The halotolerance and phylogeny of cyanobacteria with tightly coiled trichomes (*Spirulina* Turpin) and the description of *Halospirulina tapeticola* gen. nov., sp. nov.

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The morphologies, halotolerances, temperature requirements, pigment compositions and 16S rRNA gene sequences of five culture collection strains and six novel isolates of cyanobacteria with helical, tightly coiled trichomes were investigated. All strains were very similar morphologically and could be assigned to the genus *Spirulina* (or section *Euspirulina sensu* Geitler), according to traditional classification. However, the isolates showed significantly different requirements for salinity and temperature, which were in accordance with their respective environmental origins. The genetic divergence among the strains investigated was large. The results indicate the drastic underestimation of the physiological and phylogenetic diversity of these cyanobacteria by the current morphology-based classification and the clear need for new taxa. Three of the isolates originated from hypersaline waters and were similar with respect to their high halotolerance, broad euryhalinity and elevated temperature tolerance. By phylogenetic analyses, they were placed in a tight monophyletic cluster apart from all other cyanobacteria. Thus it is proposed to reclassify highly halotolerant cyanobacteria with tightly coiled trichomes in *Halospirulina* gen. nov., with the type species *Halospirulina tapeticola* sp. nov.

Keywords: cyanobacteria, phylogeny, halotolerance, *Halospirulina*, microbial mats

INTRODUCTION

Cyanobacteria with tightly coiled trichomes are frequently found in thermal freshwater environments as well as in brackish, marine and hypersaline waters (Anagnostidis & Golubic, 1966; Castenholz, 1977; Dubinin *et al.*, 1995; Ehrlich & Dor, 1985; Gabbay-Azaria & Tel-Or, 1991; Garcia-Pichel *et al.*, 1994; Pentecost, 1994; Tomaselli *et al.*, 1995;

Wilmotte, 1991). Under favourable conditions they can form dense benthic populations and make major contributions to primary productivity (Anagnostidis & Golubic, 1966; Castenholz, 1977; Kruschel & Castenholz, 1998). Based on their conspicuous morphology alone, they are classified under the genus *Spirulina* Turpin (Anagnostidis & Komárek, 1988; Castenholz, 1989a; Turpin, 1829; subgenus or section *Euspirulina sensu* Geitler, 1932). On the basis of the tightness of the helix, thin cross-walls (invisible by light microscopy) and several ultrastructural features, they are morphologically distinguished from a variety of other cyanobacteria with more loosely helical or sinuous trichomes, such as the commercially produced strains of the genus *Arthrospira* Stitzenberger (Anagnostidis & Komárek, 1988; Castenholz, 1989a; Tomaselli *et al.*, 1996). The genetic distinctness of *Spirulina* (strain PCC 6313) and *Arthrospira* (strains PCC 7345 and 8005) has been confirmed on the basis

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of 16S rRNA gene sequence analysis (Nelissen *et al.*, 1994).

Depending on trichome diameter and coil shape, cyanobacteria of the genus *Spirulina* are commonly assigned to one of a few species, most frequently to *Spirulina subsalsa* Oersted, *Spirulina labyrinthiformis* Gomont and *Spirulina major* Kützing, regardless of their habitat of origin. Consequently, they are traditionally considered cosmopolitan micro-organisms with remarkable capabilities to acclimatize to broad ranges of environmental conditions (Anagnostidis & Golubic, 1966; Geitler, 1932). However, morphology-based classification may provide insufficient taxonomic resolution and cyanobacteria with similar or identical morphology may have significantly different physiology. In recent years, the analysis of 16S rRNA gene sequences has demonstrated that morphological groupings of cyanobacteria in some cases correspond to phylogenetically coherent taxa (Garcia-Pichel *et al.*, 1996), whereas in others the traditional classification drastically underestimates extant diversity (Ferris *et al.*, 1996). In bacteriology, in particular, the tolerances to and requirements for high salt concentrations and high temperatures have been recognized as important phenotypic properties correlating with phylogeny (Hiraishi & Ueda, 1994; Imhoff & Söling, 1996; Imhoff *et al.*, 1998; Overmann & Tuschak, 1997). We have demonstrated that extreme halotolerance among unicellular cyanobacteria is a physiological characteristic that can be used to define a phylogenetically coherent group of cultivated strains (Garcia-Pichel *et al.*, 1998).

For cyanobacteria with *Spirulina*-like morphology, uncertainties about the evolutionary coherence of the current generic classification have been expressed sporadically on the basis of analyses of lipid compositions (Cohen & Vonshak, 1991) or ultrastructure (Tseng & Chang, 1990). In addition, the composition of genomic DNA in the two strains for which this information is available is quite different, with G + C content determined to be 53.5 mol % in *Spirulina major* PCC 6313 (Herdman *et al.*, 1979) and 43.8 mol % in *Spirulina subsalsa* P7 (Wilmotte *et al.*, 1997). However, a comprehensive comparative study on the physiology and phylogeny of these cyanobacteria has been lacking and, therefore, the diversity within the botanical genus *Spirulina* remains largely unexplored. The question whether morphological counterparts from different environments are related or have undergone convergent evolution is particularly interesting. We have analysed and compared the 16S rRNA gene sequences, morphologies, halotolerances, temperature requirements and pigment compositions of 11 cultures of cyanobacteria currently classified as *Spirulina* spp., including six newly isolated strains. For three additional strains, nucleotide sequence information is available from public databases. A phylogenetic pattern emerges which is in part supported by phenotypic characteristics. We propose the reclassification at the generic level of those halotolerant, euryhaline cyano-

bacteria with tightly coiled trichomes from hypersaline waters.

METHODS

Cyanobacterial strains, strain histories, cultivation and purification. Clonal strains of cyanobacteria used for this study are listed in Table 1. Freshwater medium was BG11 (Rippka *et al.*, 1979) modified by decreasing the content of NaNO₃ to 0.75 g l⁻¹. Seawater and hypersaline medium were prepared by dissolving appropriate amounts of commercial seawater salts mixture (Wiegandt) in distilled water to which nutrients, trace elements and vitamins were added according to Provasoli's Enriched Seawater formulation (Starr & Zeikus, 1987) to half strength. The mixture was acidified with HCl to pH 3 and was bubbled overnight with air to drive excess CO₂ out of solution and thus reduce the amounts of carbonate and bicarbonate in the final mixture. The pH was then raised to 8.2 by addition of NaOH and the solution was autoclaved. This procedure prevented or minimized the formation of precipitates during autoclaving (Garcia-Pichel *et al.*, 1998).

An axenic culture of strain CCC Baja-95 Cl. 2^r was obtained after purification of filaments by repeated filtration through a nylon net (approximate mesh width 10 µm) and subsequent dilution and cultivation in hypersaline medium (7% total salt concentration). Axenicity was controlled microscopically.

Growth rate measurements. All strains were grown in deep Petri dishes filled with liquid media of various salinities. Strain CCC Snake-P. Y-85 was incubated at 38 °C, receiving 20 µmol photons of constant white light m⁻² s⁻¹ from fluorescent tubes for 12 h daily. All other cultures were incubated at 25 °C, receiving 20 µmol photons of white light m⁻² s⁻¹ from fluorescent tubes. Growth rates were measured by non-invasively monitoring the increase with time of bulk phycobilin/chlorophyll *a* fluorescence in the cultures using a fluorimeter specially designed for use with cultures that do not form homogeneous suspensions (Karsten *et al.*, 1996). For each strain, the correspondence between fluorescence and biomass (dry wt) was checked ($R^2 \geq 0.8$; data not shown). Growth was followed in triplicate cultures during periods of 1–4 weeks, so that four to five doublings during exponential growth could be monitored. Linear regression analysis of the natural logarithms of the fluorescence values yielded estimates of growth rates ($R^2 \geq 0.85$). Means and standard deviations of triplicate measurements are shown.

Determination of temperature requirements. Temperature ranges were determined by visual inspection of growth in test tube cultures with liquid media after incubation for a maximum of 43 d. Strain CCC Snake-P. Y-85 was incubated in freshwater medium, strain S3 was incubated at a salinity of 7% and all others at a salinity of 3.2%. All strains received constant irradiance of 20 µmol photons of white light m⁻² s⁻¹. Temperatures tested were 4, 10, 15, 20, 25, 35, 40, 45 and 50 °C. Growing cultures were subjected to stepwise temperature shifts of a maximal 6 °C each time.

Determination of carotenoid and phycobiliprotein composition. Cultures for pigment analyses were grown at salinities and light conditions as indicated for the determination of temperature requirements. Incubation temperatures were 45 °C for strain CCC Snake-P. Y-85 and 25 °C for all others. Carotenoid complement was determined by HPLC separation and on-line UV/visible spectroscopy. Approximately 50 mg cells (wet wt) were extracted in acetone.

Table 1. Strains used in this study

Strain IAM M-223 (Ishida *et al.*, 1997; Shihira-Ishikawa, 1990) was not made available to us by the Institute of Applied Microbiology Culture Collection (University of Tokyo, Japan) despite a request.

Strain*	Possible classification†	Origin			Reference
		Site of isolation	Salinity	Isolated by:‡	
CCC Baja-95 Cl. 2 ^T	<i>Spirulina subsalsa</i>	Pacific Ocean, salina Guerrero Negro, Mexico	16 %	RWC	This work; R. W. Castenholz, personal communication
CCC Baja-95 Cl. 3	<i>Spirulina subsalsa</i>	Pacific Ocean, salina Guerrero Negro, Mexico	16 %	RWC	This work; R. W. Castenholz, personal communication
MPI S3	<i>Spirulina subsalsa</i>	Mediterranean Sea, La Trinitat salt works, Spain	10 %	EC	This work; E. Clavero, personal communication
MPI S1	<i>Spirulina labyrinthiformis</i>	Mediterranean Sea, La Trinitat salt works, Spain	10 %	EC	This work; E. Clavero, personal communication
MPI S2	<i>Spirulina subsalsa</i>	Mediterranean Sea, La Trinitat salt works, Spain	5.7 %	EC	This work; E. Clavero, personal communication
MPI S4	<i>Spirulina subsalsa</i>	Mediterranean Sea, Alfacs Bay, Ebro Delta, Spain	3.7 %	EC	This work; E. Clavero, personal communication
P7 (SAG 59.90, CCAP 1475/7)	<i>Spirulina subsalsa</i>	Mediterranean Sea, Harbour of Calvi, Corsica, France	3.8 %	AW	Wilmotte (1991, personal communication); Wilmotte <i>et al.</i> (1997)
UBMM Bo 89	<i>Spirulina subsalsa</i>	Baltic Sea, Boiensdorf, Germany	1.5 %	JR	Rethmeier (1995)
UBMM Hi 45	<i>Spirulina major</i>	Baltic Sea, Hiddensee, Germany	1.0 %	JR	Rethmeier (1995)
PCC 6313 (ATCC 29542)	<i>Spirulina major</i>	Pacific Ocean, Berkeley, USA	Brackish	MMA	Rippka <i>et al.</i> (1979); Rippka & Herdman (1992)
CCC Snake P. Y-85	<i>Spirulina labyrinthiformis</i>	‘Snake Pit’ (hot spring), Yellowstone National Park, USA	2 mS§	RWC	Castenholz (1977, personal communication)
NIVA-CYA 163	<i>Spirulina subsalsa</i>	Atlantic Ocean, Oslofjord, Drøbak, Norway	NA	RS	Aakermann <i>et al.</i> (1992); Rudi <i>et al.</i> (1997)
NIVA-CYA 164	<i>Spirulina subsalsa</i>	Atlantic Ocean, Oslofjord, Drøbak, Norway	NA	RS	Aakermann <i>et al.</i> (1992); Rudi <i>et al.</i> (1997)
SAG B256.80	‘ <i>Spirulina laxissima</i> ’	Lake Nakuru (Natron Lake), Kenya	NA	EH	Schlösser (1994)

NA, Information not available.

* Culture collections: ATCC, American Type Culture Collection, Manassas, VA, USA; CCAP, Culture Collection of Algae and Protozoa, Ambleside, UK. CCC, Castenholz Culture Collection, University of Eugene, OR, USA [the CCC has recently been consolidated with others and is now referred to as Culture Collection of Microorganisms from Extreme Environments (CCMEE), University of Eugene, OR, USA]; MPI, Max Planck Institute for Marine Microbiology, Bremen, Germany; NIVA, Culture Collection of Algae, Oslo, Norway; PCC, Pasteur Culture Collection, Paris, France; SAG, Sammlung von Algenkulturen, Göttingen, Germany; UBMM, University of Bremen, Marine Microbiology, Bremen, Germany. Designations given in parentheses indicate related strains.

† Classification based on trichome morphology; with the exception of ‘*S. laxissima*’, generic names given are *sensu* Castenholz (1989a) and Anagnostidis & Komárek (1988), and species epithets are *sensu* Anagnostidis & Golubic (1966) and Geitler (1932).

‡ Researchers that isolated the cyanobacterial strains are abbreviated with their initials: M. M. Allen (University of California, Berkeley, CA, USA), R. W. Castenholz (University of Oregon, Eugene, OR, USA), E. Clavero (CID-CSIC, Barcelona, Spain), E. Hegewald (University of Göttingen, Germany), J. Rethmeier (University of Bremen, Germany), R. Skulberg (NIVA, Oslo, Norway), A. Wilmotte (University of Liege, Belgium).

§ Conductivity.

|| In contrast to the given references, a seawater aquarium is quoted as the site of isolation in the NIVA catalogue of strains (Skulberg, 1990).

Carotenoids were identified by cochromatography and spectroscopic matching with authentic primary or secondary standards. Details on the identification and quantification of carotenoids and sources of standards have been published previously (Karsten & Garcia-Pichel, 1996). Phycobiliproteins were released from approximately 50 mg cells (wet wt) into 20 mM sodium acetate, pH 5.5 (Tandeau de Marsac & Houmard, 1988), after breaking the cells by repeated freezing and thawing (liquid nitrogen/45 °C) and subsequent ultrasonication. Lysates were clarified by centrifugation in a microcentrifuge and the presence or absence of typical absorption or fluorescence corresponding to either phycoerythrin, phycoerythrocyanin or phycocyanin was determined.

PCR amplification, cloning and sequence analysis of 16S rRNA genes. The molecular biological procedures used have been described in detail previously (Garcia-Pichel *et al.*, 1998). Briefly, cells harvested by centrifugation and suspended in TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) were directly applied as templates for PCR. To determine almost complete sequences of 16S rRNA genes from cyanobacteria in unialgal but non-axenic cultures, primers 8F (Buchholz-Cleven *et al.*, 1996) and 1528R (Garcia-Pichel *et al.*, 1998) were used for PCR amplifications and the resulting PCR products were cloned applying the pGEM-T plasmid vector system (Promega). Full-length cyanobacterial 16S rRNA genes from plasmid inserts were reamplified using the same primers as before. To avoid errors due to PCR artifacts or operon microheterogeneities, PCR products derived from 10 different plasmids were mixed and processed for sequencing. For some strains only partial sequences (approx. 560 nt) were determined by applying cyanobacteria-specific primers CYA106F and CYA781R for amplification and sequencing (Nübel *et al.*, 1997). Both DNA strands of the amplification products were sequenced as described previously (Garcia-Pichel *et al.*, 1998) by using the primers 8F, 1099F, 1175R (Buchholz-Cleven *et al.*, 1996), CYA106F, CYA359F, CYA781R (Nübel *et al.*, 1997) and 1528R. All primer designations refer to 5' ends of the respective target sites in the 16S rRNA genes (*Escherichia coli* numbering of 16S rRNA nucleotides; Brosius *et al.*, 1981) and to forward (F) or reverse (R) orientation relative to that of the rRNA.

Phylogeny reconstruction. Cyanobacterial 16S rRNA gene sequences available from GenBank and those determined in this study were aligned to the sequences in the database of the software package ARB, developed by W. Ludwig and O. Strunk, and available at <http://www.mikro.biologie.tu-muenchen.de/>. Phylogenetic trees were constructed on the basis of 73 almost complete sequences (from nt 45 to 1455 corresponding to *E. coli* numbering) [database accession nos of sequences used: Y18789, Y18790, Y18792, Y18793, AF091109, X75045, AB003164, AJ000716, 1001484, X52171, AF001480, AF053396–AF053399, AF001477, X03538, AJ007907, AF091110, AJ007374, AB003169, AB003163, X84810, X84808, Chms.sglbs (RDP), X84809, AJ000715, X78680, Z28699, AJ000708–AJ000714, AJ000724, X78681, Prcl.didem (RDP), AF013030, X70767, AF013028, AF013029, AF091321, AF091322, X70769, X70770, X75044, X84811, X84812, Chrc.7203 (RDP), AF027653–AF027655, AF062637, AF062638, X59559, X68780, AJ224447, AF067818, AF067819, AF092504, AF091150, AB003165–AB003168, J01422, X82156, AF091108, Glb.violac (RDP), M24911, D26185]. Alignment positions at which one or more sequences had gaps or ambiguities were omitted from the analyses. To evaluate the

consistency of computed tree topologies, subsets of data were analysed by using various algorithms as follows. A variety of single and multiple outgroup sequences representing phylogenetically diverse organisms were included in the analyses. To assess the influence of the most variable nucleotide positions they were excluded from some calculations by applying filters based on character frequency (see ARB manual; Ludwig *et al.*, 1998). The maximum-likelihood, maximum-parsimony and neighbour-joining methods as integrated in the ARB software package were applied. The latter calculation was based on a matrix of evolutionary distances determined using the Jukes–Cantor or Felsenstein equations. The maximum-parsimony calculation was subject to bootstrap analysis (1000 replicates). In the dendrogram presented, partial sequences (database accession numbers: Y18791, Y18792, Y18794–Y18798, Z82787, Z82788) were integrated according to the maximum-parsimony criterion without allowing them to change the topology of the tree as established with complete sequences (see ARB manual; Ludwig *et al.*, 1998).

RESULTS

Microscopic observations

Results of microscopic observations are summarized in Table 2. With the exception of '*Spirulina laxissima*' SAG B256.80 all strains studied have regularly helically coiled trichomes, thin crosswalls invisible by light microscopy and no visible sheaths (Fig. 1). Thus, they fit the description of the genus *Spirulina sensu* Castenholz (1989a) and *sensu* Anagnostidis and Komárek (1988), and the 'section' *Euspirulina* of the genus *Spirulina sensu* Geitler (1932). Coiling is either clockwise or counter-clockwise depending on the respective strains. For all these strains, motility was observed as apparent rotation along the helix axis. In PCC 6313 and UBMM Hi 45, trichome coils are open, whereas in all other strains coils are closed (Fig. 1). Trichome and helix widths vary among strains, thus different species epithets could be assigned according to traditional classification (Table 1; Anagnostidis & Golubic, 1966; Geitler, 1932). Morphological variability within strains was not noticeable during the present study, even when grown under different cultivation conditions. However, slight despiralization under unfavourable growth conditions has been described for two marine strains (strain P7, also included in this study, and strain A4; Wilmotte, 1991) and for field populations (Anagnostidis & Golubic, 1966).

Trichomes of strain SAG B256.80, designated '*Spirulina laxissima*' in the catalogue of strains of the SAG culture collection (Schlösser, 1994), are constricted at the easily discernable crosswalls. They are curved or loosely helical, but spirality is not regular; it has longer relative wavelength than in any of the other strains (Fig. 1). Motility was not observed. This strain differed from all other strains investigated in this study by maintaining buoyancy and growing suspended homogeneously in liquid medium, instead of adhering to glass walls of culture tubes or forming pellicles. The identification of this strain as *S. laxissima* West in

Table 2. Morphologies and temperature requirements

Strain	Helix shape	Trichome width (µm)*	Helix width (µm)*	Sense of coiling†	Temperature requirement (°C)
CCC Baja-95 Cl. 2 ^T	Closed	1.5	4.0	ccw	20–38
CCC Baja-95 Cl. 3	Closed	3.0	6.0	ccw	20–38
MPI S3	Closed	2.5	5.0	ccw	20–40
MPI S1	Closed	1.0	2.0	cw	15–35
MPI S2	Closed	1.7	3.0	cw	10–35
MPI S4	Closed	1.5	3.0	cw	10–35
P7	Closed	1.6	2.7	cw	10–35
NIVA-CYA 163	Closed	1.5	4.0	ccw	NA
NIVA-CYA 164	Closed	1.5	4.0	ccw	NA
UBMM Bo89	Closed	1.7	3.0	cw	10–25
IAM M-223 (IAM M-183)‡	Closed	1.5	3.0	cw	NA
CCC Snake P. Y-85	Closed	1.5	3.0	cw	35–45
UBMM Hi 45	Open	1.5	4.0	ccw	10–35
PCC 6313	Open	2.0	4.5	ccw	10–35
SAG B256.80	No helix	1.5	—	—	NA

NA, Information not available.

* Mean values of at least 20 trichomes.

† cw, clockwise; ccw, counter-clockwise.

‡ Data are from Shihira-Ishikawa (1990). That paper contains an illustration and a general description of strain IAM M-183, which is closely related to strain IAM M-223, according to the IAM catalogue of strains (second edition, 1998).

the sense of Geitler (1932) is questionable. Geitler had assigned this morphospecies to his 'section' *Euspirulina*, encompassing helically coiled cyanobacteria with crosswalls that are invisible in living specimens.

Salt requirements

The dependence of growth rates on salinity is illustrated in Fig. 2. With respect to their salt requirements for growth, the strains can be assigned to three groups which correlate with the environmental conditions in the habitats from which they were isolated: freshwater, marine and hypersaline (see Table 1). The only freshwater strain available (CCC Snake-P. Y-85) tolerated 1.6% total salts, but died at marine salinity (3.2%). Marine strains were somewhat variable with respect to salinity optima and tolerances. Typically they grew with optimum rates at 3.2% total salts (MPI S4, P7, UBMM Bo 89, UBMM Hi 45) or slightly higher (MPI S1, PCC 6313, 7%) or lower (MPI S2, 1.6%). Strains PCC 6313 and UBMM Hi 45 were remarkable in that they did not show a distinct and narrow salinity optimum, but were able to grow with close to optimum rates in freshwater medium (BG11). Thus, they can be termed euryhaline. Strain MPI S1 tolerated a salinity of 10% but died at 13%. In contrast, three strains from hypersaline environments at 25 °C grew at salinities from 1.6 (MPI S3) or 3.2% (CCC Baja-95 Cl. 2^T, CCC Baja-95 Cl. 3) to 16%. In

strain CCC Baja-95 Cl. 2^T, elevated temperature (38 °C) resulted in increased growth rates at high salinities and an increased upper salinity limit of growth (20%). This temperature effect on halotolerance had previously been observed for some unicellular, extremely halotolerant cyanobacteria (Garcia-Pichel *et al.*, 1998). Thus, the three latter strains were extremely euryhaline and were among the most halotolerant cyanobacteria that have been described, second only to some members of the *Halotheca* cluster of unicellular, extremely halotolerant strains (Garcia-Pichel *et al.*, 1998; MacKay *et al.*, 1984; Reed & Stewart, 1988).

Temperature requirements

The cyanobacterial strains analysed showed markedly different temperature requirements (Table 2). Strain CCC Snake P. Y-85 was isolated from a 50 °C site in a sulfidic freshwater hot spring (Castenholz, 1977, personal communication). In our experiments, it showed growth between 35 and 45 °C and did not grow at 50 °C. Natural populations of this organism showed an upper temperature limit of 51 °C and maximum photosynthesis rates at 45 °C (Castenholz, 1977). The strains that had shown the highest halotolerance (MPI S3, CCC Baja-95 Cl. 3, CCC Baja-95 Cl. 2^T) tolerated 40 or 38 °C, respectively, and did not grow at 15 °C and below. Thus they displayed a slightly elevated temperature requirement compared to the normal-

Fig. 1. Photomicrographs of cyanobacterial isolates. All organisms are shown at the same magnification and the bar shown applies to all panels. Bar, 10 μ m.

salinity marine strains, most of which tolerated 10–35 °C. Similarly, this characteristic was found in unicellular cyanobacteria from hypersaline environments and might be an adaptation to life in brines with low heat capacity which may easily reach high temperatures when sunlit (Castenholz, 1969; Garcia-Pichel *et al.*, 1998).

Pigment compositions

All strains analysed contained phycocyanin. In addition, phycoerythrin was detected in strains NIVA-CYA 164, P7 and UBMM Bo 89, while phycoerythrocyanin was not found in any strain. NIVA-CYA 164 had red trichomes and UBMM Bo89 had brownish to black trichomes, while all other strains looked blue-green when observed in white light. The ability for chromatic adaptation was not tested during this work; however, it has been reported to be lacking in strain P7 (Wilmotte, 1991) and in two red-pigmented strains A4 and 3F identified as *Spirulina subsalsa* status

versicolor (Tomaselli *et al.*, 1995; Wilmotte, 1991; Wilmotte *et al.*, 1997).

With respect to their carotenoid contents, cyanobacteria with *Spirulina*-like morphology were rather diverse (Tables 3 and 4). The total number of carotenoids in the different strains varied from three to nine. β -Carotene was found in all strains and echinenone in most. Two of the most halotolerant strains (MPI S3, CCC Baja-95 Cl. 3) contained the same types of carotenoids in similar ratios; however, CCC Baja-95 Cl. 2^T produced aphanyzophyll and an unidentified carotenoid with an absorption maximum at 480 nm instead of myxoxanthophyll (L-chinovosemyxol) and canthaxanthin. The carotenoid composition in the freshwater strain CCC Snake P. Y-85 and in the marine strains PCC 6313 and MPI S1 was found to be very similar. The phycoerythrin-producing strains NIVA-CYA 164, P7 and UBMM Bo 89 were different from each other with respect to their carotenoids. Strain NIVA-CYA 163 previously had been reported to contain considerable amounts of astax-

Fig. 2. Growth rates versus salinity in the cyanobacterial isolates investigated. All measurements were performed at 25 °C, except with strains CCC Snake P. Y-85 (38 °C) and CCC Baja-95 Cl. 2^T (25 and 38 °C).

Table 3. Retention time, absorption maxima and extinction coefficients (ϵ) determined at 436 nm for the carotenoids detected

Carotenoid	Retention time (min)	Absorption maxima*	ϵ (mM ⁻¹ cm ⁻¹)
Aphanzophyll	1:37	(452) 476 506	69.2†
Myxoxanthophyll‡	3:51	(455) 478 509	69.2
Isozeaxanthin	6:04	(430) 454 481	83.2
Unknown carotenoid 1	6:08	480	143.9†
Zeaxanthin	7:64	(430) 454 481	83.2
Canthaxanthin	8:45	478	143.9
Unknown carotenoid 2	8:51	(430) 448 478	83.2†
Unknown carotenoid 3	9:71	467	143.9†
Unknown carotenoid 4	14:91	(435) 453 480	83.2†
Echinonone	15:11	458 (480)	75.3
β -Carotene	19:58	(435) 453 480	125.3

* Shoulders are given in parentheses.

† Extinction coefficients unknown and arbitrarily assigned on the basis of spectral resemblance to known carotenoids.

‡ L-Chinovose-myxol.

Table 4. Carotenoid composition in cyanobacterial strains with *Spirulina*-like morphology

Strain	Total no. carotenoids	Carotenoids identified*
CCC Baja-95 Cl. 2 ^T	5	Aphanyzophyll (2·05), UC1 (1·05), echinenone (0·81)
CCC Baja-95 Cl. 3	5	Myxoxanthophyll (0·18), canthaxanthin (1·86), echinenone (0·73)
MPI S3	7	Myxoxanthophyll (0·50), canthaxanthin (0·59), echinenone (0·50)
MPI S1	6	Myxoxanthophyll (1·05), canthaxanthin (1·00), UC3 (0·14), echinenone (0·95)
MPI S2	5	Myxoxanthophyll (0·38), isozeaxanthin (2·12), UC2 (0·46)
MPI S4	3	Isozeaxanthin (1·42)
P7	6	Isozeaxanthin (1·36), zeaxanthin (0·36), UC2 (0·59), UC4 (1·41)
UBMM Bo89	3	Isozeaxanthin (0·49), UC2 (0·12),
UBMM Hi45	4	Canthaxanthin (2·57), UC3 (0·29), echinenone (2·36)
PCC 6313	6	Myxoxanthophyll (0·81), canthaxanthin (1·24), UC3 (0·14), echinenone (1·14)
CCC Snake P. Y-85	5	Myxoxanthophyll (0·33), canthaxanthin (1·22), UC3 (0·11), echinenone (0·41)
NIVA-CYA 163 [†]	9	Myxoxanthophyll (0·09), zeaxanthin (1·25), astaxanthin (0·56), echinenone (0·03)
NIVA-CYA 164 [†]	8	Myxoxanthophyll (0·02), zeaxanthin (0·44), echinenone (0·02)

* Molar amounts relative to β -carotene are given in parentheses. Carotenoids that made up less than 1% of the total were not identified. UC1, UC2, UC3 are unknown carotenoids 1, 2, and 3, respectively (see Table 3).

[†] Data are from Aakermann *et al.* (1992); myxoxanthophyll contains an unknown portion of L-fucose-myxol.

anthin (Aakermann *et al.*, 1992), which in these experiments was not detected in any other strain. Thus, from the data currently available, no obvious correlation of carotenoid composition and any other trait emerged. However, six strains that contained only small amounts of echinenone (NIVA-CYA 163, NIVA-CYA 164) or completely lacked this carotenoid (MPI S2, MPI S4, P7, UBMM Bo89) were of marine origin and clustered together in phylogenetic analyses based on 16S rRNA gene sequences (see below).

16S rRNA gene sequences and phylogeny

16S rRNA gene sequences were deposited in the EMBL database under accession numbers Y18789–Y18798. A tree based on maximum-likelihood computation is illustrated in Fig. 3. Since phylogeny reconstruction applying the maximum-likelihood method is computationally very expensive, bootstrap values were determined based on maximum-parsimony (1000 replicates). The maximum divergence among 16S rRNA gene sequences from cyanobacteria with *Spirulina* morphology was found to be 9·4% (11·3% as judged from partial sequences; not corrected for multiple base changes). The analyses unveiled three clusters of related sequences. 16S rRNA genes from the most halotolerant strains (MPI S3,

CCC Baja-95 Cl. 3, CCC Baja-95 Cl. 2^T) were 98·6% or more similar to each other, 7·7% or more different from all other cyanobacteria and consistently clustered together in reconstructed phylogenetic trees regardless of the calculation methods applied and supported by results of bootstrap analysis. The second cluster encompassed six sequences from organisms of marine origin (P7, MPI S4, MPI S2, UBMM Bo 89, NIVA-CYA 163, NIVA-CYA 164) that were 95·5% or more similar to each other. The third cluster contained the 16S rRNA gene sequences from the two strains with openly coiled trichomes (PCC 6313, UBMM Hi 45), which in the stretch analysed (nt 165–747; *E. coli* numbering) differed from each other by a single nucleotide insertion only. Applying various methods for tree calculations, all cyanobacteria with closed trichome coils consistently clustered together. PCC 6313 (together with UBMM Hi 45), however, either was positioned deeply branching from this cluster or was attracted by the node connecting *Synechococcus* sp. PCC 7002 and *Oscillatoria* sp. M-220 (not shown). The latter was the case when trees were calculated either on the basis of the maximum-parsimony algorithm or on the basis of distance matrices when nucleotide positions had been removed from the data sets that were conserved in less than 38% of the sequences in the respective alignments (Ludwig *et al.*,

Fig. 3. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences containing at least nt 45–1455 (*E. coli* numbering; Brosius *et al.*, 1981). 16S rRNA gene sequences from *E. coli* and *Bacillus subtilis* were used as outgroup sequences. Bootstrap values were determined on the basis of maximum-parsimony calculations (1000 replicates), exclusively involving almost complete gene sequences. They are indicated only for those clusters that contain *Spirulina*-like cyanobacteria. The phylogenetic affiliations of organisms represented by partial sequences (strains are indicated by asterisks; partial sequences contain at least nt 170–746 or, in the case of strains NIVA CYA 163 and 164, nt 346–845; Rudi *et al.*, 1997) were reconstructed by applying the parsimony criteria without changing the overall tree topology (see ARB manual; Ludwig *et al.*, 1998). Strains investigated in this study are framed. The scale bar indicates 10% estimated sequence divergence.

1998). Strain SAG B256.80 (*Spirulina laxissima*) was not closely related to any other strain included in this study.

DISCUSSION

Ecology, evolution and phylogeny

Applying various methods for phylogeny reconstruction and including various subsets of 16S rRNA gene sequence data, all trees computed assigned the cyanobacteria with closed trichome helices (i. e. excluding strains PCC 6313 and UBMM Hi 45) to a single cluster (Fig. 3). The genetic divergence among these strains nevertheless was unexpectedly high, with (almost complete) 16S rRNA gene sequences differing by up to 9.4% (not corrected for multiple base changes at individual nucleotide positions), suggesting that *Spirulina* morphology is an early and stable development in the evolution of cyanobacteria. This hypothesis is in accordance with the observation of microfossils resembling these recent cyanobacteria which date back 850 million years (Schopf, 1996).

Strains PCC 6313 and UBMM Hi 45, representing cyanobacteria that are coiled more openly and thus would be identified as *S. major* according to morphology-based taxonomy (Fig. 1; Geitler, 1932; Rippka & Herdman, 1992), seem to be only loosely affiliated to this cluster, however. Although it seems likely that all strains with open and closed trichome helices have a common ancestor, as suggested by the majority of phylogenetic trees calculated, bootstrap analysis failed to support this hypothesis. The short internodal branch in the phylogenetic tree (Fig. 3) indicates that the separation of this group from other cyanobacteria is based on information provided by only a few nucleotide positions (Ludwig *et al.*, 1998). Thus, the indication of a monophyletic origin of all *Spirulina*-like strains is of only low significance and the possibility that *Spirulina* morphology evolved convergently in two separate lineages of cyanobacteria cannot be dismissed with confidence.

Spirulina-like strains able to grow at salinities of 13% and above were found to be closely related to each other independently of their geographic origin. Phylo-

genetic analyses placed these three strains within a tight cluster clearly distinct from other cyanobacteria, including the extremely halotolerant, unicellular cyanobacteria (Fig. 3; Garcia-Pichel *et al.*, 1998). Growth in hypersaline environments requires a number of adaptations. The ability to accumulate quaternary ammonium compounds as osmolites is probably very significant (Garcia-Pichel *et al.*, 1998). Glycine betaine or glutamate betaine is produced by all cyanobacteria that are able to grow in brines with more than 13% salinity and has also been found in highly halotolerant strains displaying *Spirulina* morphology (Gabbay-Azaria & Tel-Or, 1991; MacKay *et al.*, 1984; Reed & Stewart, 1988). Thus, the genetic divergence of these strains from their counterparts in freshwater and normal marine environments can be understood in terms of a separate evolutionary history based on the ecophysiological capability to exploit extreme environmental niches. Similar results were recently reported for unicellular cyanobacteria (Garcia-Pichel *et al.*, 1998) and anoxygenic phototrophic bacteria of the families *Chromatiaceae* (Imhoff *et al.*, 1998) and *Ectothiorhodospiraceae* (Imhoff & Söling, 1996).

Consequences for classification

Our data disprove the traditional opinion of broad ecological euryvalence and ubiquitous distribution of few closely related species of cyanobacteria with *Spirulina* morphology (Anagnostidis & Golubic, 1966). Ecologically distinct organisms thriving in different habitats have different physiological capabilities and different evolutionary histories that are reflected in genetic divergence. The sequence divergence among 16S rRNA genes from cyanobacteria currently assigned to the genus *Spirulina* (9.4%, uncorrected) is significantly larger than that typical for genera of other prokaryotes (Amann *et al.*, 1992; Ash *et al.*, 1993; Vandamme *et al.*, 1996; Wisotzkey *et al.*, 1992). In addition, large differences in G+C content of genomic DNA have been reported (53.5 mol% versus 43.8 mol% in strains PCC 6313 and P7, respectively; Herdman *et al.*, 1979; Wilmotte *et al.*, 1997). This extent of genetic divergence is almost as large as that found among all heterocystous cyanobacteria so far investigated, the 16S rRNA genes of which differ in sequence by a maximal 10.4% (our calculation) and the DNA compositions of which span 38–47 mol% (Herdman *et al.*, 1979). Nevertheless, cyanobacteria able to form heterocysts currently are classified as two different orders, *Nostocales* and *Stigonematales* (Anagnostidis & Komárek, 1990; Castenholz, 1989b, c; Komárek & Anagnostidis, 1989) with a total of 80 different genera (Anagnostidis & Komárek, 1990; Komárek & Anagnostidis, 1989). This discrepancy reflects a drastic underestimation of the genetic and physiological diversity of cyanobacteria by traditional morphology-based classification, especially when cyanobacteria with less complex morphologies are concerned. Obviously, the

conspicuous shape of the cyanobacteria investigated in this study is evolutionarily too conserved to be an appropriate and sufficient taxonomic character for their classification at the genus level. Instead, to acknowledge the diversity of these micro-organisms, criteria are needed to define physiologically and phylogenetically coherent new taxa (Anagnostidis & Komárek, 1988; Castenholz, 1992).

The differential salt requirements or tolerances of bacteria reflect their adaptations to different habitats and to separate evolutionary developments, and therefore, these characteristics are established as important criteria in bacteriological classification (Imhoff *et al.*, 1998). For example, the specific salt response can be used to distinguish major phylogenetic branches of anoxygenic phototrophic bacteria (Imhoff *et al.*, 1998). We have recently reported that unicellular cyanobacteria that grow with close to optimum rates at salinities of 15% or above form a single or possibly two phylogenetic lineages within the cyanobacterial radiation (Garcia-Pichel *et al.*, 1998). Here we demonstrate that some cyanobacteria with *Spirulina*-like morphology that are able to grow at salinities of 13% or higher are closely related to each other, but are only distantly affiliated to their morphological counterparts from freshwater and moderate marine habitats and are clearly distinct from all other cyanobacteria from which 16S rRNA gene sequences are available. In addition, these strains are physiologically similar in that they are extremely euryhaline but unable to grow in freshwater medium and they tolerate relatively high temperatures (at least 38 or 40 °C, respectively). We propose the separation of those strains from the genus *Spirulina* and the reassignment of highly halotolerant cyanobacteria with helically coiled trichomes to the new genus *Halospirulina* gen. nov., which can be defined on the basis of basic morphology and high halotolerance (see below). The three strains of this cluster display different helix diameters (4–6 µm), have different carotenoids and their 16S rRNA gene sequences differ by a maximal 1.4%.

Strains PCC 6313 and UBMM Hi 45 have openly coiled trichomes and thus are morphologically similar to each other and distinct from all other strains. Interestingly, these strains were the only ones included in this study that were euryhaline in that they tolerated freshwater as well as marine salinity. This is in accordance with the phylogenetic analysis based on 16S rRNA gene sequences, which places these strains separate and deeply branching from the cluster of cyanobacteria with tightly coiled trichomes in which single helix-turns touch each other (closed helices). PCC 6313 and UBMM Hi 45 match the morphology-based definition of *Spirulina major* Kützinger ex Gomont (Anagnostidis & Golubic, 1966; Geitler, 1932) which is the type species of the genus *Spirulina* according to the botanical code (lectotype; Castenholz, 1989a). If strain PCC 6313 would be accepted as the type strain for *Spirulina major* at the species and genus level as previously suggested (Rippka & Herdman, 1992), then

probably all other strains (except UBMM Hi 45) need to be reclassified and assigned to newly created genera. However, this is not the intention here and will require future studies. Additional physiological and genetic characters need to be investigated and the suitability of helix tightness as a taxonomic criterion needs to be confirmed. Possibly, morphological variability within strains may complicate the use of the latter since slight despiralization depending on growth conditions has been described (Wilmotte, 1991). Hindák (1985) even reported the occurrence of completely uncoiled filaments in a culture of *S. major*. Other studies on the variability of helix tightness, however, have been restricted to cyanobacteria that have to be assigned to the genus *Arthrospira* according to current classification (Hindák, 1985; Jeeji-Bai, 1985; Jeeji-Bai & Seshadri, 1980; Lewin, 1985).

Description of *Halospirulina* gen. nov.

Halospirulina gen. nov. (Ha.lo.spi.ru.li'na. Gr. n. *hals*, *halos* salt; L. dim. fem. n. *spirulina* a small coil; N.L. fem. n. *Halospirulina* salt-tolerant small coil).

Halotolerant, euryhaline cyanobacteria with trichomes coiled into a tight, closed helix, able to grow at salinities between 3 and 13 ‰ or above, but not at freshwater salinities. Trichome widths are typically between 1.5 and 3 µm, and helix widths vary between 4 and 6 µm. The cross walls are thin and invisible in live specimens. No sheath is visible under light microscopy. Gliding motility present, involving rotation. Found in sunlit hypersaline environments. Tolerate temperatures for growth of at least 38 °C. Type species is *Halospirulina tapeticola*.

Description of *Halospirulina tapeticola* sp. nov.

Halospirulina tapeticola sp. nov. (ta.pe.ti'co.la. L. n. *tapete* mat; L. suff. *cola* dweller; M.L. fem. n. *tapeticola*, microbial-mat dweller).

Halotolerant, euryhaline cyanobacteria with trichomes coiled into a tight, closed helix, able to grow at salinities between 3 and 20 ‰, but not at freshwater salinities. Trichome and helix widths are 1.5 and 4 µm, respectively. The cross walls are thin and invisible in live specimens. No sheath is visible under light microscopy. Gliding motility present, involving rotation. The axenic type strain is CCC Baja-95 Cl. 2^T, which was isolated from a microbial mat in a hypersaline evaporation pond of a salina at the Pacific coast of Baja California, Mexico. This strain is available from the Culture Collection of Microorganisms from Extreme Environments, Eugene, OR, USA, as CCC Baja-95 Cl. 2^T, and has been deposited in the Pasteur Culture Collection, Paris, France.

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