried out for the gold-labelled lectins from *Glycine max* and the FITC-labelled lectins from *Lens culinaris* and *Tetragonolobus purpureas.* Dilutions tested were 1:50, 1:100 and 1:200. Cultures used as inoculum were grown at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$ for six days.

No differences were found in lectin-binding between the three strains under any of the environmental conditions tested. While the lectins from *Glycine max* bound strongly to cell surface structures of all three strains, neither of the two FITC-labelled lectins showed binding to any of the strains at any dilution tested. A dilution of 1:100 was used subsequently.

The influence of environmental factors on the formation of binding sites for the lectins (ie cell surface polysaccharides) was investigated on the same set of strains (D0918/H, D0923, D0925) and lectins (*Glycine max, Lens culinaris, Tetragonolobus purpureas*). Environments tested were: 20 °C and 30 µmol photon $m^{-2}$ $s^{-1}$, 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$, 30 °C and 70 µmol photon $m^{-2}$ $s^{-1}$, laboratory conditions (17-25 °C with approx. 30 µmol photon $m^{-2}$ $s^{-1}$).

The results showed that neither culture age nor growth temperature or light intensity influence the binding to the lectins tested. While neither of the two FITC-labelled lectins showed binding to any of the strains, the gold-labelled lectin from *Glycine max* bound to all three strains grown under any of the growth environments tested.

## 8.3.2 Variation in lectin-binding specificity

The screening of the 35 strains, the five duplicate strains and six different morphotypes was carried out using cultures grown at 30 °C and 30 µmol photon $m^{-2}$ $s^{-1}$ until they reached the early phase of fast growth (generally after six days). The lectins from *Glycine max*, *Helix pomatia* and *Triticum vulgaris* proved to bind to trichomes at the constrictions between

neighbouring cells (Fig. 8.12A), or to the slime (if present) excreted by the cells (Fig. 8.12B). These features, however, were not regarded as lectin-binding, which was defined as binding to the glycocalyx at the cell surface.

The results of the screening of the 35 *Arthrospira* strains revealed qualitative and quantitative differences between strains (Table 8.3). Duplicate strains and the different morphotypes of a strain show broadly similar results (Table 8.3B, C). However, strain D0911 showed lectin-binding to lectins from *Helix pomatia* and *Triticum vulgaris*, while its duplicate strains D0875 and D0876 lacked this character (Table 8.3B). A second exception was strain D0918; the straight morphotype showed lectin binding to lectins from *Helix pomatia* and *Triticum vulgaris*, while the helical one did not (Table 8.3C).

Table 8.3 Results of the screening for the ability of lectins to bind to the cell surface of *Arthrospira* spp. G = *Glycine max*, H = *Helix pomatia*, Tr = *Triticum vulgaris*, L = *Lens culinaris*, Te = *Tetragonolobus purpureas* (+) = unsure whether 'true' binding of lectins or contamination of components from Zarrouk's medium; + to +++++ = relative intensity of lectin-binding as judged by intensity of fluorescence.

8.3A. Variation between *Arthrospira* strains.

| | G | H | Tr | L | Te |
|---|---|---|---|---|---|
| D0867 | +++ | +++ | +++ | - | - |
| D0872/H | +++ | +++ | +++ | - | - |
| D0873 | +++++ | +++++ | +++++ | - | - |
| D0880 | +++++ | ++ | - | - | - |
| D0881 | - | - | - | - | - |
| D0882 | - | - | - | - | - |
| D0884 | - | - | - | - | - |
| D0885/H1 | +++ | + | - | - | - |
| D0890 | +++ | - | - | - | - |
| D0891 | ++ | ++ | + | - | - |
| D0895 | +++ | + | - | - | - |
| D0896 | ++ | ++ | ++ | - | - |
| D0897 | - | - | - | - | - |
| D0899 | + | - | - | - | - |
| D0900 | - | - | ++ | - | - |
| D0904 | +++ | + | ++ | - | - |
| D0905 | +++ | ++ | ++ | | |
| D0907 | + | +++ | - | - | - |
| D0909 | - | - | - | - | - |
| D0910/H | ++++ | +++ | +++ | - | - |
| D0911 | +++ | + | + | - | - |
| D0913 | +++++ | ++ | ++ | - | - |
| D0914/H | +++++ | ++ | ++ | - | - |
| D0915 | - | - | - | - | - |
| D0916 | +++ | + | ++ | - | - |
| D0918/H | +++ | - | - | - | - |
| D0919 | +++++ | +++ | +++ | - | - |
| D0920 | +++ | ++ | ++ | - | - |
| D0921 | - | - | - | - | - |
| D0922 | ++++ | +++ | +++ | - | - |
| D0923 | +++++ | ++++ | (+) | - | - |
| D0925 | +++++ | ++++ | ++++ | - | - |
| D0929 | +++ | + | + | - | - |
| D0930 | ++ | + | + | - | - |
| D0933 | (+) | (+) | (+) | - | - |

8.3B. Analysis of duplicate strains.

| | G | H | Tr | L | Te |
|---|---|---|---|---|---|
| **D0873** | ++++ | ++++ | ++++ | - | - |
| D0879 | ++ | + | + | - | - |
| D0875 | (+) | - | - | - | - |
| D0876 | (+) | - | - | - | - |
| **D0911** | +++ | + | + | - | - |
| **D0880** | ++++ | ++ | - | - | - |
| D0887 | ++++ | + | ++ | - | - |
| D0906/H | +++ | ++ | ++ | - | - |

8.3C. Analysis of different morphotypes of the same strain.

| | G | H | Tr | L | Te |
|---|---|---|---|---|---|
| **D0872/H** | +++ | +++ | +++ | - | - |
| D0872/S | +++ | +++ | +++ | | |
| **D0885/H1** | +++ | (+) | - | - | - |
| D0885/H2 | ++ | (+) | - | - | - |
| **D0906/H** | +++ | ++ | ++ | - | - |
| D0906/S | +++ | + | + | - | - |
| **D0910/H** | ++++ | +++ | +++ | - | - |
| D0910/S1 | ++++ | ++ | ++ | - | - |
| **D0914/H** | ++++ | ++ | ++ | - | - |
| D0914/S | ++++ | ++ | +++ | - | - |
| **D0918/H** | +++ | - | - | - | - |
| D0918/S | +++ | ++ | + | - | - |

Analysis of the differences in the intensity with which the lectins bind to *Arthrospira* strains are summarized in Table 8.4A. The lectins from *Glycine max* showed binding to more strains and quantitatively stronger than any of the other lectins tested.

The analysis of the variation of *Arthrospira* strains concerning their ability to lectin-binding shows, that all of the *Arthrospira* strains, which bind to the lectins from *Glycine max*, also bind to the lectins from *Helix pomatia* (Table 8.4B). None of the three strains (D0890, D0899, D0918/H), which proved to bind lectins from *Glycine max*, but not lectins from *Helix pomatia*, showed lectin-binding to lectins from *Triticum vulgare*. One strain (D0900) showed lectin-binding to lectins from *Triticum vulgare*, but not to the lectins from *Glycine max* (Table 8.3A, 8.4B).

Table 8.4 Analysis of the results of lectin-binding to the cell surface of *Arthrospira spp.* – Intensity of lectin-binding (A) and variation between strains (B). The number of strains showing lectin-binding for any of the three lectins is based on 33 strains as those cases, where it was unsure whether lectins were binding, are not included.

8.4A Variation in the intensity of lectin-binding.

| | Total | + | ++ | +++ | ++++ | +++++ |
|---|---|---|---|---|---|---|
| G | 25 | 2 | 3 | 12 | 2 | 6 |
| H | 22 | 7 | 7 | 6 | 1 | 1 |
| Tr | 19 | 4 | 8 | 5 | 1 | 1 |
| L | 0 | 0 | 0 | 0 | 0 | 0 |
| Te | 0 | 0 | 0 | 0 | 0 | 0 |

8.4B Variation between lectins.

| | Total | Lectin-binding to G | Lectin-binding to H | Lectin-binding to Tr |
|---|---|---|---|---|
| Lectin-binding to lectins from G | 25 | - | 22 | 18 |
| Lectin-binding to lectins from H | 22 | 22 | - | 18 |
| Lectin-binding to lectins from Tr | 19 | 18 | 18 | - |

## 8.4 Discussion

### 8.4.1 Fatty acid composition

Cohen *et al.* (1987) investigated the fatty acid composition of 18 *Arthrospira* strains. The results obtained demonstrated that all strains tested showed the same fatty acid composition. However, great diversity was found in the quantitative distribution of the fatty acids. While the proportion of 16:0 fatty acids was relatively constant, the percentage of unsaturated and polyunsaturated $C_{18}$ fatty acids varied greatly.

Similar results were obtained from the screening program of the set of *Arthrospira* tested in the presented work. However, the use of a wider range of strains and investigation of the influence of more environmental conditions proved to elucidate new parameters influencing the desaturation of fatty acids of *Arthrospira* strains.

Cylindrical bodies may be formed by lipid membranes (Section 6.4.2). However, a comparison between the occurrence of cylindrical bodies and the fatty acid composition of a strain can not be drawn, as cultures were grown under different environments in both experiments and environmental factors have been shown to influence both fatty acid composition (Cohen et al., 1987; Section 8.2.2) and presence of cylindrical bodies (Van Eykelenburg, 1980).

None of the six *Spirulina* strains tested contained γ-linolenic acid, thus confirming γ-linolenic acid as a chemotaxonomic marker to differentiate between the genera *Spirulina* and *Arthrospira* as suggested by Tomaselli et al. (1996).

#### 8.4.1.1 Fatty acid content

Great variation was observed for the overall fatty acid content between the three replicates of all *Arthrospira* strains. Reasons for the high standard errors are most likely to be found in the methodology for determining the biomass. Increasing production and excretion of polysaccharides, which could not be washed off easily, were observed to be a typical character of environmental stress such as high cell density in non-continuous cultures, high light intensity or low temperature stress (usual observation). The aliquots of cultures used for determining the biomass for the calculation of overall fatty acid content were always higher than 3 mg, thus allowing reproducible weighing on a micro-scales (M. Christmas,

pers. comm.). However, small differences in the amount of polysaccharides excreted from the cells may have been sufficient to cause great variation between replicates of the same strain. Even higher standard deviation were found for cultures grown under any of the other environments tested making conclusions about the changes of the cell content of the fatty acid fraction unreliable.

The values for the overall fatty acid content of *Arthrospira* strains obtained in the presented work are generally higher than the values Cohen et al. (1987) found. However, the two data sets cannot be compared as both growth environments and most of the strains tested were different in this study to that of Cohen et al. (1987).

8.4.1.2 Is D0887 a duplicate strain of D0880 and D0906/H?

D0880 and D0906 (both straight and helical morphotypes) exhibit very similar fatty acid composition under all the environments tested. Furthermore, Cohen *et al.* (1987) found similar (low) γ-linolenic acid levels for strain UTEX 2340, which is a direct subculture of strain D0880 (Starr & Zeikus, 1993). However, under any of the environments tested D0887, supposed to be a third duplicate of D0880, showed marked quantitative differences to strains D0880 and D0906 in the content of polyunsaturated $C_{18}$ fatty acids.

The fact that D0887 exhibits a straight trichome conformation while D0880 and D0906/H are helical trichomes does not seem to be a likely explanation for the differences in the levels of polyunsaturated $C_{18}$ fatty acids between those subclones. Analysis of the fatty acid composition of cultures of straight and helical trichomes of four strains including D0906 (D0872, D0906, D0910, D0918) showed no differences in the fatty acid composition between the different morphotypes (both morphotypes of strains D0906 and D0918 were additionally investigated under the four different environments tested and no differences in the fatty acid composition were found).

Scheldeman et al. (1999) have recently reported the comparative amplified ribosomal DNA restriction analysis (ARDRA) of the ITS region of (Section 1.3.2) the same set of *Arthrospira* strains. The results showed that all *Arthrospira* strains group into two clusters. D0880, D0906 (/H and /S) and D0887 were found to belong to the same cluster, thus strengthening the possibility that the strains derived indeed from the same stock. Therefore, assuming that no false labelling of the cultures occurred at any of the culture collections, an explanation may be provided by factors like mutation and genetic drift.

The fact that the content of linoleic acid of strains D0880 and D0906/H decreases with lower temperature or increasing light intensity while the γ-linolenic acid content increases, indicates that the delta-6 desaturase activity represents the rate-limiting factor in the formation of γ-linolenic acid, which is especially obvious under a growth environment of 30 °C and low light intensity. Therefore, mutations responsible for the differences in γ-linolenic acid between those strains would have had to affect the control mechanism regulating delta-6-desaturase activity, *desD* expression or the delta-6 desaturase turnover in *Arthrospira* strain D0880 (and its subclones D0906 and UTEX 2340). The molecular factors and signalling pathways which regulate the delta-6-desaturase activity, *desD* expression and/or the delta-6 desaturase turnover in *Arthrospira* strains, which would have to be affected by the 'hypothetical' mutation, are unknown.

### 8.4.1.3 Environmental factors influencing fatty acid desaturation

The analysis of the fatty acid composition of strains grown under different environmental conditions showed that the composition is highly influenced by the environment. Most strains showed similar changes in the fatty acid composition in response to the environmental changes investigated, but several strains responded in a unique way.

*Temperature*

Decrease in growth temperature led generally to higher γ-linolenic acid and linoleic acid contents in photoautotrophic cultures. These results agree with the "classic" understanding of the biological role of temperature-induced changes in the fatty acid composition of cyanobacteria (and plants) as established by Murata and coworkers (for recent review see Nishida & Murata, 1996).

*Light*

As discussed earlier (Section 7.5.1.3), the irradiance used for the analysis of the influence of high light flux was limited to 70 µmol photon $m^{-2}$ $s^{-1}$. To obtain healthy cultures for fatty acid analysis under this high light flux, it was necessary to adapt the cultures used for inoculation slowly to the high irradiance. Increase of the light intensity at 30 °C proved to trigger increasing levels of γ-linolenic acid in all but two (D0918/H, D0920; Fig. 8.7) *Arthrospira* strains tested. These results do not agree with the data presented by Cohen et al.

(1987) who found that increasing light intensity does not have "much" (Cohen et al., 1987) effect on the fatty acid desaturation of *Arthrospira* strains but on the overall content of fatty acids. There are three possible explanations for the different results of Cohen et al. (1987).

Firstly, Cohen et al. (1987) tested the impact of light intensity on the fatty acid composition of two *Arthrospira* strains, which are not included in this study. These strains may, similar to strains D0918/H and D0920, not have shown any change in their fatty acid composition in response to the increase in light intensity.

Secondly, the cultures tested by Cohen et al. (1987) for their response to different light intensities were grown at 32 °C or above compared to 30 °C used in this study. The higher growth temperature used by Cohen et al (1987) may have influenced the response of the two strains tested.

Thirdly, the presented work examined the response of *Arthrospira* strains to an increase in light intensity from 10 µmol photon $m^{-2}$ $s^{-1}$ to 60 or 70 µmol photon $m^{-2}$ $s^{-1}$ at 20 °C or 30 °C, respectively. Cohen et al. (1987), however, analyzed the fatty acid composition of cultures of the two *Arthrospira* strains grown at light intensities of 75, 150 and 300 µmol photon $m^{-2}$ $s^{-1}$. A light intensity of 75 µmol photon $m^{-2}$ $s^{-1}$ may have saturated the photosynthetic machinery of the cell while any further increase in the light intensity did not lead to any further changes in the fatty acid composition. However, due to the different experimental conditions it is difficult to compare the two results.

Although this is the first report on the fatty acid composition of *Arthrospira* cultures grown dark-heterotrophically, increased fatty acid desaturation under light limiting conditions has been observed before (Hirano et al., 1990; Tanticharoen et al., 1993). However, an explanation for the biological function of increased desaturation of membrane fatty acids under dark-heterotrophic conditions has not been reported so far. In contrast to increased desaturation caused by low temperature or high light intensity the lack of any obvious environmental stress for membrane fatty acids under dark-heterotrophic condition at 30 °C excludes the explanation of a protective response.

All attempts to grow cultures at 20 °C and 70 µmol photon $m^{-2}$ $s^{-1}$ caused photodamage within the first 3 days of incubation as judged by the change from the dark blue-green pigmentation to a green and partially yellow pigmentation. This may be due to higher susceptibility to photoinhibition under low temperature stress (Wada et al., 1994; Tasaka et al., 1996). Therefore, 60 µmol photon $m^{-2}$ $s^{-1}$ were used as high light environment at 20 °C as this was found to avoid photoinhibition. Despite increasing light intensity fewer strains

showed elevated levels of γ-linolenic acid with increasing light intensity compared to a similar shift of the light intensity under 30 °C. As care was taken to ensure that only cultures were used for preparation of the fatty acid fraction that were seemingly in a "healthy" state, ie dark blue-green pigmentation, it is assumed that the data obtained represent genuine characters of the strains and are not a result of photoinhibition.

The high γ-linolenic acid content of *Arthrospira* strains grown under dark-heterotrophic conditions may be of special economic importance as it may allow high yield production of γ-linolenic acid under specifically controlled and sterile conditions in bioreactors. If parallel to the extraction of γ-linolenic acid other valuable compounds of *Arthrospira* cultures are being extracted then Spirulina production in bioreactors may increase to economic competitiveness (Tanticharoen et al., 1994).

### 8.4.1.4 Variation in the fatty acid composition

The analysis of the variation in the fatty acid composition allows two main conclusions. Firstly, the growth environment that most differentiates between strains is 30 °C and 10 μmol photon $m^{-2}$ $s^{-1}$ (Fig. 8.7; Table 8.2A). In contrast, decreasing the growth temperature from 30 °C to 20 °C leads to a much lower variation between strains under either of the two light environments tested at this temperature. As all strains respond similarly to the low temperature stress the variation between strains decreases. It seems that the cyanobacterial cell does not have an alternative response to low temperature stress than to increase the content of unsaturated fatty acids in order to maintain membrane fluidity (Section 1.6.1.1). An increase in light intensity at 20 °C does not lead to much greater variation between strains. This inflexibility of the cell to respond to a second stress (in addition to the low temperature stress) may also be responsible for the greater susceptibility of the cell to photoinhibition, which was observed under this environment (Section 8.4.1.3).

The value of the dark-heterotrophic growth conditions for discriminating between strains is more difficult to assess as only seven of the ten strains were tested for this environment, and two of those without any replicates (D0920, D0925). However, the data for the seven strains indicates that dark-heterotrophic growth leads to more variation between strains than does low temperature acclimation.

Secondly, the fatty acids that most differentiate between strains are the polyunsaturated $C_{18}$ fatty acids. Although the palmitic acid content of a strain shows great variation between the

environments tested, it still does not allow to distinguish between strains, as all strains proved to behave similarly (Fig. 8.7A).

8.4.1.5 Reproducibility

Repeat experiments on the fatty acid composition of several of the *Arthrospira* strains investigated showed that the data are highly reproducible. The data of those cultures, which were grown under an environment that provides sufficient light intensity, were found to be reproducible to a higher degree (Fig. 8.8B). While under light-limiting conditions minor differences in the light intensity may lead to relatively big changes in the fatty acid composition, only very minor differences in the fatty acid composition were found when the light intensity is not limiting. This agrees with the observation of photodamage occurring in cultures, which are shifted from 30 to 70 µmol photon $m^{-2}$ $s^{-1}$ without adaptation to the higher light flux and indicates that saturation of the photosynthetic machinery (of stationary 30-mL cultures) is reached at 30 °C at a light intensity of about 70 µmol photon $m^{-2}$ $s^{-1}$ (Section 7.5.1.3).

8.4.1.6 Use of fatty acid composition as chemotaxonomic marker

Main characters of good taxonomic markers are reproducibility and polymorphisms between species or strains (O'Brien & Colwell, 1987).

Due to too high a variation in the weight determination of the biomass used for fatty acid analysis the overall fatty acid content does not provide a useful marker (Section 8.4.1.1).

The growth environment of 10 µmol photon $m^{-2}$ $s^{-1}$ in combination with 30 °C led to the greatest variation between strains and, therefore, is the 'environment of choice' for the taxonomic determination of *Arthrospira* strains by use of the fatty acid profile and has been applied in this study on cultures of all strains, duplicate strains and different morphotypes. Furthermore, the combination of the variation between strains, and for a particular strain between different growth environments, provides strains with a chemtaxonomic "fingerprint" which is characteristic for several of the strains. Especially the great variation in the content of the polyunsaturated $C_{18}$ fatty acids makes them very useful chemotaxonomic markers. γ-Linolenic acid and linoleic acid represent also the second and third most abundant fatty acid species in *Arthrospira* strains and their content can thus be determined with some accuracy

and high reproducibility (Section 8.4.1.5). Therefore, in the framework of a polyphasic approach (Van Damme *et al.*, 1996) to elucidate the taxonomic relationship within the genus *Arthrospira* analysis of the fatty acid composition provides a valuable chemotaxonomic marker.

### 8.4.2 Lectin-binding

#### 8.4.2.1 Methodology

When observed by UV-light in combination with an IGS-filter, all trichomes of a culture in Zarrouk's medium show epifluorescence similar to that caused by gold-labelled lectins binding to the cell surface of *Arthrospira* strains. The reason for this behaviour is unknown, but components of the trace element solution of Zarrouk's medium may be a possible reason.

Lectin-binding was regarded as binding of lectins to the glycocalyx at the cell surface, but not to excreted slime. The slime is formed by polysaccharides excreted from the cyanobacterial cell (Geesey, 1982), and seems to contain appropriate binding sites for the lectins used. Similarly, lectin binding to the constriction between two neighbouring cells was also not scored as lectin-binding, as this is the site of the cell wall pores, where the polysaccharides are excreted (Hoiczyk & Baumeister, 1998). The lectins bind, therefore, most likely to the excreted polysaccharides at the sites of the pores, but not to the glycocalyx. This is confirmed by the fact that all strains showed this feature, also indicating that lectin-binding to excreted polysaccharides does not show variation between strains and is, therefore, not a useful taxonomic marker.

The incubation time of the samples in the silver enhancement solution after treatment with the lectins enhances the visualization of the gold-particles when observed with polarized UV-light. An exposure time of approx. 10 min is recommended by the manufacturer (Amersham). The extended exposure time (15-18 min), however, seems to enhance further the visualisation of gold-labelled lectins leading to clearer results where visualisation of lectins is problematic.

8.4.2.2 Variation in lectin-binding

Due to the lack of either of the two FITC-labelled lectins to bind to the cell surface of *Arthrospira* strains, only the three gold-labelled lectins proved to be useful for taxonomic purposes. However, despite similar binding specificities of the lectins used (Table 3.3), some variation of lectin-binding was observed between the different lectins. The data obtained provide thus biochemical characters useful for the identification of *Arthrospira* strains. Although the intensity of lectin-binding (ie fluorescence) was analysed semi-quantitatively, it is still the matter of subjective judgement and may change with the incubation time used in silver enhancement solution. Therefore, to achieve higher reproducibility, it is suggested that only the ability of lectin-binding is scored for taxonomic purposes, but not the intensity.

8.5 Summary

i) The 35 *Arthrospira* strains showed qualitatively similar fatty acid compositions. Quantitative variation between strains were, however, apparent in the unsaturated fatty acid fraction.

ii) D0880 and D0906, supposed to be duplicate strains deriving from the same clone, showed a similar fatty acid composition under all environments tested while D0887, supposed to be a further duplicate strain, showed a quantitatively different fatty acid composition under all environments tested.

iii) There are no differences in the fatty acid composition between helical and straight morphotypes of a strain.

iv) None of the six *Spirulina* strains tested contained γ-linolenic acid, making it a good marker to distinguish from *Arthrospira* strains.

v) The environmental changes tested had generally all a marked influence on the levels of polyunsaturated fatty acids, although the type of response was not always the same. The growth environment of 10 μmol photon $m^{-2}$ $s^{-1}$ in combination with 30 °C led to the greatest

variation between strains and, therefore, is the "environment of choice" for the taxonomic determination of *Arthrospira* strains by use of the fatty acid profile.

vi) The combination of variation in the fatty acid composition between strains and the environmentally induced variation in the fatty acid composition provide a chemotaxonomic "fingerprint", which is in many cases characteristic for a particular strain.

vii) Increasing light intensity at 30 °C and decreasing growth temperature led generally to increased desaturation of $C_{18}$ fatty acids.

viii) The increased levels of γ-linolenic acid in dark heterotrophic cultures of *Arthrospira* strains have not been reported previously and may represent a new avenue for the production of γ-linolenic rich Spirulina biomass under sterile and controlled environmental conditions in bioreactors.

ix) The variation in lectin-binding properties of *Arthrospira* strains makes this character useful for taxonomic purposes.

# CHAPTER 9 PYROLYSIS MASS SPECTROMETRY

## 9.1 Introduction

PyMS has been shown to be a useful technique for the identification of prokaryotes including cyanobacteria (Section 1.7), thus offering a further approach to elucidate the relationship of *Arthrospira* strains. A *Spirulina* strain (D0917), able to grow on solidified Zarrouk's medium, was included in the analysis to assess the relative distance to other genera.

## 9.2. Pyrolysis mass spectrometric analysis

### 9.2.1 *Arthrospira* and *Spirulina*

Pyrolysis mass spectrometry revealed that 26 of the 35 *Arthrospira* strains tested are very similar (98%) on the basis of the whole-cell composition (Fig. 9.1; Cluster 1). The remaining 11 strains clustered into two further groups of three (Cluster 2) and six (Cluster 3) different strains each. While the three strains of the second cluster are 93.4% similar to Cluster 1 and 96.9% similar among themselves, the third cluster shows a similarity of 80.9% to Cluster 1 and its six strains are 97.2% similar to each other (Fig. 9.1). *Spirulina* sp. strain D0917 was found to be the only *Spirulina* strain able to grow on Zarrouk's medium. Therefore, this strain was included in the experiment. The two samples of *Spirulina* sp. strain D0917, one grown on ASW:BG, the other one on Zarrouk's medium, formed a fourth cluster, which shows only 50% similarity to the *Arthrospira* strains tested. The impact of the different growth media on the cell compounds is resembled in the dissimilarity of 15% between the two samples of *Spirulina* sp. strain D0917.

### 9.2.2 Duplicates and different morphotypes of a strain

*Duplicate strains*

The three sets of duplicate strains are close in the dendrogram (Fig. 9.1). D0873 and D0911 and its duplicate strains (D0879 and D0875 / D0876, respectively) are included in

the Cluster 1, thus being very similar on the basis of whole-cell composition (≥ 97 % similarity). Similarly, strains D0880 (Cluster 2) is identical to its duplicate strain D0906 (helical and straight morphotype) on the basis of the whole-cell composition. Strain D0887, supposed to be an other duplicate strain of D0880, however, is included in Cluster 1, thus showing only 93.4% similarity to D0887.

*Different morphotypes*

Five of the *Arthrospira* strains and strain D0906, which all produced both helical and straight trichomes (Section 2.2), were also included in the whole-cell fingerprint analysis. The two morphotypes of five of the strains proved to be identical (99.9%). Exceptions were the two morphotypes (H and S) of strain D0872, which were only 84.4% similar. While the helical morphotype lies within the main cluster, the straight morphotype does not belong clearly to any of the four clusters.

The two different helical morphotypes of strain D0885 (D0885/H1, D0885/H2) are grouped in different clusters (Cluster 2 and 1, respectively), sharing a similarity of only 80%. The straight morphotype of strains D0885 (D0885/S; Cluster 1) and the helical clone D0885/H2, which it derived from, however, proved to be identical (Fig. 9.1).

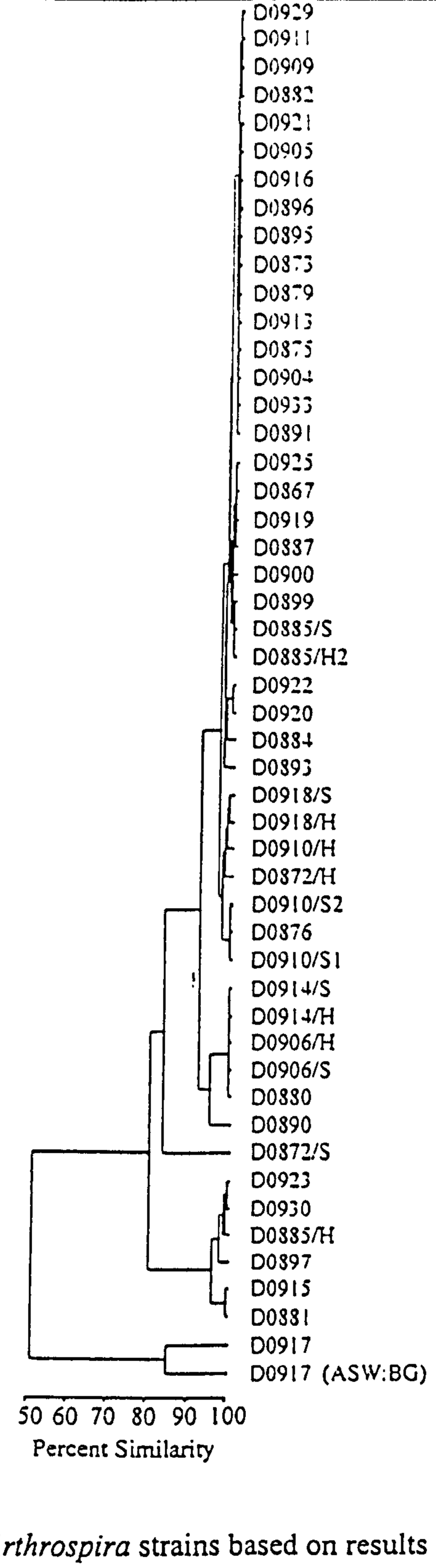



Fig. 9.1 Relationship of *Arthrospira* strains based on results from pyrolysis mass spectrometry.

PyMS was carried out on the 35 strains plus the five duplicate strains and eight different morphotypes. *Spirulina* sp. strain D0917 was included in the experiment as outgroup. The strains were grown on solidified (1% (w/v) agar) Zarrouk's medium at 30 °C and 25 µmol photon $m^{-2}$ $s^{-1}$ for 10 days, except one culture of *Spirulina* sp. strain D0917, which was grown on solidified ASW:BG medium.

## 9.3 Discussion

### 9.3.1 Methodology

The number of samples possible to analyze in one PyMS run is limited to 300. PyMS results can not be compared from different runs. As it was attempted to analyze all 35 *Arthrospira* strains as well as the five duplicate strains, eight different morphotypes and a *Spirulina* strain grown on two media, a compromise had to be made concerning the number of replicates to be analyzed: either three replicate cultures of each strain and two samples analyzed from each agar plate or two replicate cultures of each strain with three samples analyzed from each agar plate. It has been found that the latter method provides a better average of the diversity of a culture, as it ensures that cells from as many as possible different areas of the agar plate are being analyzed (A. Ward, pers. comm.). Although the agar plates were rotated 90 °C each day of incubation in one direction to ensure that all areas of the plate are exposed to the same (light) environment (Section 3.8), it can not be excluded that the biomass is not equally distributed on the agar plates causing cells to be exposed to slightly different microenvironments. For example, cells of colony forming strains will encounter different light intensities dependent on the size of a colony. Collection of biomass through three streaks from different areas of the agar plate ensures that much of the diversity of a culture is being analyzed.

### 9.3.2 Relationship of *Arthrospira* strains and distance to *Spirulina* sp.

PyMS was used in this study only to examine differences between samples analysed. The distances between the samples are all relative to one another (Magee, 1993). Therefore, editing outliers from the data set generally leads to spreading out of the remaining groups. Sequentially editing of all outliers or newly formed ones from the data set effectively leads to "zooming" in on the remaining groups. Similarly, the distances between groups are not absolute but are relative at each stage of the analysis when the data is presented in form of a dendrogram.

The *Arthrospira* strains tested in this study proved to be very closely related. Omission of outliers did not result in better resolution of the clusters. The whole-cell composition of *Arthrospira* strains belonging to the three different clusters was similar and most of the

strains within each of the clusters are nearly identical. In contrast, including *Spirulina* sp. strain D0917 in the analysis proves that this genus is very different from the genus *Arthrospira* as concluded from the results of PyMS. Although the influence of the growth medium (ASW:BG; Zarrouk's medium) used for the culture of *Spirulina* sp. strain D0917 has a great impact on the whole-cell composition, both *Spirulina* samples are clustering relatively closely when compared to the relative distance to *Arthrospira* strains, confirming the distance between the two genera.

### 9.3.3 Relationship of duplicate strains and different morphotypes

*Duplicate strains*

PyMS of the three sets of duplicate strains confirmed in all but one case their culture history. Strain D0880 and the two morphotypes of its duplicate strain D0906 are identical in their whole-cell composition. In contrast, the relatively big distance to D0887, another duplicate strain of D0880 (Section 2.2), rises further doubt on the fact that strain D0887 is a duplicate strain of D0880 and D0906. D0887 showed great differences in the fatty acid composition to the latter two, indicating that it may be the result of false-labelling during its history of culture and not a real duplicate strain (Section 8.2).

The two duplicate strains (D0875, D0876) of D0911 are grouped into the main cluster, thus showing a high degree of similarity in their whole-cell composition. The results obtained by PyMS confirm their identity, although there are small differences in the whole-cell composition between D0875/D0911 and D0876. D0876 showed minor differences also in several other phenotypic characters (Sections 6.3; 7.2.1.1; 8.2.1). The strain history of D0876 (Section 2.2) shows, that, although it was deposited at UTEX as a duplicate strain to D0875, it has been cultured over more than 30 years parallel to strain D0875. During this time period small changes in the whole-cell composition or other phenotypic characters may have been the result of genetic drift. However, according to Rippka and Herdman (1992), strain D0876 has been incorporated into PCC as strain PCC 7345 (= D0911). Therefore, it is somewhat surprising that D0875 and D0911 are more similar than D0876 and D0911 and may be explained by false labelling of one of the duplicate strains.

*Different morphotypes*

Analysis of the whole-cell composition of helical and straight morphotypes of *Arthrospira* strains shows that both morphotypes of the same strain are identical in five of the six cases tested.

The helical and straight morphotype of strain D0872 are not grouped into the same cluster. Although the culture of strain D0872 contained already both morphotypes at the time of receiving, it is assumed that the straight morphotype is indeed a true subclone derived from the helical one. The fatty acid composition confirms this assumption (Section 8.2). The different clustering of the two morphotypes may well be due to an artefact, eg a technical problem during pyrolysis or contamination of the pyrolysis chamber (P. Sisson, pers. comm.). This explanation may be confirmed by the fact that the straight morphotype does not group within any of the three clusters.

Compared to great similarity of helical and straight morphotypes, the two different helical morphotypes of strain D0885 showed great variation in their whole-cell composition. Trichomes of the two subclones differ in cell width and helix characters of the trichome (Section 5.2; Appendix A, B). In contrast to the loss of helical trichome conformation, which may be caused by only small structural changes within the peptidoglycan layer, the differences in trichome width may well be based on great quantitative differences in the whole-cell composition (eg the ratio of peptidoglycan to other cellular compounds). Quantitative differences in the amount of a particular compounds between strains affect the results obtained by PyMS (A. Ward, pers. comm.).

## 9.4 Summary

i) Pyrolysis mass spectrometry revealed that the 35 *Arthrospira* strains, five duplicates and eight different morphotypes tested are very similar on the basis of their whole-cell composition.

ii) The culture history of duplicate strains is confirmed in all but one case by analysis using PyMS. Strain D0887 clusters distinctly to its duplicate strains D0880 and D0906.

iii) The helical and straight morphotypes of the same clone are identical on the basis of their whole-cell composition.

iv) *Spirulina* strain D0917 was found distinctive from *Arthrospira* strains by PyMS, independent of the growth medium.

# CHAPTER 10 ANALYSIS OF PHENOTYPIC CHARACTERS

## 10.1 Introduction

Some of the characters tested (ie helix characters) showed an obvious correlation to the molecular grouping based on ARDRA of the ITS, and, in some instances, also to botanical species (Sections 5.2.2). A computer-aided numerical analysis was carried out to elucidate the taxonomic relationship of *Arthrospira* strains based on the whole data set of phenotypic characters.

## 10.2 Characters and coding of data

Data for 28 characters were scored in form of binary characters. Only those characters and character states, which were not the consequence of another, were included in the numerical analysis. The characters and their character states and the coded data are summarized in Table 10.1 and Appendix B, respectively. (The raw data on the characters are added in Appendix A.)

Characters were scored as absent (0) in those cases where the data were either equivocal concerning the presence or absence of a feature (ie ability to lectin-binding of strains D0923, D0933; Section 8.3.2), or no data were available (ie growth on alternative N-sources (characters 21, 22) of strains D0929, D0930, D0933; Section 7.3). To test the impact of this decision in one example, the analysis was carried out additionally on the same set of strains, but without the two characters for alternative N-sources. Characters which are common among all strains (ie presence of gas vacuoles) were not included in the analysis. The results of the first screening of all strains in four replicates were coded in the case of growth on glucose and fructose in the dark (characters 19, 20).

Table 10.1 List of morphological characters and character states included in the numerical analysis.

| Character | Character state |
|---|---|
| 1. Tendency to mutate to straight trichomes | Observed (1) |
| | Not observed(0) |
| 2. Trichome length | > 0.8 mm [1)] |
| | ≤ 0.8 mm [1)] |
| 3. Trichome width | > 8 µm [1)] |
| | ≤ 8 µm [1)] |
| 4. Cell length | > 3.5 µm [1)] |
| | ≤ 3.5 µm [1)] |
| 5. Diameter of trichome end (= width of end cell) | > 5.5 µm [1)] |
| | ≤ 5.5 µm [1)] |
| 6. End cell | Capitate/"knob"-like (1) |
| | Conical/rounded (0) |
| 7. Calyptra | Present (1) |
| | Absent (0) |
| 8. Type of trichome helix | Fusiform/dumbbell-shaped or barrel-shaped/fusiform (1) |
| | Regular (0) |
| 9. Orientation of coiling at 30 °C | Clockwise (1) |
| | Anti-clockwise (0) |
| 10. Reversion in helix orientation at increased temperature (≥ 32 °C) | Present (1) |
| | Absent (0) |
| 11. Variability in helix dimensions (ratio of lowest to highest value of ratio of pitch to diameter of coil) | > 0.75 (1) [1)] |
| | ≤ 0.75 (0) [1)] |
| 12. Attenuation of helix at apices of trichome | Fast diminishing (1) |
| | Slowly diminishing (0) |
| 13. If granules present, then | Mainly localized at cross walls, but also distributed in cell (1) |
| | Distributed in cell (0) |
| 14. Gas vacuoles | Localized at cross walls (1) |

| | |
|---|---|
| | Distributed in cell (0) |
| 15. Motility on 1% (w/v) agar | Highly motile (1) [2a)] |
| | Less motile (0) [2b)] |
| 16. Macroscopic growth appearance on agar | (Distinct) Colony (1) |
| | Other forms (0) |
| 17. Growth into 1% (w/v) agar | Present (1) |
| | Absent (0) |
| 18. Cylindrical bodies in cultures grown at 30 °C and 50 µmol photon $m^{-2}$ $s^{-1}$ | Present (1) |
| | Absent (0) |
| 19. Growth on glucose in the dark | Growth in all or some of the replicates (1) |
| | No growth in any of the replicates (0) |
| 20. Growth on fructose in the dark | Growth in all or some of the replicates (1) |
| | No growth in any of the replicates (0) |
| 21. Best growth on nitrate | Yes (1) |
| | No (0) |
| 22. Growth on urea better or similar to that on either ammonium or nitrate | Yes (1) |
| | No (0) |
| 23. Palmitic acid content (as percentage of total fatty acids) | > 46% (1) |
| | ≤ 46% (0) |
| 24. Linolenic acid content (as percentage of total fatty acids) | > 17% (1) |
| | ≤ 17% (0) |
| 25. γ-Linoleic acid content (as percentage of total fatty acids) | > 26% (1) |
| | ≤ 26% (0) |
| 26. Binding of lectins from *Glycine max* [3)] | Yes (1) |
| | No (0) |
| 27. Binding of lectins from *Helix pomatia* [3)] | Yes (1) |
| | No (0) |
| 28. Binding of lectins from *Triticum vulgaris* [3)] | Yes (1) |
| | No (0) |

[1)] The average of the range is determining the scoring; e.g. trichome width of 7.5 – 8.5 µm is scored as "0" (Section 3.9.1)

[2a)] Growth as feathery spread = high motility; [2b)] growth as colony or lawn = less motile

[3)] (+) is scored as absent (0)

## 10.3 Numerical analysis

Non-hierarchical analysis (PCA) resulted in approximately equal distribution of the strains resolved on a two-dimensional scatter plot (data not shown). Therefore, a hierarchical approach was employed to facilitate the recognition of clusters.

Calculation of a similarity matrix applying the simple matching coefficient, followed by clustering of the strains based on UPGMA, resulted in two groups of *Arthrospira* strains, which are referred to as phenotypic cluster I and II (Fig. 10.1). Similar clusters were also obtained when the analysis was carried out on the same set of strains, but without the characters for ability to grow on alternative nitrogen sources (characters 21, 22). Exceptions were four strains of phenotypic cluster I (D0900, D0909, D0915, D0933), which clustered within phenotypic II when the two characters were edited from the data set. The highest similarity coefficient (0.86) was found for strains D0919 and D0923, the lowest (0.29) for strains D0907 and D0925 and strains D0918/H and D0921. These strains shared 24 (D0919/D0923) or 8 (D0907/D0925, D0918/H /D0921) of the 28 characters. All pairs of strains, which cluster closely together in the phenogram, are supported by a high value for the $r_{cs}$ ($> 0.7$). However, the separation of the strains into the two phenotypic clusters does not resemble the actual similarity matrix well ($r_{cs} = 0.51$). Furthermore, strains with a high similarity value are resolved into different phenotypic cluster (eg similarity value of 0.64 for D0891, D0914/H), while other strains with a lower similarity value cluster closely (eg similarity value 0.5 for D0921, D0930).

Fig. 10.1 Taxonomic relationship of *Arthrospira* strains.

Fig. 10.1A The phenograms is based on data for the 33 helical strains and was calculated using the $S_{SM}$ and the UPGMA clustering method.

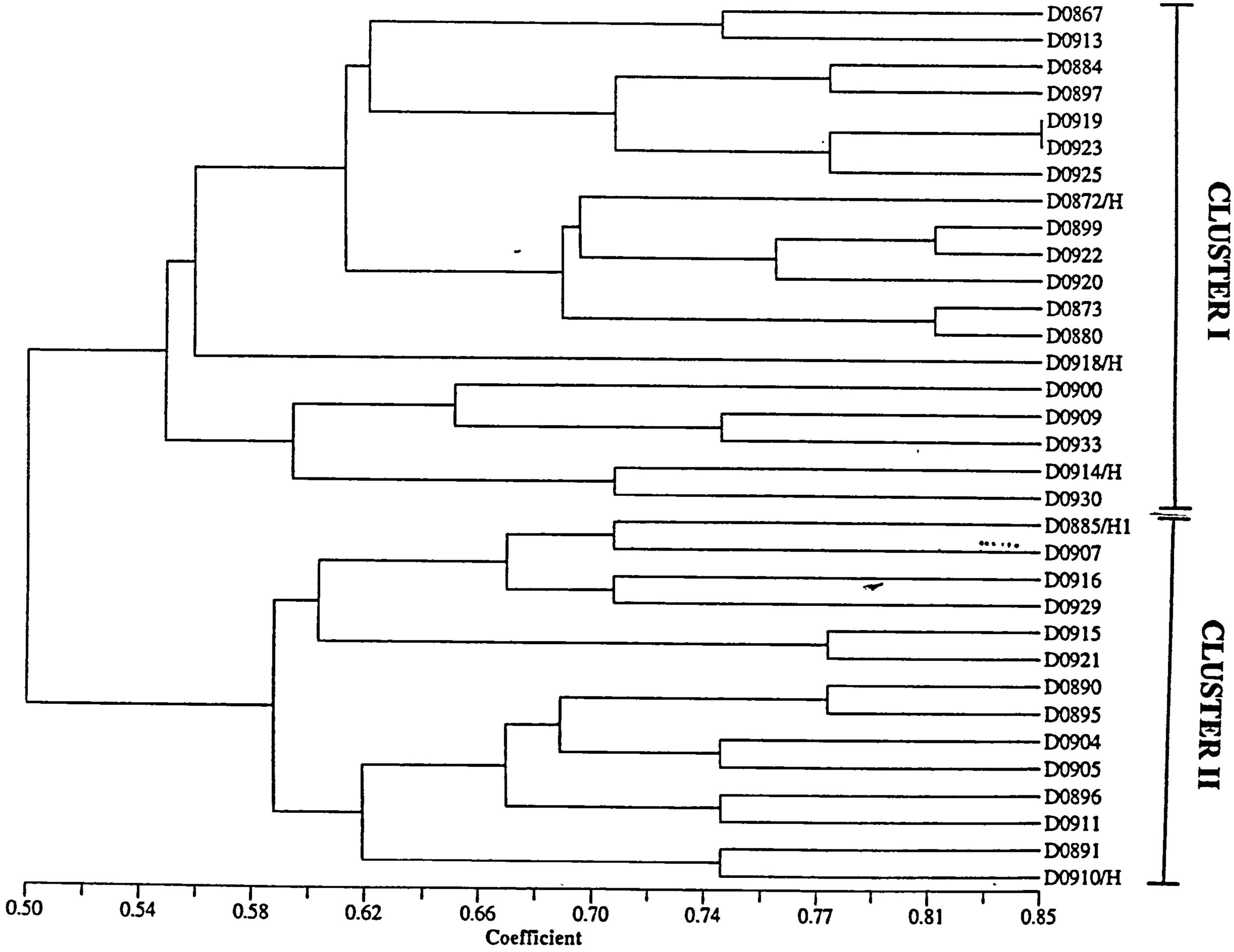
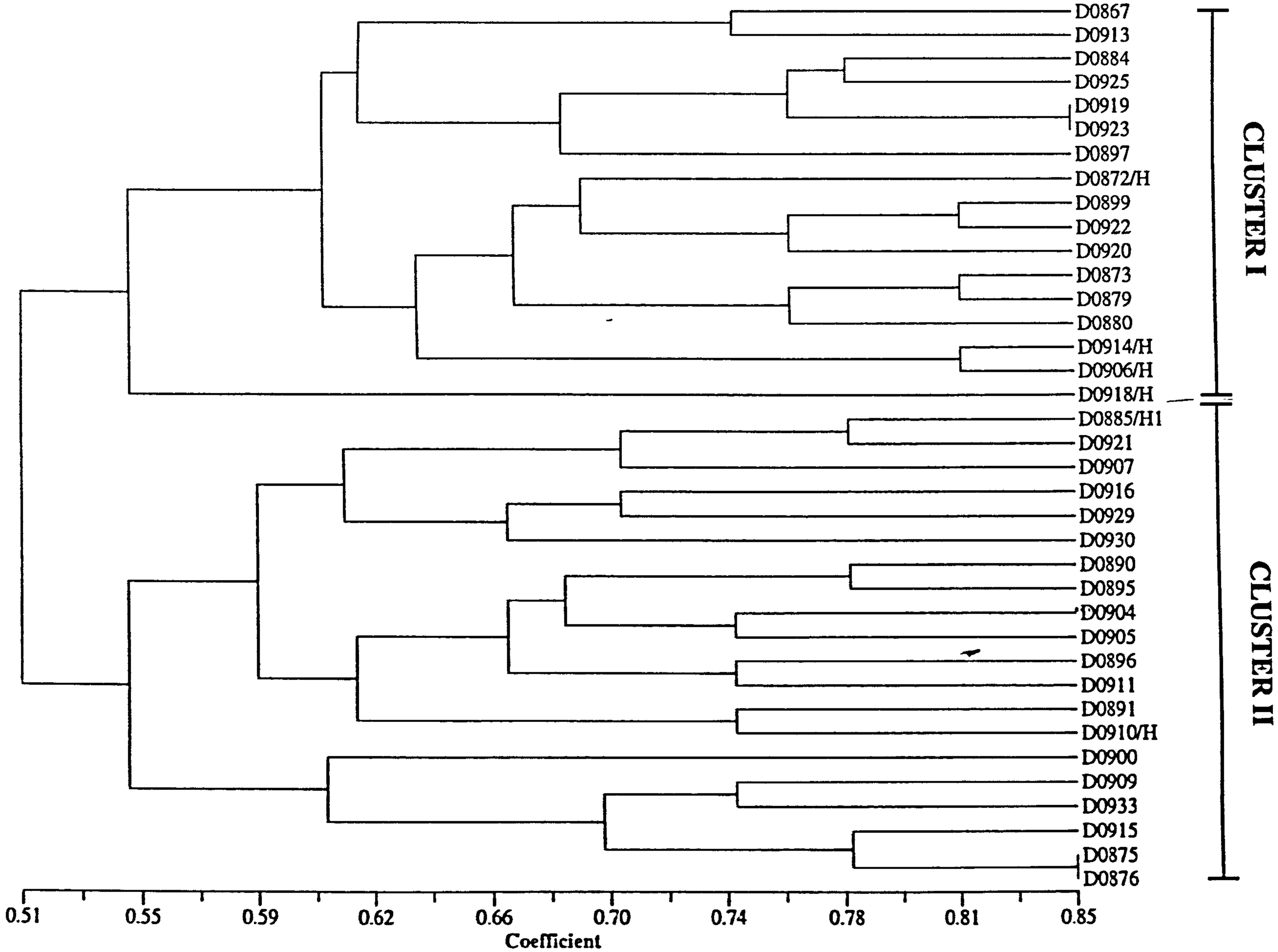

D0867
D0913
D0884
D0897
D0919
D0923
D0925
D0872/H
D0899
D0922
D0920
D0873
D0880
D0918/H
D0900
D0909
D0933
D0914/H
D0930
D0885/H1
D0907
D0916
D0929
D0915
D0921
D0890
D0895
D0904
D0905
D0896
D0911
D0891
D0910/H
CLUSTER I
CLUSTER II
0.50
0.54
0.58
0.62
0.66
0.70
0.74
0.77
0.81
0.85
Coefficient

Fig. 10.1 Taxonomic relationship of *Arthrospira* strains based on 28 characters.

Fig. 10.1B The phenograms is based on data for the 33 helical strains plus the four helical duplicate strains and was calculated using the $S_{SM}$ and the UPGMA clustering method.


D0867
D0913
D0884
D0925
D0919
D0923
D0897
D0872/H
D0899
D0922
D0920
D0873
D0879
D0880
D0914/H
D0906/H
D0918/H
D0885/H1
D0921
D0907
D0916
D0929
D0930
D0890
D0895
D0904
D0905
D0896
D0911
D0891
D0910/H
D0900
D0909
D0933
D0915
D0875
D0876
CLUSTER I
CLUSTER II
0.51
0.55
0.59
0.62
0.66
0.70
0.74
0.78
0.81
0.85
Coefficient

Comparison of the two phenotypic clusters of the phenogram with the clusters based on the characters describing the helical trichome shape (Section 5.2.2) shows a high correlation (Table 10.2). Most of the strains belonging to phenotypic cluster I show an irregular trichome helix, while strains of phenotypic cluster II have a regular trichome helix.

Table 10.2. Comparison of the two phenotypic clusters with the clusters based on only those characters which describe the helical trichome shape (Section 5.2.2). Only strains (33) with a helical trichome morphology (Section 2.1) are included. (No duplicate strains included.)

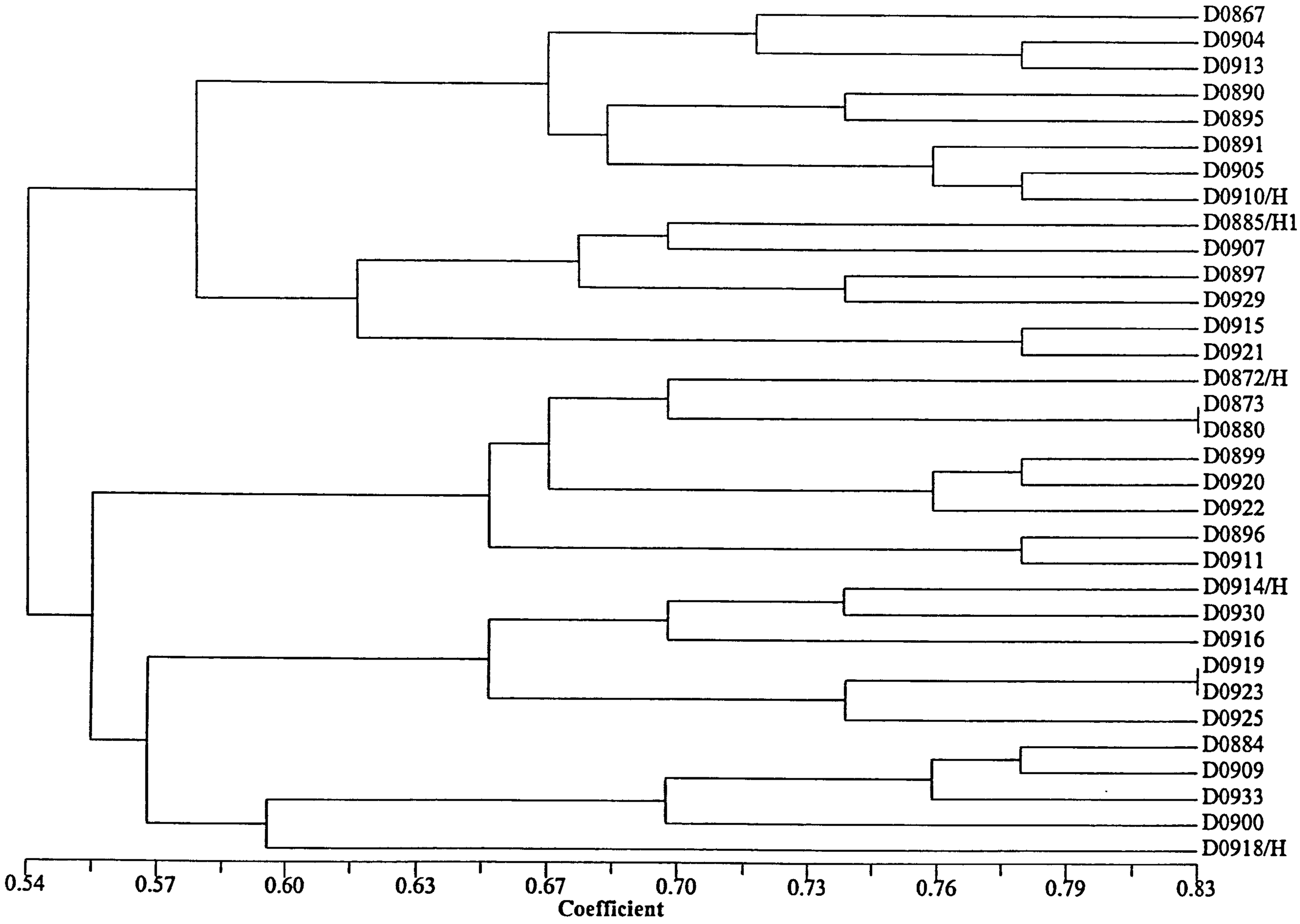
| Strains belonging to | Phenotypic cluster I | Phenotypic cluster II | Of total |
|---|---|---|---|
| Irregular trichome helix | 16 | 1 | 15 |
| Regular trichome helix | 3 | 13 | 18 |
| Of total | 19 | 14 | 33 |

A further analysis (same set of 28 characters) was carried out on the 33 strains plus four strains with helical trichome morphology (D0875, D0876, D0879, D0906/H), which are duplicates of strains included in the set of 33 helical strains. The four strains group within the same cluster as their duplicate strains (Fig. 10.1B). Some duplicate strains (D0873 and D0879, D0875 and D0876) cluster very closely, while others (D0880 and D0906/H, D0875/D0876 and D0911) are more distantly related. Inclusion of the duplicate strains in the analysis led, however, to some changes in the tree topology compared to the results for the data set of 33 strains. The three strains (D0909, D0930, D0933), which showed a regular trichome helix but clustered within phenotypic cluster I, are now resolved in phenotypic cluster II.

The numerical analysis was repeated without the characters that describe the trichome helix and/or the motility on substrate in order to analyze the influence of these characters on the phenogram. The correlation of the clusters obtained from this analysis to the phenotypic clusters based on the whole data set is relatively low, though the 23 remaining characters still group the strains into two clusters (Fig. 10.2).

Fig. 10.2 Taxonomic relationship of *Arthrospira* strains based on characters other than those describing the trichome helix and motility.

The phenograms is based on data for 23 characters of the 33 helical strains and was calculated using the $S_{SM}$ and the UPGMA clustering method.


D0867
D0904
D0913
D0890
D0895
D0891
D0905
D0910/H
D0885/H1
D0907
D0897
D0929
D0915
D0921
D0872/H
D0873
D0880
D0899
D0920
D0922
D0896
D0911
D0914/H
D0930
D0916
D0919
D0923
D0925
D0884
D0909
D0933
D0900
D0918/H
0.54
0.57
0.60
0.63
0.67
0.70
0.73
0.76
0.79
0.83
Coefficient

## 10.4 Discussion

### 10.4.1 Characters and character states

The analysis of 28 independent characters represents a high number of tests for strains of one genus of cyanobacteria, which has not been achieved previously. Despite this, this study does not meet the requirements for numerical analysis of phenotypic characters concerning the number of tests as suggested by Trüper and Schleifer (1992). However, the characters examined describe a variety of phenotypic features, thus analyzing a great portion of the morphological, ultrastructural, physiological and biochemical features of a strain. The formation and regulation mechanisms of each of the features represents a complex biological system. Therefore, the 28 independent characters studied in this work represent a large part of the genome, though each feature contributes only one or a few characters for taxonomic purposes.

It was not possible to determine in all cases which character state represents the presence or absence of a character. Therefore, $S_{SM}$ was chosen as coefficient, as it is based on the proportion of characters that have the same state (both negative or positive) in the pair of OTUs to be compared (Sackin & Jones, 1993).

The characters analyzed in this study are, presumably, of different taxonomic value. However, in the absence of an obvious basis for objective weighing of characters, assessing their "weight" would carry a great risk of misjudgement caused by subjectivity.

Morphological characters represent half of the characters used for the numerical analysis. All of them were determined by examining 20 individual cells or filaments at three different points during the time course of the project using light microscopy. Most of these characters can be measured with some accuracy (eg trichome width, helix dimensions). 'Cell length' may represent the only exception, as it is dependent on the stage of the cell cycle. Most of the qualitative characters can also be determined easily (eg type of trichome helix, helix orientation, location of gas vacuoles and granules within the cells) and many of them have been useful to characterize species of other genera of cyanobacteria (Whitton et al., 1998). 'The tendency to mutate to straight trichomes' may represent an exception as it can not be excluded that further strains will show the mutation in the future. However, observing cultures of the strains over a time period of 30 months provides a basis on which it is reasonable to judge whether a strain is likely to mutate to the straight morphotype or not.

The ability of *Arthrospira* strains to glide on substrate is correlated to the trichome helix (Section 5.3.1). Therefore, one could argue that this character should not be included in the analysis as it depends on the character state of another one (Section 10.4.1). However, the gliding mechanism has been shown to be a complex mechanism based on a combination of features (eg slime excretion, presence of surface fibrils wound helically along the trichome and $Ca^{2+}$-binding proteins; Section 1.3.5). The fact that motility is not purely dependent on the helical trichome shape is also confirmed by the occurrence of several exceptions (ie regularly and irregularly helical forms with medium degree of motility; Section 5.3.1). Therefore, motility on substrate is not a consequence of trichome shape, but trichome shape affects the ability to glide.

In contrast to the ability to glide on substrate, it is difficult to assess the motility of *Arthrospira* strains in liquid medium (Section 5.4.3.2) and to distinguish it from diffusion. Furthermore, motility in liquid medium may be based on gliding of trichomes using other trichomes as 'substrate' (Castenholz, 1967). This is the only explanation offered so far in the literature, though surface structures (ie glycocalyx) could also enable the trichomes to swim (Section 5.4.3.2). The fact that highly motile strains on substrate (D0904, D0905) show also a high motility in liquid medium supports the hypothesis of lateral gliding. Therefore, this character is not included in the analysis as it may be highly correlated to the ability to glide on substrate.

In the case of dark heterotrophic growth on sugars, it was not possible to code the partial lack of the ability to grow in the dark on sugars (Section 7.2.1). Main reason for this was the fact that these results were not reproducible and some strains lost their ability to grow on sugars. Furthermore, it is still unclear whether the lack of all replicates to behave in the same way is due to differences in the microenvironment or the genetic loss of ability to heterotrophic growth by genetic drift. The solution applied in this study was to include only the results of the first screening in the numerical analysis. Furthermore, none of the strains, which failed to grow on glucose or fructose in all four replicates of the first screening experiment, grew in any of the replicates of any repeat experiments. Therefore, the character states were defined to distinguish between strains on the basis of their "original" lack of ability or ability to grow (in some or all of the replicates tested) on glucose and fructose, thus avoiding the potential wrong interpretation of the lack of all replicates to behave in the same way.

Editing the data for the ability to grow on alternative N-sources (characters 21, 22) from the data set lead to the change of four strains from phenotypic cluster I to cluster II. The overall structure of the phenogram was, however, not affected. Therefore, it can be assumed that coding the missing data for three strains (D0929, D0930, D0933) as being absent does not have a major impact on the overall topology of the phenogram.

### 10.4.2 Taxonomic grouping

Numerical analysis of the phenotypic characters resulted in a phenogram composed of two clusters and highly resolved subclusters. The clustering of the strains into two phenotypic clusters does, however, not represent well the similarity (Appendix B) between the strains as concluded from the low values for the $r_{cs}$. An explanation for this may be provided by the fact that strains within cluster I are closely related to strains within cluster II, but are separated due to the hierarchical system of the phenogram. Further evidence for this scenario is provided by the high similarity values for strains separated into the two phenotypic clusters (Appendix B). The same reason may be responsible for the fact that a few strains change their position within the phenogram from one phenotypic cluster to the other when changes are made to the data set (ie editing of characters, addition of strains). However, despite the low value for the $r_{cs}$ there seems to be a basis for the formation of two phenotypic clusters. It seems likely that the characters describing the trichome helix and the motility on substrate contribute most to the grouping of the strains into the two phenotypic clusters, as these characters show a high correlation to the phenotypic clusters (Sections 5.2.2; 5.3.1). Analysis of the data set with the data for the same characters except those describing the helical trichome shape (three) or motility on substrate (two), confirms this scenario (Fig. 10.2). However, there is still some correlation visible to the clusters based on the whole data set, indicating that the phenogram based on the whole data set does not just represent the taxonomic relationship based on a few characters.

The inclusion of the duplicate strains in the analysis led to a change in the overall taxonomic topology. This could only be avoided using WPGMA instead of UPGMA, which would necessitate the weighing of characters. There is, however, no objective means for weighing characters of such a great diversity as used in this analysis.

In general, the duplicate strains cluster closely together. The more distant relationship between strains D0975/D0976 and D0911 reflects the fact that some of their characters

changed differently during independent culture (e.g. fatty acid composition, Section 8.2.1). The use of different growth media for the culture of these strains at the two locations (Section 1.2.5.2) is likely to have also contributed to the development of the phenotypic differences.

Although often reported to occur, no attempts have been made so far to include the different morphotypes of *Arthrospira* strains in the taxonomic considerations. Due to the loss of many features it was not possible to include them in the numerical analysis of this study.

### 10.4.3 Comparison with the data from PyMS

The data from PyMS could not be transferred into a binary code and, thus, be included in the numerical analysis. The phenogram based on PyMS shows that most of the duplicate strains and different morphotyes derived from the same stock are very similar in their whole cell content, thus confirming the success of the approach. However, there is no obvious correlation between the phenogram based on PyMS and that based on the numerical analysis of the 28 phenotypic characters. The reason for this may lie in the fact that the two data sets, which formed the bases for the two phenograms, do not share common features that are being analyzed. Of the 28 characters only seven may have significant influence on the whole cell composition: three characters concerning the fatty acid content, three characters describing the potential cell surface structures as binding sites for lectins and the presence of cylindrical bodies. However, there is no obvious correlation between the clusters based on PyMS and the fatty acid content and composition of the strains (Section 8.2), and the cell surface polysaccharides responsible for lectin-binding represent a very small proportion of the cell biomass. The presence of cylindrical bodies has been shown to be dependent on the environment (Van Eykelenburg, 1980). Therefore, no comparison can be made between the occurrence of cylindrical bodies and the clusters, as the cultures for the analysis of their ultrastructure or PyMS were grown under different environments (Sections 3.8, 3.4.3). Therefore, there is no obvious reason for a high correlation between the two phenograms.

## 10.5 Summary

i) Numerical analysis of 28 phenotypic characters resolved the 33 helical strains into two phenotypic clusters. The phenotypic clusters show, however, a low $r_{cs}$. Pairs of strains, which cluster closely together, are represented well in the phenogram ($r_{cs} > 0.7$).

ii) The two phenotypic clusters show a high correlation to the characters describing the helical trichome shape: strains belonging to phenotypic cluster I show an irregular trichome helix, while strains of phenotypic cluster II have regularly helical trichomes.

iv) Two of the four duplicate strains with helical trichomes cluster very closely to the strain, which they derive from, but all four strains group within the same phenotypic cluster as their duplicates.

# CHAPTER 11 DISCUSSION

## 11.1 Introduction

The aim of this project was to analyze the phenotypic variation within the genus *Arthrospira* and to use this information to elucidate the taxonomy of the genus.

Based on the literature it was clear that the morphological similarity between strains and the morphological variability within a strain necessitates that a wide range of features is being included in the analysis. Therefore, data for 28 characters describing morphological, ultrastructural, physiological and biochemical features were collected and analyzed using numerical methods.

It was also clear that the taxonomy of *Arthrospira* strains has to be investigated using a molecular approach. This part has been carried out at the University of Liège (Dr A. Wilmotte) parallel to the phenotypic characterization. Comparison of the results from the phenotypic and the molecular studies allows now to evaluate the taxonomy of the economically most important cyanobacterium.

## 11.2 Numerical analysis

The low $r_{cs}$ obtained for the separation of the strains into two phenotypic clusters indicates that the phenogram does not represent very well the actual similarity for the strains. Due to the hierarchical approach of clustering, intermediate strains may have been separated by grouping them into one of the two phenotypic clusters. Sackin and Jones (1993) suggest that there may still be "some structure showing through the 'noise' " of a phenogram with a low $r_{cs}$. The high degree of correlation between the data for the characters describing the trichome helix and and the motility on substrate (Section 5) and the grouping of the strains into two phenotypic clusters, suggests that the 'structure' is formed by these characters. However, the phenogram based on the data set without the three characters describing the trichome helix and the two characters describing the motility on substrate results in a phenogram, shows still similarity to the phenogram that is based on the whole data set. Therefore, the taxonomic relationship of *Arthrospira* strains as concluded from the analysis of phenotypic features resembles a genuine relationship of the strains within each of the two phenotypic clusters, but not one that is based on only a few characters shared among them.

## 11.3 Comparison with the molecular grouping and the species concept

The comparison with the data from ARDRA of the ITS (Scheldeman et al., 1999) reveals a high correlation between the 'phenotypic' and the 'molecular' clusters (Table 11.1). Furthermore, all but one strain (D0891) with fusiform or dumbell-shaped trichomes are grouped within cluster I. Although cluster I contains five strains of molecular cluster II, only two of them (D0909, D0930) show a regular trichome helix. A further strain (D0933) with regular trichome helix belongs to both phenotypic and molecular cluster I.

Table 11.1. Comparison of the taxonomic relationship of *Arthrospira* strains based on phenotypic and genotypic characters. Molecular data of *Arthrospira* strains are taken from Scheldeman et al. (1999) and only strains (33) with helical trichome morphology are included in the analysis. (No duplicate strains are included.)

| Strains belonging to | Phenotypic cluster I | Phenotypic cluster II | Of total |
|---|---|---|---|
| Molecular cluster I | 14 | 1 | 15 |
| Molecular cluster II | 5 | 13 | 18 |
| Of total | 19 | 14 | 33 |

The high correlation between the clusters from ARDRA of the ITS and the numerical analysis of phenotypic characters indicates a genuine relationship between genotype and (some of ) the phenotypic characters tested. Most of the strains, which are included in the phenotypic cluster I, show a fusiform trichome helix, while almost all of the strains, which are included in phenotypic cluster II, have regularly helical trichomes (Section 10.3). The occurrence of some exceptions may indicate, that mutations within the ITS-region do not (necessarily) occur parallel to mutations affecting genes, that are involved in the formation of the irregular trichome morphology.

The comparison of the results from the numerical analysis with the species analyzed in this project shows that there is no clear correlation between the phenotypic clustering and the botanical species, though there is some tendency of strains designated as *Arthrospira maxima* and *A. fusiformis* to belong to cluster I (Table 11.2).

Table 11.2. Comparison of the botanical species with the taxonomy of *Arthrospira* strains based on numerical analysis of phenotypic characters. Only strains (12) with a binomial (Table 2.1A) are included. (No duplicate strains included.)

| Strains belonging to | *A. maxima* (out of 2) | *A. fusiformis* (out of 3) | *A. platensis* (out of 6) | *A. indica* (out of 1) |
|---|---|---|---|---|
| Molecular cluster I | 2 | 2 | 3 | 0 |
| Molecular cluster II | 0 | 1 | 3 | 1 |

The lower level of correlation between the taxonomic relationship of *Arthrospira* strains as determined in this study and the taxonomic nomination of the 12 strains with binomials may be due to the fact, that morphological characters of the helix shape of three strains were determined differently in this work and by mainstream taxonomists. In this work, the fusiform helix shape, a typical character of *Spirulina fusiformis* Woron. (Section 1.2.3), was not found for strains D0909 and D0910/H (Section 5.2.1, Appendix A), though these strains were designated as *Arthrospira fusiformis* (Table 2.1A). Despite the lack of the fusiform helix shape , strain D0909 groups within phenotypic cluster I, but *Arthrospira fusiformis* strain D0910/H is a member of phenotypic cluster II. Furthermore, the description of *Arthrospira indica* Desikachary et Jeeji Bai 1992, which emphasizes the fusiform helix shape as characteristic for the species, does not correspond to *A. indica* strain D0929, an isolate with regular trichome helix (Section 5.2.1, Appendix A). These features are responsible for the position of *Arthrospira indica* strain D0929 within phenotypic cluster I as concluded from the results of the numerical analysis of a 'hypothetical' strain D0929 with fusiform helix, great helix variability and strong attenuation towards the apices, but otherwise the strain specific data of D0929 as determined in this study (data not shown). It is unclear as to whether *Arthrospira* strains can loose the genetic basis for the irregular helix shape.

Taking the differently determined morphology of these strains into account, and considering the species description of *Arthrospira platensis* Gomont 1892 (Fig. 1.3A), which states a regular helix as being characteristic for the species, the following scenario can be concluded. *Arthrospira maxima*, *A. fusiformis* and *A. indica* form phenotypic cluster I (Table 11.3). Members of this group show an often highly variable, but always irregular (fusiform or dumbbell-shaped) trichome helix with fast diminishing helix

attenuation towards the apices and, generally, low motility on substrate, while strains designated as *Arthrospira platensis* form the phenotypic cluster II (Table 11.3) and are of regular helix shape with slowly diminishing helix attenuation towards the apices and highly motile (Table 10.2; Section 5.2).

Table 11.3. Comparison of species as concluded from species description and morphological data with the taxonomic relationship of *Arthrospira* strains based on phenotypic characters. Only strains (13) with a binomial are included.

| Strains belonging to | *A. maxima* (out of 2) | *A. fusiformis* (out of 3) | *A. platensis* (out of 8) | *A. indica* (out of 1) |
|---|---|---|---|---|
| Phenotypic cluster I | 2 | 3 | 3 | 1 |
| Phenotypic cluster II | 0 | 0 | 3 | 0 |

A further question to be answered concerns the occurrence of gas vacuoles and their taxonomic value for the *Arthrospira*. Several taxonomists (Fott and Karim, 1973; Hindák, 1985; Komárek and Lund, 1990) reported that *Arthrospira platensis* and *A. jenneri* are benthic and, therefore, do not possess gas vacuoles (Section 5.4.1.1). There are, however, only two species descriptions (of the 18 examined in this work), which either state the presence (*A. indica*: Desikachary & Jeeji Bai, 1992) or absence (*A. tenuis*: Brühl & Biswas, 1922) of gas vacuoles. The six strains designated as *Arthrospira platensis* or *Spirulina platensis*, which were included in this study are gas vacuolated and thus are excluded from the genus *Arthrospira platensis* (and *A. jenneri*) sensu Fott and Karim (1973), Hindák (1985), Komárek and Lund (1990). However, one of the 35 *Arthrospira* strains examined in this project (D0885/H1, Fig. 5.3A), showed a trichome morphology very similar to that of *Arthrospira jenneri* represented in Gomont (1892) (Fig; 1.3A), but contained also gas vacuoles. Furthermore, this strain did not show either great variation in its helix dimensions, or fusiform or dumbbell-shaped helices, and therefore does not belong to the species *A. fusiformis*, *A. indica* or *A. maxima*. An alternative explanation may be provided by assuming that the presence of gas vacuoles is not a stable character, but may change in laboratory cultures, ie gas vacuoles are formed by benthic *Arthrospira* strains under laboratory conditions. Such a phenomenon has, however, never been observed for any

cyanobacterium. In conclusion, the occurrence of benthic species lacking gas vacuoles can not be answered, unless such strains are being made available to the scientific community for independent examination.

## 11.4 Genetic drift of cultures

### 11.4.1 Loss of helical trichome shape

The occurrence of straight trichomes in *Arthrospira* 'laboratory' cultures, possibly followed by loss of the helical morphotype, is a well documented phenomenon in culture collections (eg Schlösser, 1994). During this study mutations leading to different phenotypes occurred several times (Section 2.1). Assuming that the loss of helical morphology is caused by randomly occurring mutations, there is no obvious reason for the lack of similar mutations in nature. In contrast, UV-light absent in the laboratory culture conditions, may cause even higher mutation rates in the natural environment.

The loss of helical morphology makes the trichomes of *Arthrospira* very similar to those of *Oscillatoria* species, if not indistinguishable. Any attempt to unequivocally determine the straight trichomes as either *Oscillatoria* sp. or straight *Arthrospira* mutants would need to involve detailed analysis. However, it may be assumed that the helical morphotype of *Arthrospira* spp. is the more abundant one in the natural environment as "*Oscillatoria* spp." have never been reported to be more abundant in, for example, African alkaline lakes than *Arthrospira* spp. (eg Iltis, 1969). This indicates that helical trichome morphology leads to some advantage in nature. Booker and Walsby (1979) found that helical filaments of *Anabaena flos-aquae* are more buoyant than straight filaments. Furthermore, the authors also report that the helical morphotype of *Anabaena flos-aquae* is the more common one in nature, but the straight morphotype is stable in laboratory cultures (Booker and Walsby, 1979).

A similar reason may be responsible for the advantage of the helical morphotype of *Arthrospira* strains over the straight one in the natural environment. Despite the presence of gas vacuoles in either of the morphotypes, a low mixing rate of the water column of a lake may limit the exposure of straight trichomes to sun light, thus favouring the growth of helical trichomes due to their better buoyancy.

In contrast, the straight morphotype does not experience this disadvantage in culture and, due to its faster growth, can outcompete the helical one.

A further explanation may be provided by the fact that the natural environment of *Arthrospira* undergoes changes which reflect on the genetic variation within the population. For example, during periods of algal bloom the phenotype with the fastest growth rate may dominate, while after high rainfall a phenotype able to deal with low nutrient levels may be favoured. Therefore, the regular or irregular changes in environmental parameters help to maintain a genetic pool, thus avoiding the selection of one specific phenotype. Or, alternatively, changes in the environment may select for a phenotype with a wide ecological tolerance, but slower growth under any of the environmental conditions. In contrast, laboratory conditions were stable throughout the culture period of this study and, therefore, will have selected for the phenotype growing fastest under these conditions. In fact, the straight morphotype was found to grow faster than the helical one (Section 5.2.3.1). In addition, serial subculturing by transfer of a small inoculum to fresh culture medium seems likely to enhance the selection process.

### 11.4.2 Reversal of helix orientation

However, while it is easy to speculate on reasons why straight forms might be selected in culture but not in nature, it is not easy to do so for the helix orientation, as a selective advantage of either of the helix orientations is not obvious for either natural or laboratory environment. It is also unclear whether the reversal of the helix orientation is correlated to the taxonomic position of a strain, though there is some evidence for it. The fact that only strains with a fusiform or dumbbell-shaped trichome helix showed the reversal of helix orientation by any of the factors, that were found to influence it, may indicate that helix reversal is correlated to certain properties of the cell wall and, thus, the taxonomic position of a strain. In fact, the helix orientation of all strains of phenotypic cluster II is anti-clockwise helix orientation (except strain D0891, which, however, shows fusiform and dumbbell-shaped trichome helix). Furthermore, reversal of helix orientation occurred only from clockwise to anti-clockwise, and strains with clockwise helix orientation are only present in phenotypic cluster I.

The change of a strain to a different morphotype represents also a problem for subsequent experiments. The solution applied in this project was to use the earliest

morphotype isolated or the more complex morphological clone of a pair (ie the helical type of a mixed culture) as the reference strain. However, this does not provide a guide for a character such as helix orientation, where the original phenotype is lost due to genetic drift and can not be maintained parallel to the new one (Sections 5.2.3.3).

The discovery of helix reversal also raises the question as to whether *Arthrospira amethystina* and *A. aeruginea* are two different species. The difference on which Buell (1938) based the introduction of two different species, was, besides the helix orientation, the "grey to violet color" of *A. aeruginea*, while in *A. amethystina* the "purple tint is not noticeable" (Buell, 1938). The difference in colour was thought to be due to the presence of phycoerythrin in *A. amethystina* (Buell, 1938). The presence of phycoerythrin in *A. amethystina*, however, may also indicate that this species grew under a different environment, which triggered the production of this pigment due to chromatic adaptation (Bennet & Bogorad, 1973). If so, the different environment may have influenced the helix orientation by, for example, either of the two mechanism (temperature, mechanical force) described here.

The observation of the influence of mechanical forces on helical filaments may also help to understand why taxonomists have represented trichomes of *Arthrospira* species showing both helix orientations within a trichome (eg Hindák, 1985: *A. fusiformis*). Mechanical impact on the trichome in its natural environment or through isolation by the taxonomist may have caused the formation of such a trichome state. However, it is still unclear which helix orientation is the most common one in the natural environment of those species, which are represented showing both helix orientation within a trichome (eg Hindák, 1985: *A. fusiformis*) or between different trichomes (eg Woronichin, 1934).

### 11.4.3 Physiology

The long history of culture in a medium, which is most likely far removed from the natural one of most of the strains tested, means that they will have been subject to strong selective pressures. Selection might have occurred for the ability to tolerate or benefit from particular environmental factors, such as high phosphate and inorganic medium composition. As a consequence, many strains may have shifted genetically concerning (some of) their physiological features, such as surface phosphatase activity and ability to utilize organic carbon sources for growth. If it should be confirmed that the loss of dark

heterotrophy in *Arthrospira* strains is caused by genetic drift, it represents a strong argument for the use of heterotrophic media for the isolation and culture of new *Arthrospira* isolates from natural samples, or cyanobacteria in general. The use of heterotrophic media may also be the only way to isolate and identify strains, which are able to utilize organic carbon sources to an extent, that is of economic importance.

## 11.5 Concluding remarks - Recommendations on taxonomic determination of *Arthrospira* strains and the species concept

This study has analyzed a wide variety of phenotypic features of *Arthrospira* strains in order to elucidate their taxonomic relationship. A parallel study carried out at the University of Liège has conducted a molecular analysis of the same set of strains. The combined data obtained from both studies have been analyzed for taxonomic purposes and, thus, brought new insights into the taxonomy of the genus *Arthrospira*. The results of either of the two approaches show that the strains tested are resolved within only two clusters. The new information also allows to revise the existing species concept within the genus *Arthrospira*. Furthermore, the presented work does not only provided a database of information on phenotypic features and the taxonomic relationship of a set of strains, but also identified which methodologies are useful to characterize any (new) strain and to determine its taxonomic position.

### 11.5.1 Phenotypic characterization

So far, morphological characters have been the most common means of determining the botanical species a strain belongs to, as no other data is provided in species descriptions.

Generally the helix characters seem to be the feature with the highest correlation to the taxonomic clusters based on both the analysis of phenotypic characters and of molecular characters (Section 5.2.2, 10.3). Therefore, a first test to determine the taxonomic position of a strain should be the investigation of its helix morphology. Other characters, such as shape of end cell or trichome width are morphological characters necessary to determine the botanical species and are also useful for determining the cluster a strain belongs to. Furthermore, the investigation of morphological characters also has the advantage that no reference strain is necessary in the analysis. Although influenced by environmental

factors, the morphology is generally more stable than, for example, physiological or biochemical characters.

The macroscopic growth appearance on solidified medium proved also to be characteristic for the two clusters. Its high correlation to the phenotypic clusters makes the investigation of motility on substrate a useful approach for the analysis of the taxonomic cluster, which a strain belongs to, though it can not be applied to straight morphotypes.

The use of biochemical characters proved to be diagnostic for many of the strains, though there is no clear correlation between their biochemical data and their taxonomic relationship (Section 8). The analysis of the fatty acid profile of a strain can, however, help to determine duplicate strains and different morphotypes of a clone (Section 8.2.1). If an extended set of lectins with binding specificity to the cell surface structure of *Arthrospira* cells are being used, then this approach, using, may also lead to a similar, but faster, strain-specific fingerprint as does the analysis of the fatty acid composition of *Arthrospira* strains.

In some cases it may be unsure whether a strain with very small trichomes belongs to the genus *Arthrospira* or *Spirulina*. Though it is laborious, analysis of the pore system in the cell wall by TEM determines in those cases unequivocally the genus to which the strain belongs.

### 11.5.2 Molecular approach

To solve the question of the identity of a strain it will be necessary to apply a molecular approach. Although often used to resolve closely taxonomically related organisms, whole genome fingerprinting techniques, such as amplified fragment length polymorphism (AFLP) or randomly amplified polymorphic DNA (RAPDs), have been proven to be difficult to establish or show a low degree of reproducibility (Wilmotte, pers. comm.). In contrast, sequence analysis of genes or gene fragments (eg PCR products) represents a methodology, which can be established easily and provides a large set of characters (each base of a nucleotide sequence represents a character). Increasing information on sequence data facilitates the development of PCR primers specific for a group of organisms at most taxonomic levels. Therefore, the isolation of clones and production of axenic cultures of those is not required, thus allowing investigation the whole genetic pool of a sample without the potential loss of a genetic clone due to failure to isolate it. The increasing

familiarity of this approach and the decrease in cost for the application of the molecular methodology has led to a "multi-loci" approach being favoured for the taxonomic analysis of closely related organisms, eg at the population level (Hayes & Barker, 1997). A similar approach involving intensive sequence analysis of a wide variety of alleles seems to be the most appropriate way to further clarify the taxonomic position of *Arthrospira* strains. It will be interesting to see whether, similarly to the data for many of the phenotypic characters tested in this study, the different molecular alleles show a low level of correlation. Furthermore, using a multi-loci approach it may be possible to investigate whether horizontal gene transfer has taken place. If the latter proves to be the case, it may also help to understand the low degree of phenotypic and genetic diversity of *Arthrospira* strains originating from four continents.

### 11.5.3 Species concept

Due to the rules for the description of species according to the botanical code, the species which have been introduced so far are valid unless there is unequivocal proof that they are duplicate strains (or subspecies) of others. This work in collaboration with the molecular analysis of almost the same set of strains has shown that there is a high correlation between the phenotypic and the molecular data, though there are some strains that cluster differently dependent on the approach employed to solve their taxonomic relationship. These studies provided strong evidence that the two clusters of either of the approaches used, represent genuine taxonomic groups within the genus *Arthrospira*. Therefore, it is recommended that two new taxa within the genus *Arthrospira* are introduced, for example in the order of subgenera, one that is constituted of strains with regular trichome helix and one with strains of irregular trichome helix. This solution would allow to determine with some accuracy the taxonomic position of an isolate within the genus *Arthrospira* using purely morphological tests, while ARDRA of the isolate would determine unequivocally which the molecular cluster the isolate belongs to.

# SUMMARY

i) A study on phenotypic features of *Arthrospira* strains was carried out in order to elucidate the taxonomy of the genus *Arthrospira*. (A parallel study was carried out at the University of Liège conducting a molecular characterization of the same set of strains.)

ii) For this purpose 35 *Arthrospira* strains and five duplicate strains of those were collected. Additionally, eight different morphotypes were isolated from subcultures of these strains.

iii) A method for the production of clonal axenic cultures was established and successfully applied to all strains.

iv) All attempts to develop a method for long-term storage of *Arthrospira* strains failed, presumably due to the presence of gas vacuoles.

v) The loss of the helical trichome shape is presumably caused by a mutation, which seems to affect the peptidoglycan layer of the cell wall, while straightening of the trichome helix through osmotic pressure is reversible. The straight morphotype grew faster than the helical one, thus outcompeting the latter under constant laboratory conditions.

vi) The helix orientation of three strains was found to reverse due to genetic drift, temperature or mechanical forces. A short-term high temperature pulse was sufficient for the helix reversal to take place. The observation of reversal of helix orientation also means that the separation of *Arthrospira amethystina* and *A. aeruginea* as two different species based on their helix orientation may have to be reconsidered.

vii) The macroscopic appearance on solidified medium can be used as a measure of motility of *Arthrospira* strains and can be used for taxonomic purposes. It is unsure whether the cell surface fimbriae, which have been identified in this study using a modified fixation protocol for TEM, are involved in motility in liquid medium.

viii) Cylindrical bodies were easily recognizable due to their size and shape. Furthermore, cylindrical bodies were not present in all of the strains when grown under the same environment and were therefore a useful ultrastructural character for taxonomic purposes.

ix) Many *Arthrospira* strains could utilize glucose and fructose for growth in the dark. Glucose was utilized by more strains than fructose. The fact that many strains grew only in some of the replicates tested may indicate that dark heterotrophic growth requires closely defined conditions and/or that the ability to grow dark heterotrophically can be affected by genetic drift.

x) All strains tested behaved the same under photoheterotrophic conditions: glucose and maltose were utilized for growth (presence of DCMU), but not sucrose and fructose. Fructose was toxic at higher concentrations (20 mM).

xi) Growth in the presence of sucrose required an adaptation process which involved cell lysis of most of the cells of the inoculum. A few cells survive the sucrose upshock by presumably deposition of sucrose or sucrose breakdown products into intra-thylakoidal vacuoles, and subsequent growth. This adaptation process does, however, not lead to the ability to utilize sucrose under either photo- or dark heterotrophic conditions.

xii) Culture in Zarrouk's medium may have caused genetic drift of *Arthrospira* strains. For example, the high P content may have been responsible for the loss of surface phosphatase activity. Furthermore, the inorganic composition of the medium may have selected for a phenotype that is best adapted to it, but unable to utilize sugars for growth.

xiii) Fatty acids were a good marker for identification purposes, also for distinguishing *Arthrospira* strains from *Spirulina* sp. The fatty acid profiles of a strain grown under several different environments provided it with a chemotaxonomic "fingerprint". The data were also supported by the high reproducibility of the results.

xiv) Duplicate strains and different morphotypes of the same clone showed high similarity of the fatty acid composition.

xv) Increasing light intensity and dark heterotrophic growth at 30 °C led generally to increased desaturation of $C_{18}$ fatty acids.

xvi) Analysis of lectin-binding can help to identify a strain under investigation.

xvii) The results of PyMS showed that most of the strains were very similar in their whole cell composition, which was very different to that of *Spirulina*. The results were independent of the growth medium.

xviii) Data for 28 characters of those strains that showed a helical trichome morphology were analyzed by numerical methods. The straight morphotypes could not be included in the numerical analysis due to the loss of too many characters. Numerical analysis using the $S_{SM}$ group the strains into two phenotypic clusters which show a high correlation to the molecular clusters based on ARDRA of the ITS.

ixx) Characters, which describe the helical trichome morphology and the motility on substrate, were highly correlated to each other and to the phenotypic clusters based on the analysis of the whole data set. Analysis of the morphology of the trichome helix should therefore be the first step for the determination of the taxonomic position of *Arthrospira* strains.

xx) Several strains included in this study were assigned to the wrong species as concluded from comparison of cultures of these species and their species descriptions. As a consequence, the botanical species *Arthrospira maxima*, *A. fusiformis* and *A. indica* form phenotypic cluster I, while most of the species designated as *A. platensis* belong to phenotypic cluster II.

xxi) It is suggested that two new subgenera are introduced within the genus *Arthrospira*. One subgenus would summarize strains with often highly variable, but always irregular (fusiform or dumbbell-shaped) trichome helix showing fast diminishing helix attenuation towards the apices and generally low motility on substrate, while a second subgenus would be formed by strains with regular helix shape, slow diminishing helix attenuation towards the apices and high motility on substrate.

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# APPENDIX

Appendix A. Data for the morphological characters of *Arthrospira* srains.

Data were collected for the 35 strains, plus the five duplicate strains and eight different morphotypes of the set of 35 strains. Data from species descriptions have been added. (References to species descriptions are given in reference list.)

| Strain or species | Tendency to loose helical trichome morphology | Orientation of coiling at 30 °C | Trichome length (µm) at 30 °C | | Trichome width (µm) | | Cell length (µm) | | Ratio of cell length to width | | Diameter of trichome end (µm) | | Attenuation of trichome | | Length of pitch (µm) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | min | max | min | max | min | max | min | max | min | max | min | max | min | max |
| D0867 | no | cw | 0.5 | 1.5 | 9.5 | 11 | 4.0 | 5.5 | 1.8 | 2.5 | 5.5 | 7 | 1.5 | 1.8 | 65 | 93 |
| D0872/H | yes | cw | 0.8 | 1.3 | 8.5 | 9.5 | 4 | 5 | 1.8 | 2.4 | 5 | 6 | 1.6 | 1.9 | 110 | 135 |
| D0873 | no | acw | 0.8 | 1.5 | 8 | 9 | 3.5 | 5 | 1.6 | 2.4 | 6 | 6.5 | 1.2 | 1.3 | 80 | 90 |
| D0880 | no | acw | 1 | 1.5 | 9.5 | 10.5 | 3.5 | 5 | 1.9 | 2.9 | 6 | 6.5 | 1.4 | 1.7 | 25 | 43 |
| D0881 | yes | | 0.8 | 2 | 9 | 10 | 3.5 | 4.5 | 2.3 | 2.9 | 5.5 | 6.5 | 1.5 | 1.8 | | |
| D0882 | yes | | 0.2 | 0.5 | 6.5 | 7.5 | 3 | 4 | 1.6 | 2.3 | 5.0 | 5.5 | 1.1 | 1.4 | | |
| D0884 | no | acw | 0.2 | 0.8 | 7.5 | 8.5 | 3 | 4.5 | 1.6 | 2.7 | 5 | 6 | 1.2 | 1.6 | 10 | 20 |
| D0885/H1 | no | acw | 0.5 | 0.8 | 10.0 | 11.0 | 4.0 | 5.0 | 2.1 | 2.6 | 6 | 6.5 | 1.6 | 1.8 | 42.5 | 50 |
| D0890 | no | acw | 0.7 | 1.5 | 7.0 | 8.5 | 3 | 4 | 2.1 | 2.3 | 5 | 6 | 1.4 | 1.6 | 65 | 95 |
| D0891 | no | cw | 0.7 | 1.5 | 8.5 | 9.5 | 3 | 4 | 2.2 | 2.6 | 5.5 | 6 | 1.4 | 1.5 | 25 | 38 |
| D0895 | no | acw | 0.8 | 1.5 | 7.5 | 8.5 | 3 | 4 | 1.8 | 2.4 | 4.5 | 5.5 | 1.4 | 1.8 | 100 | 110 |
| D0896 | no | acw | 0.5 | 1.5 | 7 | 8 | 3.5 | 4.5 | 1.7 | 2.1 | 5.5 | 6.5 | 1.2 | 1.3 | 75 | 87 |
| D0897 | no | acw | 0.25 | 0.9 | 8 | 8.5 | 3 | 4 | 2.1 | 2.3 | 5 | 5.5 | 1.5 | 1.7 | 60 | 65 |
| D0899 | no | acw | 0.25 | 0.5 | 7.5 | 8.5 | 3.0 | 4.5 | 1.7 | 2.5 | 4 | 5.5 | 1.4 | 1.9 | 20 | 30 |
| D0900 | no | acw | 0.4 | 1.2 | 7 | 8 | 3.5 | 4.5 | 1.5 | 2.1 | 5 | 6 | 1.3 | 1.6 | 42.5 | 55 |
| D0904 | no | acw | 0.2 | 2.5 | 8 | 9 | 3.5 | 4 | 2.1 | 2.5 | 5 | 6 | 1.3 | 1.7 | 70 | 88 |
| D0905 | no | acw | 2 | 3 | 7.5 | 8.5 | 3 | 4 | 1.8 | 2.4 | 4.5 | 5.5 | 1.3 | 1.7 | 68 | 77.5 |
| D0907 | no | acw | 0.5 | 1 | 8 | 8.5 | 3 | 4 | 1.8 | 2.7 | 5 | 6.5 | 1.2 | 1.7 | 55 | 65 |
| D0909 | no | acw | 0.45 | 0.8 | 6.5 | 8 | 2.5 | 4 | 2.1 | 2.8 | 4.5 | 5 | 1.3 | 1.8 | 60 | 75 |
| D0910/H | yes | acw | 0.6 | 2 | 7 | 8 | 3 | 4 | 1.7 | 2.5 | 4.5 | 5.5 | 1.4 | 1.5 | 50 | 62.5 |
| D0911 | no | acw | 0.6 | 1.5 | 6.5 | 7.5 | 2.5 | 3.5 | 2.1 | 2.8 | 5.5 | 6.5 | 1.3 | 1.5 | 60 | 65 |
| D0913 | no | acw | 0.5 | 1.2 | 7.5 | 8.5 | 3.5 | 4 | 1.9 | 2.4 | 5 | 6 | 1.4 | 1.6 | 45 | 63 |
| D0914/H | yes | acw | 0.3 | 0.6 | 6 | 7 | 2.5 | 3.5 | 1.2 | 2.4 | 4.0 | 4 | 1.5 | 1.8 | 20 | 33 |
| D0915 | no | acw | 1 | 3 | 7.5 | 9 | 4 | 5 | 1.5 | 1.9 | 4.5 | 5 | 1.5 | 1.9 | 60 | 100 |
| D0916 | no | acw | 0.5 | 0.8 | 7.5 | 8.5 | 3 | 4 | 2 | 2.4 | 5.5 | 6.5 | 1.2 | 1.5 | 75 | 85 |
| D0918/H | yes | cw | 0.02 | 0.1 | 7 | 8 | 2.5 | 4 | 1.7 | 2.8 | 4.5 | 5.5 | 1.2 | 1.6 | 10 | 17.5 |
| D0919 | no | acw | 0.35 | 1.2 | 11.5 | 13 | 4 | 6 | 2.1 | 3 | 6.5 | 7.5 | 1.6 | 1.9 | 12.5 | 17.5 |
| D0920 | no | acw | 0.25 | 0.45 | 8 | 9 | 3 | 5 | 2.8 | 2.6 | 4 | 5.5 | 1.7 | 2 | 17.5 | 25 |
| D0921 | no | acw | 0.6 | 2 | 9 | 10 | 3.5 | 4 | 2.2 | 2.7 | 6 | 6.5 | 1.4 | 1.5 | 75 | 83 |
| D0922 | no | acw | 0.25 | 0.6 | 7 | 8 | 3 | 4 | 1.8 | 2.7 | 4.5 | 6 | 1.2 | 1.5 | 30 | 37.5 |
| D0923 | no | acw | 0.1 | 0.35 | 9 | 10 | 3 | 5 | 2 | 2.9 | 5.5 | 6.5 | 1.3 | 1.8 | 12.5 | 17.5 |
| D0925 | no | acw | 0.4 | 0.8 | 7.5 | 8.5 | 3.5 | 4 | 1.8 | 2.3 | 5 | 6 | 1.5 | 1.7 | 10 | 22.5 |
| D0929 | no | acw | 0.25 | 0.7 | 8.0 | 9 | 3 | 4.0 | 2.1 | 2.3 | 5 | 6.5 | 1.5 | 1.9 | 45 | 50 |
| D0930 | no | acw | 0.45 | 0.65 | 7.5 | 8.5 | 3 | 4.5 | 1.9 | 2.5 | 5 | 6 | 1.8 | 2.2 | 75 | 87.5 |
| D0933 | no | acw | 0.8 | 2 | 6.5 | 7.5 | 3 | 4.5 | 0.4 | 0.7 | 4.5 | 5.5 | 1.1 | 1.5 | 25 | 37.5 |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D0875 | no | acw | 700 | 1600 | 8 | 9 | 3 | 4.5 | 1.7 | 3 | 4 | 5.5 | 1.6 | 2 | 67 | 87 |
| D0876 | no | acw | 800 | 10 | 12 | 13 | 5.5 | 6.5 | 2.3 | 2.2 | 4.5 | 6 | 1.5 | 2 | 40 | 50 |
| D0879 | no | acw | 900 | 2300 | 9.5 | 10.5 | 3.5 | 4.5 | 2 | 2.7 | 6.0 | 7 | 1.3 | 1.7 | 107.5 | 140 |
| D0887 | yes | | 2000 | 5000 | 9.0 | 11.0 | 2 | 5 | 1.8 | 2.8 | 4 | 5.5 | 1.2 | 1.8 | | |
| D0872/S | yes | | 400 | 3000 | 9 | 10.5 | 4 | 5 | 1.8 | 2.3 | 5.5 | 6.5 | 1.5 | 2 | | |
| D0885/H2 | yes | acw | 900 | 1600 | 9 | 9.5 | 4.0 | 5.0 | 1.8 | 2.3 | 4 | 4.5 | 2 | 2.3 | 100 | |
| D0885/S | yes | | | | | | | | | | | | | | | |
| D0906/H | yes | acw | 600 | 1200 | 9 | 10.5 | 3.5 | 5 | 2 | 3 | 4.5 | 5.5 | 1.8 | 2 | 45 | 52.5 |
| D0906/S | yes | | 900 | 1700 | 9.5 | 11 | 4.5 | 5.5 | 1.9 | 2.2 | 5 | 6 | 1.7 | 2.1 | | |
| D0910/S1 | yes | | 2000 | 5000 | 8 | 9.5 | 3.5 | 4.5 | 1.8 | 2.6 | 6 | 7.5 | 1.2 | 1.4 | | |
| D0910/S2 | yes | | 800 | 2000 | 8 | 9 | 3 | 4.5 | 1.6 | 2.8 | 5 | 6 | 1.3 | 1.6 | | |
| D0914/S | yes | | 600 | 2500 | 6 | 7 | 2 | 5 | 1.8 | 2.2 | 4 | 4 | 1.5 | 1.9 | | |
| D0918/S | yes | | 150 | 350 | 7.5 | 8 | 2 | 3 | 2.1 | 3 | 5.5 | 6 | 1.2 | 1.5 | | |
| *A.jenneri* Gomont 1892 | | | | | 5 | 8 | 4 | 5 | >1.0 | >2.0 | | | | | 21 | 31 |
| *A.platensis* Gomont 1892 | | | | | 6 | 8 | 2 | 6 | >1.0 | >1.0 | | | | | 43 | 57 |
| *A.miniata* Gomont 1892 | | | | | 2 | 3 | 3.5 | 7 | >1.0 | <3.0 | | | | | 11 | 13 |
| *A.maxima* Setch. et N.L.Gardner 1917 | | | | | 7 | 9 | 5 | 7 | | | | | | | 70 | 80 |
| *A.breviarticulata* Setch. et N.L.Gardner 1917 | | | | | 14.5 | 16 | 2.5 | 3.5 | | | | | | | | |
| *A.skujae* Magrin, Senna et Komárek 1997 | | | | | 3.1 | 4.1 | 3.1 | 9.3 | 0.4 | 0.6 | | | | | 7.4 | 13 |
| *S.attenuata* Umezaki 1952 | | | | | 5 | 6 | 1.5 | 2.5 | 2 | 3 | | | | | 13 | 20 |
| *A.tenuis* Brühl et Biswas 1922 | | | | | 2.0 | 2.0 | 2 | 3 | ca. 1 | <1 | | | | | | |
| *A.curta* Lemmerm. 1909 | | | 19 | 19 | 4.0 | 4.0 | | | | | | | | | | |
| *S.aeruginea* H.F.Buell 1938 | acw | | | | 3.9 | 5.7 | 3.5 | 5 | | | | | | | 14 | 24 |
| *S.amethystina* H.F.Buell 1938 | cw | | | | 3.9 | 5.7 | 3.5 | 5 | | | | | | | 14 | 24 |
| *A.laxissima* Setch 1924 | | | | | 10 | 12 | | | 0.3 | 0.5 | | | | | | |
| *A.gomontiana* Setch. 1895 | | | | | 2.5 | 3 | 4 | 5 | | | | | | | 16 | 18 |
| *S.argentina* Freng. 1937 | | | 708 | 708 | 9 | 10.5 | 3.35 | 3.75 | | | | | | | 63 | 75 |
| *S.allansonii* Welsh 1961 | | | 90 | 280 | 5 | 6.5 | 2.4 | 4.5 | | | | | | | 60 | 60 |
| *S.fusiformis* Woron. 1934 | | | 10 | 325 | 6.8 | 6.8 | | | | | | | | | 1.8 | 24 |
| *A.indica* Desikachary et Jeeji Bai 1992 | | | | | 4 | 11.2 | | | | | 3.5 | 7 | | | 16 | 66 |
| D0868 | | | 200 | 1000 | 1 | 1 | | | | | | | | | 1.5 | 1.5 |
| D0869 | | | 200 | 800 | 1.5 | 1.5 | | | | | | | | | 4.0 | 4 |
| D0870 | | | 200 | 1000 | 1.5 | 1.5 | | | | | | | | | 4.0 | 4 |
| D0871 | | | 800 | 1000 | 0.8 | 1 | | | | | | | | | 1.5 | 1.5 |
| D0877 | | | 400 | 1000 | 1.7 | 2 | | | | | | | | | 4.5 | 5 |
| D0878 | | | 100 | 400 | 1.5 | 1.5 | | | | | | | | | 3.5 | 4 |
| D0917 | | | 700 | 1000 | 1 | 1.3 | | | | | | | | | 2 | 2.5 |

| Strain or species | Diameter of trichome helix | | Ratio of length of pitch to diameter of helix | | Variation in helix dimensions (ratio of lowest to highest value of ratio of pitch to diameter of | Type of trichome helix | Attenuation of helix at apices of trichome | Trichome apical cell | Granules | Gas vacuoles |
|---|---|---|---|---|---|---|---|---|---|---|
| | min | max | min | max | | regular | Fast diminishing | Trichome apical cell | | |
| D0867 | 25 | 32.5 | 2.3 | 3.5 | 0.66 | irregular/fusiform | fast diminishing | conical | at cross walls | at cross walls |
| D0872/H | 37.5 | 55 | 2.9 | 3.6 | 0.6 | irregular/fusiform | fast diminishing | capitate | at cross walls | at cross walls |
| D0873 | 37.5 | 50 | 1.5 | 2.1 | 0.71 | fusiform | slowly diminishing | conical/capitate | at cross walls | distributed |
| D0880 | 35 | 42.5 | 0.7 | 1.1 | 0.64 | fusiform/dumbbell-shaped | fast diminishing | capitate | distributed | distributed |
| D0881 | | | | | | | | rounded/conical/calyptrate | at cross walls | at cross walls |
| D0882 | | | | | | | | conical/calyptrate | at cross walls | at cross walls |
| D0884 | 47.5 | 55 | 0.2 | 0.4 | 0.5 | fusiform/dumbbell-shaped | fast diminishing | rounded/conical | at cross walls | at cross walls |
| D0885/H1 | 17.5 | 20 | 2.4 | 2.9 | 0.83 | regular | slowly diminishing | conical/calyptrate | at cross walls | at cross walls |
| D0890 | 35 | 53 | 1.8 | 2.4 | 0.75 | regular | slowly diminishing | rounded/conical/calyptrate | at cross walls | at cross walls |
| D0891 | 50 | 55 | 0.5 | 1.3 | 0.38 | fusiform/dumbbell-shaped | slowly diminishing | rounded/conical/calyptrate | at cross walls | at cross walls |
| D0895 | 42.5 | 52.5 | 1.9 | 2.4 | 0.79 | regular | slowly diminishing | rounded/conical | distributed | at cross walls |
| D0896 | 25 | 35 | 2.6 | 3.1 | 0.84 | regular | slowly diminishing | rounded/conical/capitate | distributed | at cross walls |
| D0897 | 42.5 | 55 | 1.3 | 2.6 | 0.5 | fusiform | fast diminishing | capitate | distributed | at cross walls |
| D0899 | 27.5 | 30 | 0.7 | 1 | 0.7 | fusiform | fast diminishing | capitate | distributed | at cross walls |
| D0900 | 33 | 48 | 1.3 | 1.5 | 0.87 | fusiform | slowly diminishing | rounded/conical/calyptrate | distributed | at cross walls |
| D0904 | 25 | 30 | 2.3 | 2.8 | 0.82 | regular | slowly diminishing | rounded/conical/calyptrate | at cross walls | distributed |
| D0905 | 22.5 | 27.5 | 2.8 | 3 | 0.93 | regular | slowly diminishing | rounded/conical | at cross walls | distributed |
| D0907 | 37.5 | 40 | 1.3 | 1.7 | 0.76 | regular | slowly diminishing | rounded/conical | distributed | at cross walls |
| D0909 | 33 | 46 | 1.5 | 1.6 | 0.94 | regular | slowly diminishing | capitate/calyptrate | distributed | at cross walls |
| D0910/H | 25 | 35 | 1.8 | 2.1 | 0.9 | regular | slowly diminishing | conical/calyptrate | at cross walls | at cross walls |
| D0911 | 35 | 40 | 1.6 | 1.7 | 0.94 | regular | slowly diminishing | capitate | distributed | distributed |
| D0913 | 30 | 35 | 1.1 | 1.9 | 0.58 | fusiform | fast diminishing | rounded/conical | at cross walls | at cross walls |
| D0914/H | 25 | 33 | 0.6 | 1 | 0.6 | fusiform/dumbbell-shaped | fast diminishing | capitate/calyptrate | at cross walls | at cross walls |
| D0915 | 20 | 38 | 1.6 | 3.2 | 0.5 | regular | slowly diminishing | rounded/conical | at cross walls | distributed |
| D0916 | 30 | 35 | 2.4 | 2.8 | 0.86 | regular | slowly diminishing | capitate/calyptrate | at cross walls | distributed |
| D0918/H | 42.5 | 50 | 0.2 | 0.4 | 0.5 | fusiform/dumbbell-shaped | fast diminishing | rounded/capitate | distributed | distributed |
| D0919 | 50 | 62.5 | 0.2 | 0.3 | 0.67 | fusiform | fast diminishing | rounded/conical/capitate | at cross walls | at cross walls |
| D0920 | 27 | 33 | 0.6 | 0.8 | 0.75 | fusiform | fast diminishing | capitate | distributed | distributed |
| D0921 | 30 | 35 | 2.3 | 2.7 | 0.85 | regular | slowly diminishing | rounded/conical | at cross walls | at cross walls |
| D0922 | 25 | 32.5 | 0.9 | 1.3 | 0.69 | fusiform | fast diminishing | rounded/conical | distributed | at cross walls |
| D0923 | 47.5 | 55 | 0.2 | 0.3 | 0.67 | fusiform/dumbbell-shaped | fast diminishing | capitate/calyptrate | at cross walls | at cross walls |
| D0925 | 42.5 | 50 | 0.2 | 0.4 | 0.5 | barrel-shapedfusiform | fast diminishing | capitate/calyptrate | at cross walls | at cross walls |
| D0929 | 27.5 | 32.5 | 1.4 | 1.8 | 0.77 | regular | slowly diminishing | rounded/conical | distributed | distributed |
| D0930 | 27.5 | 37.5 | 2 | 3.2 | 0.62 | regular | slowly diminishing | capitate/calyptrate | distributed | at cross walls |
| D0933 | 18.7 | 22.5 | 1.2 | 1.6 | 0.75 | regular | slowly diminishing | capitate/calyptrate | distributed | distributed |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| D0875 | 42 | 48 | 1.5 | 1.9 | 0.79 | regular | slowly diminishing | round/conical/capitate | at cross walls | at cross walls |
| D0876 | 50 | 70 | 0.8 | 0.7 | 0.88 | regular | slowly diminishing | round/conical | at cross walls | at cross walls |
| D0879 | 32.5 | 47.5 | 2.4 | 3.9 | 0.62 | regular | slowly diminishing | round/conical/capitate | distributed | at cross walls |
| D0887 | | | | | | | | capitate/calyptrate | at cross walls | at cross walls |
| D0872/S | | | | | | | | round/conical/capitate | at cross walls | at cross walls |
| D0885/H2 | 22 | 28 | 3.9 | 4.6 | 0.85 | regular | slowly diminishing | capitate/calyptrate | at cross walls | at cross walls |
| D0885/S | | | | | | | | capitate/calyptrate | at cross walls | at cross walls |
| D0906/H | 45 | 52.5 | 0.8 | 1.2 | 0.67 | fusiform/dumbbell-shaped | slowly diminishing | capitate/calyptrate | at cross walls | distributed |
| D0906/S | | | | | | | | capitate/calyptrate | at cross walls | distributed |
| D0910/S1 | | | | | | | | rounded/conical/calyptrate | at cross walls | distributed |
| D0910/S2 | | | | | | | | rounded/conical/calyptrate | distributed | at cross walls |
| D0914/S | | | | | | | | capitate/calyptrate | at cross walls | at cross walls |
| D0918/S | | | | | | | slowly diminishing | rounded/conical | distributed | distributed |
| *A.jenneri* Gomont 1892 | 9 | 15 | | | | | slowly diminishing | | at cross walls | |
| *A.platensis* Gomont 1892 | 26 | 36 | | | | | slowly diminishing | | at cross walls | |
| *A.miniata* Gomont 1892 | 4.0 | 4 | | | | | slowly diminishing | | distributed | |
| *A.maxima* Setch. et N.L.Gardner 1917 | 40 | 60 | | | | | slowly diminishing | capitate | at cross walls | |
| *A.breviarticulata* Setch. et N.L.Gardner 1917 | | | | | | | slowly diminishing | not capitate | distributed | |
| *A.skujae* Magrin, Senna et Komárek 1997 | 7.4 | 11.8 | | | | regular | slowly diminishing | not capitate | distributed | |
| *S.attenuata* Umezaki 1952 | 50 | 80 | | | | | slowly diminishing | not calyptrate | distributed | |
| *A.tenuis* Brühl et Biswas 1922 | 20 | 35 | | | | | | | distributed | absent |
| *A.curta* Lemmerm. 1909 | | | | | | regular | | not capitate | distributed | |
| *S.aeruginea* H.F.Buell 1938 | 9.5 | 18 | | | | | | | at cross walls | |
| *S.amethystina* H.F.Buell 1938 | 9.5 | 18 | | | | | | | at cross walls | |
| *A.laxissima* Setch 1924 | | | | | | | | not capitate | distributed | |
| *A.gomontiana* Setch. 1895 | 6 | 6 | | | | | | not capitate | distributed | |
| *S.argentina* Freng. 1937 | 33 | 49 | | | | | | | distributed | present |
| *S.allansonii* Welsh 1961 | 39 | 60 | | | | | | | | |
| *S.fusiformis* Woron. 1934 | 50 | 65 | | | | 1 | fast diminishing | | | |
| *A.indica* Desikachary et Jeeji Bai 1992 | 30 | 66 | | | | | fast diminishing | capitate/calyptrate | at cross walls | |
| D0868 | 3.0 | 3 | | | | | | | | |
| D0869 | 4.0 | 4.0 | | | | | | | | |
| D0870 | 4.0 | 4.0 | | | | | | | | |
| D0871 | 3.0 | 3.0 | | | | | | | | |
| D0877 | 4.0 | 4 | | | | | | | | |
| D0878 | 3.5 | 4.0 | | | | | | | | |
| D0917 | 3 | 3.5 | | | | | | | | |

Appendix B. Coded data for the 28 characters used for numerical analysis and similarity matrix calculated from it.

i) Data are shown for 28 characters for the set of 37 strains which includes four helical strains that are duplicates of others also included in the set (Section 10.2). The coded data is given in form of the input format for calculation of $S_{SM}$ using the mvsp software package. The order of the characters in horizontal direction is the same as listed in Table 10.1.

```
*L 37 28
a b c d e f g h i j k l m n o p q r s t u v w x y z aa ab
D0867     0 1 1 1 1 0 0 1 1 0 0 1 1 1 0 0 1 1 1 1 0 1 1 0 1 1 1 1
D0872/H   1 1 1 1 0 1 0 1 1 0 0 1 1 1 0 1 0 0 1 0 1 1 1 1 0 1 1 1
D0873     0 1 1 1 1 1 0 1 0 0 0 0 1 0 0 0 0 0 1 0 0 1 1 1 0 1 1 1
D0880     0 1 1 1 1 1 1 1 0 0 0 1 0 0 0 0 0 1 1 0 0 1 1 1 0 1 1 0
D0884     0 0 0 1 0 1 0 1 0 0 0 1 1 1 0 1 0 1 1 1 0 1 0 1 0 0 0 0
D0885/H1  1 0 1 1 1 0 1 0 0 0 1 0 1 1 1 0 1 1 1 1 0 0 0 0 1 1 1 0
D0890     0 1 0 0 0 0 1 0 0 0 1 0 1 1 0 0 0 0 1 1 1 1 1 1 1 1 0 0
D0891     0 1 0 0 1 0 1 1 1 0 0 0 1 1 0 1 0 1 1 1 1 0 1 1 1 1 1 1
D0895     0 1 0 0 0 0 0 0 0 0 1 0 0 1 0 0 1 1 1 1 0 1 1 1 1 1 1 0
D0896     0 1 0 1 1 1 0 0 0 0 1 0 0 1 1 0 1 0 1 1 1 1 1 0 0 1 1 1
D0897     0 0 1 0 0 1 0 1 0 0 0 1 0 1 0 1 0 1 1 1 0 0 0 0 1 0 0 0
D0899     0 0 1 1 0 1 0 1 0 0 0 1 0 1 0 0 0 1 1 0 1 1 1 1 0 1 0 0
D0900     0 0 0 1 0 0 1 1 0 0 1 0 0 1 0 1 0 0 1 1 0 1 1 1 0 0 0 1
D0904     0 1 1 1 0 0 1 0 0 0 1 0 1 0 1 0 0 0 1 1 0 1 1 0 1 1 1 1
D0905     0 1 0 0 0 0 0 0 0 0 1 0 1 0 1 0 1 1 1 1 1 0 1 0 1 1 1 1
D0907     0 0 1 0 1 0 0 0 0 0 1 0 0 1 0 0 1 1 1 0 1 0 1 0 1 1 1 0
D0909     0 0 0 0 0 1 1 0 0 0 1 0 0 1 0 1 0 1 1 0 0 1 0 1 1 0 0 0
D0910/H   1 1 0 0 0 0 1 0 0 0 1 0 1 1 0 1 0 1 1 1 1 0 0 0 1 1 1 1
D0911     0 1 0 0 1 1 0 0 0 0 1 0 0 0 0 1 0 0 1 1 1 1 1 1 1 1 1 1
D0913     0 1 0 1 0 0 0 1 0 0 0 1 1 1 0 0 0 0 1 1 1 1 0 0 1 1 1 1
D0914/H   1 0 0 0 0 1 1 1 0 0 0 1 1 1 0 0 0 1 1 0 0 0 0 1 1 1 1 1
D0915     0 1 1 1 0 0 0 0 0 0 0 0 1 0 0 0 0 1 0 1 1 0 0 1 1 0 0 0
D0916     0 0 0 0 1 1 1 0 0 0 1 0 1 0 0 0 1 1 1 1 0 0 0 0 0 1 1 1
D0918/H   1 0 0 0 0 0 0 1 1 1 0 1 0 0 0 1 0 1 1 1 0 1 0 1 0 1 0 0
D0919     0 0 1 1 1 1 0 1 0 0 0 1 1 1 0 1 0 1 1 1 0 1 0 0 1 1 1 1
D0920     0 0 1 1 0 1 0 1 0 0 0 1 0 0 0 1 0 1 1 1 1 0 1 1 0 1 1 1
D0921     0 1 1 1 1 0 0 0 0 0 1 0 1 1 1 0 1 1 1 1 1 0 0 0 1 0 0 0
D0922     0 0 0 0 0 0 0 1 0 0 0 1 0 1 0 0 0 1 1 0 1 1 1 1 0 1 1 1
D0923     0 0 1 1 1 1 1 1 0 1 0 1 1 1 0 1 0 1 1 1 0 1 0 0 0 1 1 0
D0925     0 0 0 1 0 1 1 1 0 1 0 1 1 1 0 1 0 0 1 1 0 1 0 1 0 1 1 1
D0929     0 0 1 0 1 0 0 0 0 0 0 0 0 0 0 1 0 1 1 1 0 0 0 0 1 1 1 1
D0930     0 0 0 1 0 1 1 0 0 0 0 0 0 1 0 0 1 0 1 0 0 0 0 0 1 1 1 1
D0933     0 1 0 1 0 1 1 0 0 0 0 0 0 0 0 0 0 1 1 0 0 0 0 1 0 0 0 0
D0879     0 1 1 1 1 1 0 0 0 0 0 0 0 1 0 0 0 0 1 1 0 0 1 1 0 1 1 1
D0875     0 1 0 1 0 1 0 0 0 0 1 0 1 1 0 1 0 0 1 1 0 0 0 1 1 0 0 0
D0876     0 1 1 1 0 0 0 0 0 0 1 0 1 1 0 1 0 1 1 0 0 0 0 1 1 0 0 0
D0906/H   1 1 1 1 0 1 1 1 0 0 0 1 1 0 0 0 0 1 0 0 0 0 0 1 0 1 1 1
```

ii) Similarity matrix calculated from the data for the 28 characters using the $S_{SM}$.

| | D0867 | D0872/H | D0873 | D0880 | D0884 | D0885/H1 | D0890 | D0891 | D0895 | D0896 | D0897 | D0899 | D0900 | D0904 | D0905 | D0907 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D0867 | 1 | | | | | | | | | | | | | | | |
| D0872/H | 0.64 | 1 | | | | | | | | | | | | | | |
| D0873 | 0.68 | 0.75 | 1 | | | | | | | | | | | | | |
| D0880 | 0.64 | 0.64 | 0.82 | 1 | | | | | | | | | | | | |
| D0884 | 0.54 | 0.61 | 0.57 | 0.61 | 1 | | | | | | | | | | | |
| D0885/H1 | 0.61 | 0.32 | 0.43 | 0.46 | 0.43 | 1 | | | | | | | | | | |
| D0890 | 0.50 | 0.50 | 0.54 | 0.50 | 0.54 | 0.54 | 1 | | | | | | | | | |
| D0891 | 0.68 | 0.61 | 0.57 | 0.54 | 0.50 | 0.50 | 0.68 | 1 | | | | | | | | |
| D0895 | 0.64 | 0.43 | 0.54 | 0.57 | 0.54 | 0.61 | 0.79 | 0.61 | 1 | | | | | | | |
| D0896 | 0.61 | 0.54 | 0.64 | 0.54 | 0.43 | 0.57 | 0.61 | 0.50 | 0.68 | 1 | | | | | | |
| D0897 | 0.54 | 0.46 | 0.43 | 0.54 | 0.79 | 0.50 | 0.46 | 0.50 | 0.54 | 0.36 | 1 | | | | | |
| D0899 | 0.57 | 0.71 | 0.68 | 0.79 | 0.75 | 0.39 | 0.57 | 0.46 | 0.57 | 0.54 | 0.68 | 1 | | | | |
| D0900 | 0.46 | 0.54 | 0.57 | 0.54 | 0.71 | 0.43 | 0.68 | 0.57 | 0.61 | 0.57 | 0.57 | 0.61 | 1 | | | |
| D0904 | 0.64 | 0.50 | 0.68 | 0.57 | 0.39 | 0.68 | 0.71 | 0.54 | 0.64 | 0.68 | 0.39 | 0.43 | 0.61 | 1 | | |
| D0905 | 0.61 | 0.39 | 0.50 | 0.39 | 0.36 | 0.64 | 0.68 | 0.64 | 0.75 | 0.71 | 0.43 | 0.39 | 0.43 | 0.75 | 1 | |
| D0907 | 0.61 | 0.39 | 0.50 | 0.54 | 0.36 | 0.71 | 0.61 | 0.57 | 0.75 | 0.64 | 0.57 | 0.61 | 0.43 | 0.54 | 0.71 | 1 |
| D0909 | 0.32 | 0.39 | 0.43 | 0.54 | 0.71 | 0.50 | 0.68 | 0.50 | 0.68 | 0.43 | 0.71 | 0.61 | 0.71 | 0.46 | 0.43 | 0.57 |
| D0910/H | 0.50 | 0.50 | 0.39 | 0.36 | 0.46 | 0.68 | 0.71 | 0.75 | 0.64 | 0.54 | 0.54 | 0.36 | 0.54 | 0.64 | 0.75 | 0.61 |
| D0911 | 0.50 | 0.57 | 0.68 | 0.57 | 0.46 | 0.39 | 0.71 | 0.68 | 0.71 | 0.75 | 0.46 | 0.50 | 0.61 | 0.64 | 0.68 | 0.61 |
| D0913 | 0.75 | 0.68 | 0.64 | 0.54 | 0.64 | 0.50 | 0.68 | 0.64 | 0.61 | 0.64 | 0.57 | 0.61 | 0.57 | 0.68 | 0.64 | 0.50 |
| D0914/H | 0.54 | 0.61 | 0.57 | 0.61 | 0.64 | 0.57 | 0.54 | 0.64 | 0.54 | 0.36 | 0.64 | 0.61 | 0.50 | 0.46 | 0.50 | 0.50 |
| D0915 | 0.50 | 0.43 | 0.54 | 0.50 | 0.61 | 0.54 | 0.64 | 0.54 | 0.57 | 0.39 | 0.61 | 0.57 | 0.46 | 0.57 | 0.61 | 0.54 |
| D0916 | 0.54 | 0.32 | 0.57 | 0.54 | 0.50 | 0.71 | 0.54 | 0.57 | 0.61 | 0.64 | 0.50 | 0.39 | 0.50 | 0.61 | 0.71 | 0.64 |
| D0918/H | 0.46 | 0.54 | 0.43 | 0.54 | 0.71 | 0.36 | 0.46 | 0.50 | 0.54 | 0.29 | 0.64 | 0.61 | 0.57 | 0.32 | 0.36 | 0.36 |
| D0919 | 0.79 | 0.64 | 0.68 | 0.64 | 0.75 | 0.61 | 0.43 | 0.61 | 0.50 | 0.54 | 0.75 | 0.64 | 0.54 | 0.57 | 0.46 | 0.54 |
| D0920 | 0.57 | 0.71 | 0.68 | 0.71 | 0.68 | 0.39 | 0.43 | 0.61 | 0.50 | 0.54 | 0.68 | 0.79 | 0.61 | 0.50 | 0.54 | 0.54 |
| D0921 | 0.61 | 0.32 | 0.43 | 0.39 | 0.50 | 0.79 | 0.61 | 0.50 | 0.61 | 0.64 | 0.57 | 0.46 | 0.43 | 0.61 | 0.71 | 0.71 |
| D0922 | 0.61 | 0.68 | 0.64 | 0.68 | 0.64 | 0.36 | 0.61 | 0.64 | 0.68 | 0.57 | 0.57 | 0.82 | 0.64 | 0.46 | 0.57 | 0.64 |
| D0923 | 0.64 | 0.57 | 0.61 | 0.71 | 0.75 | 0.61 | 0.43 | 0.54 | 0.43 | 0.46 | 0.68 | 0.64 | 0.54 | 0.50 | 0.32 | 0.46 |
| D0925 | 0.54 | 0.68 | 0.64 | 0.61 | 0.79 | 0.43 | 0.54 | 0.57 | 0.46 | 0.50 | 0.57 | 0.61 | 0.71 | 0.54 | 0.36 | 0.29 |
| D0929 | 0.61 | 0.39 | 0.57 | 0.54 | 0.50 | 0.64 | 0.46 | 0.64 | 0.61 | 0.50 | 0.71 | 0.46 | 0.50 | 0.61 | 0.64 | 0.71 |
| D0930 | 0.54 | 0.46 | 0.57 | 0.54 | 0.50 | 0.64 | 0.54 | 0.50 | 0.61 | 0.64 | 0.57 | 0.54 | 0.57 | 0.61 | 0.57 | 0.64 |
| D0933 | 0.36 | 0.43 | 0.61 | 0.71 | 0.68 | 0.46 | 0.57 | 0.46 | 0.57 | 0.46 | 0.61 | 0.64 | 0.61 | 0.50 | 0.46 | 0.46 |

| | D0909 | D0910/H | D0911 | D0913 | D0914/H | D0915 | D0916 | D0918/H | D0919 | D0920 | D0921 | D0922 | D0923 | D0925 | D0929 | D0930 | D0933 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| D0867 | | | | | | | | | | | | | | | | | |
| D0872/H | | | | | | | | | | | | | | | | | |
| D0873 | | | | | | | | | | | | | | | | | |
| D0880 | | | | | | | | | | | | | | | | | |
| D0884 | | | | | | | | | | | | | | | | | |
| D0885/H1 | | | | | | | | | | | | | | | | | |
| D0890 | | | | | | | | | | | | | | | | | |
| D0891 | | | | | | | | | | | | | | | | | |
| D0895 | | | | | | | | | | | | | | | | | |
| D0896 | | | | | | | | | | | | | | | | | |
| D0897 | | | | | | | | | | | | | | | | | |
| D0899 | | | | | | | | | | | | | | | | | |
| D0900 | | | | | | | | | | | | | | | | | |
| D0904 | | | | | | | | | | | | | | | | | |
| D0905 | | | | | | | | | | | | | | | | | |
| D0907 | | | | | | | | | | | | | | | | | |
| D0909 | 1 | | | | | | | | | | | | | | | | |
| D0910/H | 0.61 | 1 | | | | | | | | | | | | | | | |
| D0911 | 0.61 | 0.64 | 1 | | | | | | | | | | | | | | |
| D0913 | 0.43 | 0.68 | 0.61 | 1 | | | | | | | | | | | | | |
| D0914/H | 0.64 | 0.68 | 0.46 | 0.64 | 1 | | | | | | | | | | | | |
| D0915 | 0.54 | 0.57 | 0.50 | 0.61 | 0.46 | 1 | | | | | | | | | | | |
| D0916 | 0.57 | 0.68 | 0.61 | 0.50 | 0.64 | 0.46 | 1 | | | | | | | | | | |
| D0918/H | 0.57 | 0.46 | 0.46 | 0.50 | 0.57 | 0.46 | 0.43 | 1 | | | | | | | | | |
| D0919 | 0.54 | 0.57 | 0.57 | 0.75 | 0.68 | 0.50 | 0.61 | 0.54 | 1 | | | | | | | | |
| D0920 | 0.46 | 0.50 | 0.64 | 0.61 | 0.61 | 0.57 | 0.54 | 0.61 | 0.71 | 1 | | | | | | | |
| D0921 | 0.50 | 0.61 | 0.46 | 0.57 | 0.36 | 0.75 | 0.57 | 0.29 | 0,54 | 0.39 | 1 | | | | | | |
| D0922 | 0.57 | 0.54 | 0.61 | 0.71 | 0.71 | 0.46 | 0.50 | 0.64 | 0.61 | 0.75 | 0.36 | 1 | | | | | |
| D0923 | 0.54 | 0.50 | 0.43 | 0.61 | 0.61 | 0.43 | 0.61 | 0.61 | 0.86 | 0.64 | 0.46 | 0.54 | 1 | | | | |
| D0925 | 0.57 | 0.54 | 0.54 | 0.71 | 0.71 | 0.39 | 0.57 | 0.64 | 0.75 | 0.68 | 0.29 | 0.64 | 0.82 | 1 | | | |
| D0929 | 0.57 | 0.68 | 0.68 | 0.57 | 0.57 | 0.61 | 0.71 | 0.57 | 0.75 | 0.68 | 0.57 | 0.57 | 0.61 | 0.50 | 1 | | |
| D0930 | 0.64 | 0.61 | 0.54 | 0.64 | 0.71 | 0.46 | 0.71 | 0.36 | 0.61 | 0.54 | 0.50 | 0.57 | 0.54 | 0.64 | 0.64 | 1 | |
| D0933 | 0.75 | 0.50 | 0.50 | 0.46 | 0.61 | 0.71 | 0.61 | 0.54 | 0.43 | 0.57 | 0.54 | 0.54 | 0.50 | 0.54 | 0.54 | 0.68 | 1 |

Appendix C. Reprint of publication Scheldeman et al. (1999).

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# *Arthrospira* ('*Spirulina*') strains from four continents are resolved into only two clusters, based on amplified ribosomal DNA restriction analysis of the internally transcribed spacer

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**Abstract**

We present the results of a phylogenetic study, based on amplified ribosomal DNA restriction analysis of the rDNA operon, of 37 *Arthrospira* ('*Spirulina*') cultivated clonal strains from four continents. In addition, duplicates from different culture collections or markedly different morphotypes of particular strains established as clonal cultures were treated as separate entries, resulting in a total of 51 tested cultures. The strain *Spirulina laxissima* SAG 256.80 was included as outgroup. The 16S rRNA genes appeared too conserved for discrimination of the strains by amplified ribosomal DNA restriction analysis, and thus the internally transcribed spacer was selected as molecular taxonomic marker. The internally transcribed spacer sequences situated between the 16S and the 23S rRNA were amplified by polymerase chain reaction and yielded amplicons of about 540 bp. Direct use of cells for polymerase chain reaction seemed to inhibit the amplification reaction. This was overcome by the design of a crude lysis protocol and addition of bovine serum albumin in the polymerase chain reaction mix.The amplicons were digested with four restriction enzymes (*Eco*RV, *Hha*I, *Hin*fI, *Mse*I) and the banding patterns obtained were analyzed. Cluster analysis showed the separation of all the strains into two main clusters. No clear relationships could be observed between this division into two clusters and the geographic origin of the strains, or their designation in the culture collections, or their morphology. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

*Keywords:* rRNA; Internally transcribed spacer; *Arthrospira*; *Spirulina*; Genotype; Taxonomy

## 1. Introduction

The main characteristics of the genus *Arthrospira* (wrongly merged with the genus *Spirulina* by Geitler in 1932 [1]) defined in the Bergey's Manual of Systematic Bacteriology [2] are the loosely coiled tri-

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Table 1
List of strains, origin, and morphology

| Strain designation | Strain number | Origin | Morphology | Durham number | Source | Clones in other collections |
|---|---|---|---|---|---|---|
| *Arthrospira maxima* | CCAP 1475/9 | Natron lake, Chad | H | D0873 | CCAP | ATCC 53871; Lefevre 1963/M132-1; SAG 84.79; UTEX 2342 |
| *Arthrospira maxima* | SAG 84.79 | Natron lake, Chad | H | D0879 | SAG | |
| *Arthrospira maxima* | Lefevre 1963/M132-1 | Natron lake, Chad | H | D0903 | CCALA | |
| *Arthrospira platensis* | SAG 85.79 | Natron lake, Chad | H | D0880 | SAG | Laporte 1963/M132-2b; NIVA CYA 120; UTEX 2340 |
| *Arthrospira* sp. ('*platensis*') | Laporte 1963/M132-2b | Natron lake, Chad | H | D0906/H | CCALA | |
| *Arthrospira* sp. ('*platensis*') | Laporte 1963/M132-2b | Natron lake, Chad | S | D0906/S | CCALA | |
| *Arthrospira platensis* | SP-4 | Natron lake, Chad | S | D0887 | Durham | |
| *Arthrospira platensis* | SAG 85.79 (Lill) | Natron lake, Chad | H | | H. Lill | |
| *Arthrospira platensis* | SAG 86.79 | Natron lake, Chad | S | D0882 | SAG | Compère 86/79 |
| *Arthrospira platensis* | SAG 86.79 (Lill) | Natron lake, Chad | S | | H. Lill | |
| *Arthrospira* sp. | CI | Lake Bodou, Kanem, Chad | H (S) | D0918 | A. Sanang-elantoni | |
| *Arthrospira* sp. | SP-8 | Lake Simbi, Kenya | H | D0891 | Durham | |
| *Arthrospira maxima* | CCAP 1406/2 | Lake Naivasha, Kenya | H | D0867 | CCAP | |
| *Arthrospira fusiformis* | CCAP 1475/8 | Lake Chitu, Ethiopia | H | D0872/H | CCAP | |
| *Arthrospira fusiformis* | CCAP 1475/8 | Lake Chitu, Ethiopia | S | D0872/S | CCAP | |
| *Arthrospira* 'Lonar' | | Lonar Lake, Maharashtra, India | H | D0920 | R. Fox | |
| *Arthrospira* 'Titicaca' | | Lake Titicaca, Peru | H | D0922 | R. Fox | |
| *Arthrospira* sp. | SP-14 | Unknown | H | D0897 | Durham | |
| *Arthrospira* sp. | SP-16 | Unknown | H | D0899 | SAC | |
| *Arthrospira* sp. | SP-17 | Unknown | H | D0900 | SAC | |
| *Arthrospira* sp. | Strain EF-18A | Unknown | H | D0925 | Earthrise Farms | |
| *Arthrospira* sp. | PCC 9223 | Lake Santa Olalla, Donana National Park, Spain | H | D0933 | PCC | |
| *Arthrospira* sp. | PCC 7939 | India, Kenya, Mexico or Peru | H | D0912 | PCC | Records lost at PCC |
| *Arthrospira* sp. | PCC 7940 | India, Kenya, Mexico or Peru | H | D0913 | PCC | Records lost at PCC |
| *Arthrospira* sp. | PCC 8005 | India, Kenya, Mexico or Peru | H | D0914 | PCC | Records lost at PCC |
| *Arthrospira indica* | MCRC isolate straight | MCRC, Madras, India | S | | N. Jeeji-Bai | |

Table 1 (*Continued*).
List of strains, origin, and morphology

| Strain designation | Strain number | Origin | Morphology | Durham number | Source | Clones in other collections |
|---|---|---|---|---|---|---|
| *Arthrospira* sp. (*'platensis'*) | Compere 86.79 | Natron lake, Chad | H | D0905 | CCALA | SAG 86.79 |
| *Arthrospira* sp. (*'platensis'*) | Compere 1968/3786 | Lake Bodou, Kanem, Chad | H | D0904 | CCALA | |
| *Arthrospira* sp. (*'platensis'*) | Leonard and Wouters 1968 | Lake Bodou, Kanem, Chad | H | D0907 | CCALA | |
| *Arthrospira platensis* | SP-2 | Lake Chad, Chad | H | D0885/H1 | Durham | NIES-39; IAM M-135 |
| *Arthrospira platensis* | SP-2 | Lake Chad, Chad | H(S) | D0885/H2 | Durham | |
| *Arthrospira fusiformis* | Hindak 1985/1 | Lake Arenguade, Ethiopia | H | D0909 | CCALA | |
| *Arthrospira fusiformis* | Hegewald 1976/83 | Lake Nakuru, Kenya | S | D0910/S1 | CCALA | |
| *Arthrospira fusiformis* | Hegewald 1976/83 | Lake Nakuru, Kenya | S | D0910/S2 | CCALA | |
| *Arthrospira fusiformis* | Hegewald 1976/83 | Lake Nakuru, Kenya | H | D0910/H | CCALA | |
| *Arthrospira platensis* | UTEX 1926 | Saline marsh Del Mar Slough, San Diego Co., CA, USA | H | D0875 | UTEX | PCC 7345; UTEX 1928; ATCC 29408 |
| *Arthrospira platensis* | UTEX 1928 | Saline marsh Del Mar Slough, San Diego Co., CA, USA | H | D0876 | UTEX | |
| *Arthrospira platensis* | PCC 7345 | Saline marsh Del Mar Slough, San Diego Co., CA, USA | H | D0911 | PCC | |
| *Arthrospira platensis* | SAG 257.80 | Laguna Huacachina, Ica, Peru | S | D0881 | SAG | Hegewald 1977/229 |
| *Arthrospira platensis* | SAG 257.80 (Lill) | Laguna Huacachina, Ica, Peru | S | | H. Lill | |
| *Arthrospira* 'crater' | | Lake in volcano crater, Queretaro, Mexico | H | D0919 | R. Fox | |
| *Arthrospira* 'Orovilca' | | Lake Orovilca, Ica, Peru | H | D0921 | R. Fox | |
| *Arthrospira platensis* | SP-1 | Lake Texcoco, Mexico | H | D0884 | Durham | NIES-46; IAM M-185 |
| *Arthrospira* sp. | SP-7 | Lake Texcoco, Mexico | H | D0890 | Durham | |
| *Arthrospira* sp | PCC 9108 | Commercial culture facility, Cheng-hai, Yunnan, China | H | D0916 | PCC | |

Table 1 (*Continued*).
List of strains, origin, and morphology

| Strain designation | Strain number | Origin | Morphology | Durham number | Source | Clones in other collections |
|---|---|---|---|---|---|---|
| *Arthrospira platensis* | Berhampur | Berhampur, India | H | D0930 | N. Jeeji-Bai | |
| *Arthrospira indica* | MCRC isolate spiral | MCRC, Madras, India | H | D0929 | N. Jeeji-Bai | |
| *Arthrospira* sp. | SP-13 | Unknown | H | D0896 | Durham | |
| *Arthrospira* sp. | SP-12 | Unknown | H | D0895 | Durham | |
| *Arthrospira* sp. | PCC 8006 | India, Kenya, Mexico or Peru | H | D0915 | PCC | Records lost at PCC |
| *Arthrospira* sp. | Strain EF-2 | Unknown | H | D0923 | Earthrise Farms | |
| *Spirulina laxissima* | SAG 256.80 | Lake Nakuru, Kenya | | D0883 | SAG | Hegewald 1976/75 |

No horizontal lines are drawn between duplicates and/or subcultures of one strain. Line spacings separate strains from clusters I and II within *Arthrospira*, and the strains from genera *Arthrospira* and *Spirulina*. H, helical filaments; S, straight filaments; H(S), straight filaments appearing among the helical filaments; H1/H2, differences in length of the helix pitch, helix diameter, and trichome diameter (unpublished results, M. Scott, M. Mühling and B.A. Whitton); S1/S2, differences in length of filament (unpublished results, M. Scott, M. Mühling and B.A. Whitton). ATCC, American Type Culture Collection, Rockville, MD, USA; CCALA, Culture Collection of Autotrophic Organisms, Trebon, Czech Republic; CCAP, Culture Collection of Algae and Protozoa, Ambleside, Cumbria, UK; Durham, Culture Collection of Durham University, Durham, UK; IAM, Institute of Applied Microbiology, University of Tokyo, Japan; NIES, National Institute for Environmental Studies Collection, Tsukuba, Ibaraki, Japan; NIVA, Norwegian Institute for Water Research, Oslo, Norway; PCC, Pasteur Culture Collection of Cyanobacterial Strains, Paris, France; SAC, SIAM ALGAE, Samutprakarn, Thailand; SAG, Sammlung von Algenkulturen der Universität Göttingen, Germany; UTEX, Culture Collection of Algae at the University of Texas at Austin, Austin, TX, USA.

chomes of width varying between 3 and 12 μm with cross-walls visible in light microscopy. They are generally found in tropical and subtropical regions in warm water bodies with high carbonate and bicarbonate content, and elevated pH and salinity [3]. Due to their richness in amino acids and γ-linolenic acid, they are currently sold as a food supplement and health food under the name '*Spirulina*' [4,5], though the 16S rRNA sequences of these two genera show that they are not related [6]. Since the increased awareness of the nutritional potential of *Arthrospira* in the 1960s, many strains have been deposited in culture collections and used in laboratories and mass cultivation plants [7,8]. However, the taxonomic situation of this genus, with at least six currently recognized binomials (*A. fusiformis*, *A. geitleri*, *A. indica*, *A. jenneri*, *A. maxima*, *A. platensis*), is confused and conflicting hypotheses have been published [3,9,10]. A major problem is the morphological variability of the strains under different environmental conditions. For example, the degree of spiralisation may show great variation and the spontaneous appearance of straight trichomes in a previously coiled strain is a well-documented phenomenon [3].

There is clearly a need to study the genotypic relatedness of many *Arthrospira* strains to give a firmer basis for future taxonomic revisions. Two sequences of the 16S rRNA and internally transcribed spacer (ITS) from *Arthrospira* PCC 7345 and PCC8005 have already been published [6]. They show a 16S rRNA sequence similarity of 99.7%. On the other hand, the ITS sequences were less similar, having 83.6% similarity in the non-coding areas (excluding $tRNA^{Ile}$ and $tRNA^{Ala}$).

As many *Arthrospira* strains as possible were obtained from culture collections and other laboratories. Digestions by *Eco*RV, *Hin*fI, *Nde*II, and *Taq*I of the 16S rRNA gene of 16 *Arthrospira* strains taken at random yielded identical restriction patterns and thus, we concluded that the 16S rRNA could not be used to resolve the *Arthrospira* strains (data not shown). Instead, the ITS appeared to present a variability suitable for discriminating the strains. Four restriction enzymes were selected and used for the amplified ribosomal DNA restriction analysis (ARDRA) of the ITS. This technique has already been used for screening of large numbers of bacterial strains in the framework of polyphasic taxonomic studies [11] and for identification of cyanobacterial strains of very diverse phylogenetic affiliations [12], but the present study describes its first use for a detailed study of a single cyanobacterial genus. Because amplification from a few cells was not feasible, a crude lysis protocol had to be designed and polymerase chain reaction conditions had to be modified by the addition of bovine serum albumin (BSA).

## 2. Materials and methods

### 2.1. Cyanobacterial strains

Fifty-one *Arthrospira* cultures were obtained from culture collections, representing theoretically 37 unique genotypes. Several were duplicates of the same strain, present in different culture collections or obtained from different laboratories. They were treated as different entries, because the possibility of mistakes having occurred in the various collections could not be ruled out. These duplicates could also be considered as internal controls for reproducibility of the data. In addition, four strains (D0872, D0885, D0906, D0910) included different morphotypes and these were separated into axenic clonal subcultures by successive transfers and washings in drops of sterile culture medium.

As only the strains from the Pasteur Culture Collection were guaranteed to be axenic, all strains were purified and established as axenic clonal cultures as explained above.

*Spirulina* medium [13] was used for cultivation. Stock cultures of 5 ml were kept under low light (10–40 $\mu E\ m^{-2}\ s^{-1}$) and at a constant temperature of 25°C at the University of Liège and 30°C at the University of Durham. Table 1 lists the origins and morphology of the strains.

### 2.2. Lysis with proteinase K

About 1 ml of a dense culture was harvested by centrifugation in a microcentrifuge tube (16000×*g*, 15 min) and cells were washed three times with RS buffer (0.15 M NaCl, 0.01 M EDTA, pH 8.0). Twenty μl of a solution of 0.05 $\mu g\ \mu l^{-1}$ proteinase K in 1×PCR buffer of Super Taq Plus (HT Biotechnology, UK) was added and incubated at 37°C for

1 h, followed by 5 min in a boiling water bath. Five μl 5 M NaCl was added and gently mixed. A final centrifugation (8000×*g*, 5 min) was performed prior to PCR or storing the samples at −20°C.

### 2.3. PCR of 16S rRNA plus ITS

In a total volume of 50 μl, 0.5 μl of lysis mixture or DNA extraction was added to 1×PCR buffer of Super Taq Plus, 0.2 mM dNTPs, 0.4 μM primer 16S5'F, 0.4 μM primer 23S5'R, 1 mg $ml^{-1}$ BSA, and 0.8 U Super Taq Plus polymerase with a proof-reading activity (HT Biotechnology, UK). The primer sequences, derived from [14] were 'AGAGTTTGATCCTGGCTCAG' and 'TCTGTGTGCCTAGGTATCC' respectively. The thin-wall tubes were submitted to thermal cycling in the Gene Cycler (Bio-Rad, USA): 180 s at 94°C; 10 cycles of 45 s at 94°C, 45 s at 57°C, 120 s at 68°C; 25 cycles of 45 s at 90°C, 45 s at 57°C, 120 s at 68°C, and a final elongation step of 7 min at 68°C. The PCR products were visualized after 1% (w/v) agarose gel electrophoresis and stored at −20°C.

### 2.4. Purification of the PCR products

The PCR products were subjected to electrophoresis in 1% (w/v) agarose gel in 1×TAE buffer (40 mM Tris acetate, 2 mM EDTA). The bands of expected length were excised from the gels and the DNA was extracted by centrifugation on a minicolumn with glasswool [15]. Only two PCR products were loaded at opposite sides of a minigel and care was taken to avoid any cross-contamination. The DNA was precipitated with ethanol and resuspended in 20 μl of buffer $TE^{-4}$ (10 mM Tris pH 7.4, 0.1 mM EDTA pH 8.0) and stored at −20°C.

### 2.5. PCR of ITS

This PCR was performed like the first PCR (Section 2.3), except that 1 μl of the purified PCR product (Section 2.4) was generally used instead of the lysis material and primer 16S5'F was replaced by primer 16S3'F (TGYGGCTGGATCACCTCCTT). The same cycling conditions were used except that the annealing temperature was 53°C instead of 57°C, 40 s was used instead of 45 s, and 75 s instead of 120 s.

### 2.6. Digests

Ten μl of the PCR product was added to 2 μl of the 10×reaction buffer and 5 U of the restriction enzymes *Eco*RV, *Hha*I, *Hin*fI or *Mse*I (Gibco Life Sciences, USA) in a total volume of 20 μl. Incubation was carried out for 2 h in the optimum temperature of the enzyme (manufacturer's conditions). The reaction was stopped by addition of 0.4 μl 0.5 M EDTA, pH 8.0.

### 2.7. Electrophoresis

A standardized electrophoresis protocol using LSI MP agarose (Life Sciences International) at a concentration of 2% was used to maximize the reproducibility between different gels. The agarose was slowly sprinkled into TBE buffer at room temperature and stirred with a magnetic bar. The flask was weighted before heating until the agarose was completely molten. The flask was returned to its original weight by addition of distilled water. When the solution had reached 60°C, it was poured into a 15×15 cm tray and left to polymerize for 20 min. Prior to loading the gel, it was placed at 4°C for 30 min. Electrophoresis proceeded at a constant voltage of 3.8 V $cm^{-1}$ during 130 min. The gel was colored in an ethidium bromide bath (1 μg $ml^{-1}$ in TBE) for 20 min and rinsed before visualization on an UV-transilluminator.

### 2.8. Analysis with GelCompar

Gel images were saved as TIF files using a gel imaging device (Vilber Lourmat) and the included software Bio Profil v.6.0. They were loaded in the software package GelCompar 4.0 (Applied Maths, Kortrijk, Belgium), cut into gel tracks and normalized using the digest of pBR322/*Hae*III as the standard. After assigning logical bands to the normalized patterns, the four gels were combined for analysis. The similarity between the banding patterns was calculated using the Dice coefficient and the UPGMA method was used to draw a dendrogram based on the matrix of similarity coefficients. A band matching

tolerance of 0.4% was selected. The bands above 50 bp were visible and well resolved, and thus were used for the analysis. The addition of the band lengths to reach about 540 bp was performed to rule out the presence of non-specific bands and partial digests.

## 3. Results and discussion

### 3.1. Amplifications by PCR of the rRNA genes

Despite numerous trials, the cells of *Arthrospira* strains could not be used directly as template in the PCR reaction, probably due to inhibitory compounds. It was necessary to design a slightly longer lysis protocol involving proteinase K. The addition of BSA to the PCR mix was mandatory for the success of the reaction.

The PCR reactions gave products of identical lengths for all the *Arthrospira* strains, corresponding to the ca. 2000 and 540 bp found in published sequences of 16S plus ITS, and ITS alone, respectively [6]. Based on this length conservation, it is probable that all the ITS contained $tRNA^{Ile}$ and $tRNA^{Ala}$.

### 3.2. ARDRA of ITS

Using the software CUTTER (http://www.medkem.gu.se/cutter/) and the two published sequences [6], we could only find four discriminative restriction enzymes giving usable patterns. Other enzymes chos-

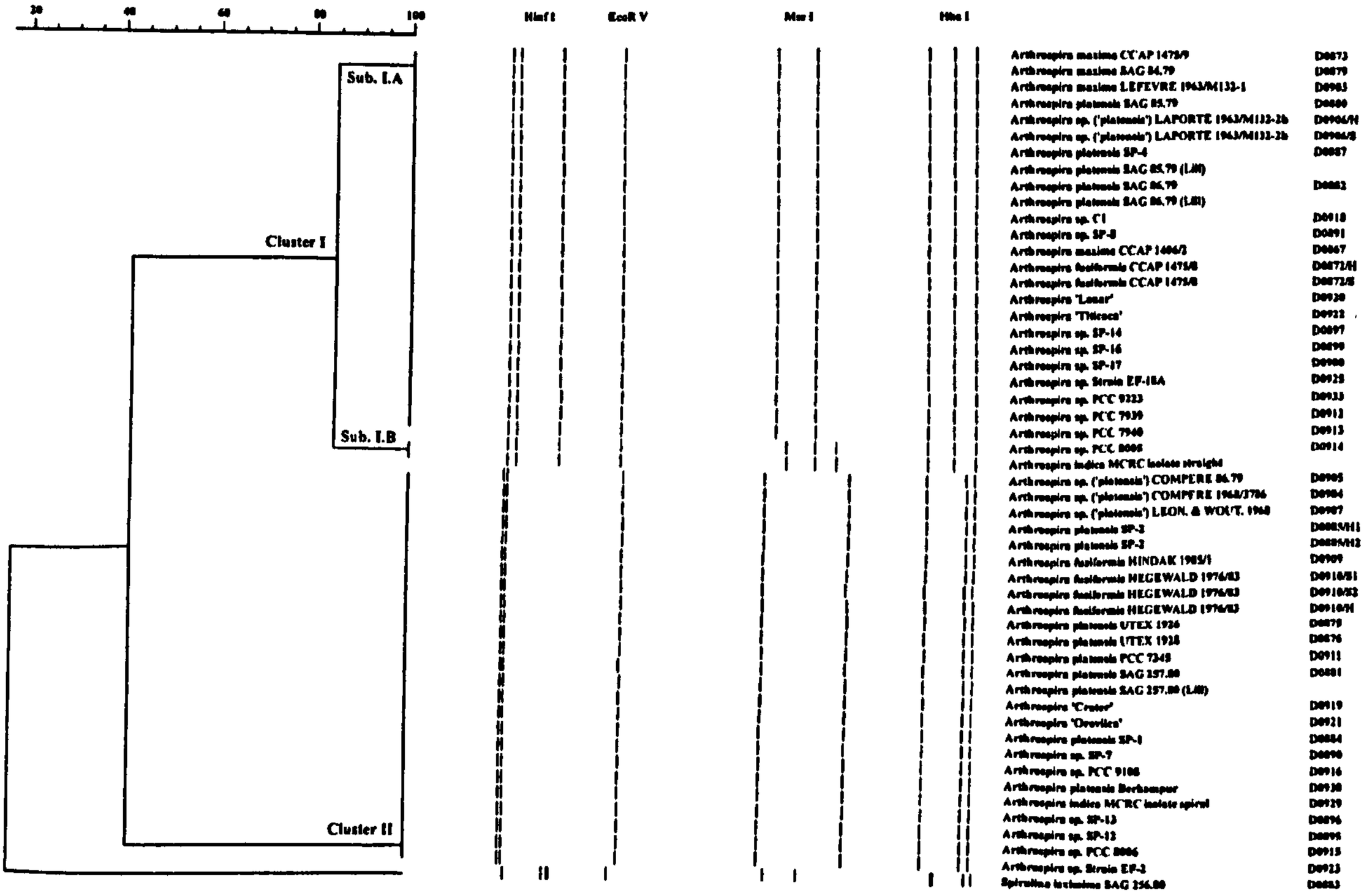



Fig. 1. Dendrogram built by UPGMA clustering of Dice correlation coefficients of normalized combined ARDRA patterns of ITS for 51 *Arthrospira* and one *Spirulina* cultures obtained with the restriction enzyme combination *Eco*RV, *Hha*I, *Hin*fI, and *Mse*I. The schematic representation of the four ARDRA patterns is given in front of the strain designations and of the numbers in the Durham Collection. The lengths of fragments longer than 50 bases are for *Hin*fI-cluster I: 260, 214, 67; *Hin*fI-cluster II: 281, 260; *Eco*RV-cluster I: 499; *Eco*RV-cluster II: 540; *Hha*I-cluster I: 290, 162, 88; *Hha*I-cluster II: 290, 113, 88; *Mse*I-subcluster IA: 389, 151; *Mse*I-subcluster IB: 305, 151, 84; *Mse*I-cluster II: 490, 52 bases.

en at random were tested in case the two published sequences were not representative of the overall diversity of the studied strains. *Nde*II and *Msp*I did not cut at all the ITS of 15 strains chosen at random, *Rsa*I, *BsiZ*I and *Ava*II cut only in conserved areas and did not differentiate the strains, *Taq*I gave too slight differences in band lengths to allow a reliable analysis, *Alu*I gave too small fragments (under 100 bases) to be visible, and *Hae*III gave partial digests which did not allow a good and reproducible analysis.

For 21 cultures, the banding patterns were obtained twice in independent experiments, starting from new cultures, and they were identical. In addition, duplicate strains from different culture collections or laboratories gave the same results, except SAG 86.79 from SAG (D0882) and from Lill's laboratory which were different from their putative 'duplicate' Compère 86.79 (D0905) from CCALA at the level of the ARDRA of ITS and the morphology. This distinctiveness at the genotypic and phenotypic levels implies that either SAG or CCALA does not hold the original strain. In the cases where different morphotypes were isolated from the same culture (D0872, D0 885, D0906, D0910), the ARDRA results were identical.

Cluster analysis resolved the 51 *Arthrospira* cultures into only two main genotypic clusters, designated I and II in Fig. 1. The *Arthrospira* cultures were clearly separated from the *Spirulina laxissima* SAG 256.80 strain (D0883) which serves as outgroup. It is interesting to note that Viti et al. [16] also found two clusters when 10 *Arthrospira* strains were studied by total DNA restriction profile analysis. However, only strain C1 (D0918) is common to both studies. It is possible that their Texcoco strains (Sosa 6, Sosa 18, 6 Mx, 3 Mx) are related to our strains D0884 and D0890, which have the same origin (Table 1). In this case, their separation of Mexican strains and Chad strain C1 into two genotypes would coincide with our observations.

The two sequenced strains which were used to choose the restriction enzymes, PCC 7345 (D0911) and PCC 8005 (D0914), belong to different clusters and appear thus representative of the whole diversity of the studied *Arthrospira* strains. Thus, the 16S rRNA gene and ITS sequence similarities between the clusters I and II are probably close to the values of 99.7 and 83.6%, respectively [6].

The cultures from SAC and Earthrise Farms (D0899, D0900, D0923 and D0925) fall into different clusters, but have ARDRA patterns identical to those of other culture collection strains. This is to be expected since culture collection facilities are both sources and recipients of these commercial grown strains.

Inside cluster I, a subgroup of two cultures could be distinguished and is designated as 'subcluster IB'. Strains PCC8005 (D0914) and *A. indica* MCRC isolate straight had *Mse*I patterns, which were different from the other members of cluster I ('subcluster IA'). The precise origin of strain PCC8005 has been lost, though it was known that it was included in a group of four strains given by N. Jeeji-Bai to the PCC (http://www.pasteur.fr/Bio/PCC). It seems possible that both these strains are in fact cryptic duplicates.

The ITS clusters do not appear to have any well-defined geographical distribution and overlap each other in a rather intriguing way. Cluster I contains strains from Chad, Ethiopia, Kenya, Madagascar, India, and Peru, whereas cluster II includes other strains from Chad, Ethiopia, Kenya, India, California, Mexico, Peru, China, and Spain. Concerning the taxonomic assignment of the strains, strains assigned to the species *A. platensis*, *A. maxima*, *A. indica* and *A. fusiformis* can be found in both clusters. In addition, morphological observations revealed that straight and helical morphotypes can be found in any of the two clusters. To a certain extent, the wide geographic distribution of the ITS clusters can be explained by migration of water birds (flamingoes, pelicans, ibis, ducks...) between lakes. However, the geographic overlaps and the apparently quite random distribution of both clusters are difficult to explain.

The conservation of the 16S rRNA sequences in the *Arthrospira* strains from four continents is remarkable. Such cosmopolitan distribution has been found for marine planktic cyanobacteria [17], *Microcoleus chthonoplastes* growing in the microbial mats of intertidal zones or pools and lagunas close to the sea [18], and marine filamentous strains with narrow trichomes (*Phormidium* or *Leptolyngbya*) [19]. However, these cyanobacteria were oceanic or their habitats could be linked by circulation of seawater. In

the present case, *Arthrospira* strains have very specialized habitats with a patchy distribution.

The ARDRA data from the 16S rRNA genes and the ITS suggest either a very recent divergence or a great genotypic conservatism, as well as complex patterns of geographic spreading. The ARDRA technique does not allow to conclude on the taxonomic status [20] of the studied *Arthrospira* strains, but it seems likely that they belong to one species (based on 16S rRNA gene) or two species (based on the ITS). The use of additional enzymes might allow to find small ITS variations inside the clusters, but would not destroy the observed dichotomy in two clusters. However, more detailed genotypic information (e.g. sequences) would be needed to make firm taxonomic conclusions.

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