

**Diversität und salinitätsabhängiges Verhalten benthischer,
cyanobakterieller Lebensgemeinschaften**

**Diversity and Salinity-Dependent Behaviour of Benthic
Cyanobacterial Communities**

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Summary

In this study cyanobacterial communities in microbial mats from hypersaline environments have been investigated. Relations between the diversity of the cyanobacteria, environmental factors, and the productivity of the communities have been given particular attention.

1. On the basis of published nucleic acid sequences a PCR method was developed that enables the specific amplification of 16S rRNA gene segments from cyanobacteria and plastids.
2. Cyanobacterial strains isolated from hypersaline microbial mats were analysed with respect to their phylogeny and halotolerance and other phenotypic properties. Phylogenetic analyses suggested that adaptations to life at extremely high salinities has been developed independently in several lineages of the cyanobacteria. High halotolerance is a conserved ecophysiological character that enables the classification of phylogenetically coherent taxa.
3. Comparative analyses of 16S rRNA gene sequences indicated that the majority of mat forming cyanobacteria in the habitats investigated is closely related to isolated strains the halotolerance of which is known. In view of the knowledge about comparable microbial mat systems from geothermal springs this is a surprising result. The distribution of the organisms along a gradient of salinity could be interpreted in the light of their ecophysiology.
4. The diversity of cyanobacteria and diatoms in a number of microbial mats was determined applying three cultivation-independent approaches. The congruence of results based on analyses of 16S rRNA genes, morphologies, and carotenoids suggested that a meaningful comparative quantification of microbial diversity in principle is possible. A positive correlation was found between the diversity of oxygenic phototrophic microorganisms and the functional stability of the respective communities.

Zusammenfassung

Im Rahmen der vorliegenden Dissertation wurden cyanobakterielle Lebensgemeinschaften in mikrobiellen Matten aus hypersalinen Standorten untersucht. Insbesondere wurden Beziehungen zwischen der Diversität der Cyanobakterien, den darauf Einfluß nehmenden Umweltfaktoren, und der Produktivität der Organismengemeinschaften studiert.

1. Auf der Grundlage von publizierten Nukleinsäuresequenzen wurde ein PCR-Protokoll entwickelt, das die spezifische Amplifizierung von 16S-rRNA-Gen-Segmenten aus Cyanobakterien und Plastiden ermöglicht.
2. Aus hypersalinen mikrobiellen Matten isolierte Cyanobakterien wurden hinsichtlich ihrer Phylogenie und Halotoleranz sowie weiterer phänotypischer Merkmale untersucht. Die phylogenetischen Analysen zeigten, daß sich die Anpassung an extrem hohe Salinitäten unter Cyanobakterien sehr wahrscheinlich mehrfach entwickelt hat, und daß sie in den verschiedenen Entwicklungslinien ein konserviertes ökophysiologisches Merkmal ist, das zur Klassifizierung phylogenetisch kohärenter Taxa geeignet ist.
3. Vergleichende Analysen von 16S-rRNA-Gen-Sequenzen zeigten, daß der größte Teil der mattenbildenden Cyanobakterien in den untersuchten Habitaten nahe mit Isolaten verwandt ist, deren Halotoleranz bekannt ist. Dies ist ein überraschendes Ergebnis angesichts des für ähnliche Mattensysteme aus geothermalen Quellen beschriebenen Kenntnisstands. Die Verteilung der Organismen entlang eines Salinitätsgradienten konnte hinsichtlich ihrer Ökophysiologie interpretiert werden.
4. Die Diversität von Cyanobakterien und Diatomeen in einer Reihe von mikrobiellen Matten wurde mit drei verschiedenen kultivierungsunabhängigen Methoden ermittelt. Die Übereinstimmung der Analysen von 16S rRNA-Genen, Morphologie, und Carotinoiden zeigte, daß eine aussagefähige, vergleichende Quantifizierung von mikrobieller Diversität prinzipiell möglich ist. Weitere Versuche ergaben, daß die Diversität der oxygen-phototrophen Mikroorganismen mit der funktionellen Stabilität der Lebensgemeinschaften positiv korreliert ist.

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Teil 1: Darstellung der Ergebnisse im Gesamtzusammenhang

A. Einleitung

Mikroorganismen spielen eine bedeutende Rolle in den Stoffkreisläufen der Biosphäre, und die Untersuchung ihrer Wechselwirkungen untereinander und mit ihrer Umwelt ist daher von großem Interesse. Die geringe Größe dieser Organismen erschwert jedoch das Studium ihrer Diversität und Ökologie. Die Isolierung von Mikroorganismen und Kultivierung in Reinkulturen ist unverzichtbar, um das physiologische Potential einzelner, genetisch homogener Populationen experimentell zu erforschen. Sodann ist die eindeutige Identifizierung dieser Organismen in ihrer natürlichen Umgebung wünschenswert, um ihre komplexen ökologischen Interaktionen verstehen zu lernen.

Die vorliegende Arbeit will einen Beitrag zum Verständnis der Diversität und Ökologie der Cyanobakterien leisten. Modellhaft wurde der Einfluß der Salinität auf die Zusammensetzung benthischer cyanobakterieller Lebensgemeinschaften in Solen einer Salzgewinnungsanlage untersucht. An im Labor kultivierten Cyanobakterien wurden ihre speziellen ökophysiologischen Anpassungen an diesen Lebensraum studiert. Analysen zur Phylogenie der besonders halotoleranten Isolate zeigten, daß sie eigene evolutionäre Entwicklungslinien darstellen. Eine quantitative Beschreibung der Diversität der Cyanobakterien und Diatomeen ermöglichte die Untersuchung möglicher Zusammenhänge zwischen der Diversität phototropher Mikroorganismen und der Produktivität ihrer natürlichen Lebensgemeinschaften.

1. Mikrobielle Matten

Mikrobielle Matten sind makroskopisch sichtbare, zusammenhängende Lebensgemeinschaften von Mikroorganismen. Sie sind mit organischem Material angereichert, erscheinen in der Regel geschichtet und werden als rezente Analoga sogenannter Stromatoliten angesehen, fossiler Sedimente, die bis zu 3,5 Milliarden Jahre alt sind (Des Marais, 1995; Karsten & Kühl, 1996). Heute treten verschiedenartige mikrobielle Matten in sehr unterschiedlichen Lebensräumen auf; die am stärksten ausgeprägten Formen findet man jedoch an Standorten mit Umweltbedingungen, die für die Mehrheit der eukaryontischen Organismen lebensfeindlich sind, so daß der Konkurrenz- und Fraßdruck durch vielzellige Pflanzen und Tiere vermindert oder ausgeschlossen ist. Solche "extremen" Lebensräume sind beispielsweise Seen in der Antarktis, Tiefseesedimente, heiße Quellen und hypersaline Seen und Lagunen (Salinen). Die

Primärproduktion in Matten, die regelmäßig von Tageslicht beschienen werden, beruht vollständig oder zum weitaus größten Teil auf den Aktivitäten photoautotropher Mikroorganismen. Auf sulfidabhängiger, anoxygener Photosynthese basierende mikrobielle Matten sind beschrieben worden (Ward *et al.*, 1989); die treibende Kraft der meisten rezenten Matten ist jedoch die sauerstoffproduzierende oxygene Photosynthese durch Cyanobakterien (Blaualgen) und eukaryontische Mikroalgen wie Diatomeen (Kieselalgen) (Castenholz, 1994). Die Primärproduzenten fixieren anorganischen Kohlenstoff und synthetisieren organische Verbindungen, die durch Ausscheidung oder als tote Substanz für verschiedenartige heterotrophe Organismen verfügbar werden. Einige von diesen nutzen den von Cyanobakterien und Diatomeen produzierten Sauerstoff, um organische Kohlenstoffverbindungen zu oxidieren. Gärende Organismen produzieren niedermolekulare organische Substanzen und Wasserstoff, die von methanogenen oder dissimilatorisch sulfatreduzierenden Prokaryonten als Wachstumssubstrate genutzt werden, wobei entsprechend Methan beziehungsweise Sulfid und Kohlendioxid, sowie weitere Schwefelverbindungen verschiedener Oxidationsstufen freigesetzt werden können. Meerwasser enthält reichlich Sulfat, so daß in mikrobiellen Matten in marinen Habitaten die Sulfatreduktion wahrscheinlich den wichtigsten anaeroben Prozeß der Kohlenstoffoxidation darstellt (Canfield & Des Marais, 1991). Das dabei entstehende Sulfid kann spontan oder durch chemotrophe und phototrophe Bakterien zu elementarem Schwefel und Sulfat reoxidiert werden.

Durch die Stoffwechselaktivitäten und photosynthetischen Pigmente der verschiedenen Mikroorganismen erscheinen viele mikrobielle Matten makroskopisch als deutlich vertikal geschichtete Systeme. Braune und grüne Schichten liegen an der Oberfläche und enthalten Diatomeen und Cyanobakterien; darunter sind oftmals weiße und rote Schichten zu erkennen, die von chemotrophen Schwefelbakterien und Schwefelpurpurbakterien gebildet werden, gefolgt von einer dicken, anoxischen, durch Eisensulfid schwarz gefärbten Schicht, die durch die Aktivität von Sulfatreduzieren entsteht. Tatsächlich allerdings überlappen die Verteilungen der verschiedenen funktionalen Gruppen von Mikroorganismen, und ihr Wechselspiel findet auf engstem Raum statt. Die photische Zone ist oftmals auf die oberen ein bis zwei Millimeter der Matte beschränkt (Jørgensen *et al.*, 1987). Hier werden sehr hohe Produktivitäten erreicht, die im Verhältnis zur Biomasse mit denen von tropischen Regenwäldern vergleichbar sind. Hohe Photosyntheseraten werden jedoch von hohen Atmungsraten begleitet, so daß die Stoffumsetzungen sich in einem dynamischen Fließgleichgewicht befinden (Jørgensen *et al.*, 1983). Gleichzeitig wurden in der Sauerstoff enthaltenden photischen Zone die höchsten Sulfatreduktionsraten gemessen (Canfield & Des Marais, 1991; Fründ & Cohen, 1992; Visscher *et al.*, 1992). Durch die dichte Anordnung der Mikroorganismen und die damit verbundene starke Lichtabsorption und starke räumliche metabolische Aktivität entstehen innerhalb der dünnen oberen Schicht der Matte steile vertikale Gradienten besonders der Sauerstoff- und Sulfidkonzentrationen sowie des pH-Wertes (Jørgensen *et al.*, 1983). Diese Gradienten hängen in starkem Maße von der photosynthetischen Sauerstoffproduktion und

damit von der Intensität des einfallenden Lichts ab, die sich im Tagesverlauf sowie jahreszeitlich und wetterabhängig verändert (Castenholz, 1994). Die in mikrobiellen Matten lebenden Organismen haben vielfältige Anpassungen an diese zeitlich stark veränderlichen chemischen und physikalischen Bedingungen entwickelt wie chemo- und phototaktische Beweglichkeit und erhöhte Resistenzen gegenüber toxischen Substanzen und schädigender Strahlung (Bebout & Garcia Pichel, 1995; Cohen *et al.*, 1975; Garcia-Pichel *et al.*, 1994; Krekeler *et al.*, 1998; Kruschel & Castenholz, 1998; Teske *et al.*, 1998).

2. Hypersaline Lebensräume

Mikrobielle Matten in hypersalinen Lebensräumen sind neben den in verschiedenen geothermalen Quellen vorkommenden bisher am umfassendsten untersucht worden. Das Interesse an salzablagernden Strukturen und Prozessen ist wissenschaftlicher (geologischer, chemischer, biologischer) und kommerzieller Natur. Fossile Salzablagerungen sind wichtige Quellen von Natriumchlorid (Steinsalz) und Kaliumsalzen (Pottasche) für die menschliche Versorgung, enthalten oftmals Ölvorräte und liefern Informationen über ozeanische und kontinentale, geologische Entwicklungen. Unter rezenten, stark salzhaltigen Gewässern unterscheidet man solche, die unter marinem Einfluß stehen, von jenen, in denen fossile Salze gelöst sind, da sie sich hinsichtlich der Zusammensetzung der Salze unterscheiden können. In heutiger Zeit sind Salzablagerungsstätten vergleichsweise selten, werden aber in der Geologie und Paläontologie als Schlüssel zum Verständnis der geologischen Vergangenheit angesehen und zur Salzgewinnung aus Meerwasser auch künstlich angelegt (Salinen). Stark konzentrierte Solen sind für die meisten vielzelligen Organismen lebensfeindlich, für bestimmte Arten und besonders für verschiedenartige Mikroorganismen sind sie jedoch die bevorzugten Lebensräume. Speziell angepasste Prokaryonten und Mikroalgen können die Fällung von Salzen und die Bildung von Sedimenten in ihrer hypersalinen Umwelt maßgeblich beeinflussen (Javor, 1989).

Erhöhte Salzkonzentrationen in der Umgebung verlangen von aquatischen Organismen Anpassungen an einen hohen osmotischen Druck und hohe Ionenkonzentrationen. Dazu sind zwei grundsätzlich verschiedene Strategien bekannt (Galinski, 1995). Halobakterien (halophile Archaeobakterien) und möglicherweise auch bestimmte halophile anaerobe Eubakterien weisen hohe intrazelluläre Konzentrationen spezifischer Ionen (K^+ , eventuell Na^+) auf, um den herrschenden osmotischen Druck auszugleichen. Gleichzeitig ist ihr gesamtes Zellinneres an die geringe Wasserverfügbarkeit angepasst. Andere Organismen einschließlich Eukaryonten entfernen Natriumionen unter Energieaufwand aus ihren Zellen und akkumulieren stattdessen organische Osmolyte wie Disaccharide (Saccharose, Trehalose), Glycerin, Glycosylglycerin oder Glycin-Betain. Diese osmoprotektiven Substanzen sind ungeladene, polare Moleküle, die auch in hohen Konzentrationen die Funktionen der Zellen nicht beeinträchtigen. Viele

Organismen, die diese Substanzen nicht selbst synthetisieren können, können ihre Halotoleranz durch die Aufnahme von Osmolyten aus der Umgebung erhöhen (Galinsky, 1995). Außer dem Gesamtsalzgehalt hat auch die spezifische Zusammensetzung der gelösten Salze Einfluß auf die Tolerierbarkeit durch Lebewesen. So ist zu erklären, daß Mikroorganismen in Krusten aus abgelagertem Salz leben können, während die übrigbleibenden Solen nahezu steril erscheinen (Javor, 1989).

Im Rahmen dieser Arbeit wurden mikrobielle Matten aus Verdunstungsbecken der in Guerrero Negro an der Pazifikküste Mexikos gelegenen Salzgewinnungsanlage der Firma Exportadora de Sal S. A. (ESSA) untersucht. Diese Anlage besteht aus einer Sequenz von 13 miteinander in Verbindung stehenden Verdunstungsbecken mit einer Gesamtfläche von etwa 200 Quadratkilometern sowie nachgeschalteten Kristallisationsbecken (Javor, 1983). In die Anlage gepumptes Meerwasser erhöht auf seinem Weg durch diese Becken durch sonnen- und windgetriebene Verdunstung seine Salinität (Gesamtsalzgehalt), bis die Salze in der Reihenfolge ihrer Löslichkeit ausfallen. Innerhalb dieses Gradienten sind bei Salinitäten von etwa 6 bis 16% mikrobielle Matten entstanden, die schon seit einigen Jahren wissenschaftlich untersucht werden (zum Vergleich: normales Meerwasser hat eine Salinität von 3,5%). Die meisten bisherigen Studien beschäftigten sich mit Stoffkreisläufen innerhalb der Matten (Kohlenstoff, Sauerstoff, Schwefel, Stickstoff), den chemisch-physikalischen Bedingungen, unter denen die entsprechenden mikrobiellen Umsetzungen stattfinden, und den Anpassungen verschiedener Mikroorganismen an die drastischen, zeitlichen und räumlichen Veränderungen der Lichtverhältnisse und der Sauerstoff- und Sulfidkonzentrationen. Übersichten über frühere Arbeiten geben Javor (Javor, 1989) und Des Marais (Des Marais, 1995). Die vorliegende Dissertation beschreibt Untersuchungen zur Diversität und Ökologie der Cyanobakterien, die die dominanten Primärproduzenten in diesen mikrobiellen Matten sind.

3. Die Diversität der Cyanobakterien

Cyanobakterien bilden eine monophyletische Gruppe gram-negativer Eubakterien, die eine oxygene Photosynthese ähnlich der der Pflanzen betreiben können. Das heißt, daß sie Lichtenergie zur Oxidation von Wasser durch zwei hintereinandergeschaltete Photoreaktionen und dadurch zur Produktion molekularen Sauerstoffs nutzen. Alle Cyanobakterien nutzen Chlorophyll *a* als Photosynthesepigment; die meisten besitzen zusätzlich Phycobiliproteine als Antennenpigmente. Die "Prochlorophyten" synthetisieren die Chlorophylle *a* (Divinyl-Chlorophyll *a* in *Prochlorococcus*) und *b*, enthalten keine Phycobiliproteine und wurden zunächst als eine zweite Gruppe Sauerstoff entwickelnder phototropher Prokaryonten angesehen (Florenzano *et al.*, 1986). Angesichts neuerer Erkenntnisse besonders zu ihrer molekularen Phylogenie erscheint diese Aufteilung jedoch willkürlich (Pinevich *et al.*, 1997; Turner, 1997). Alle Cyanobakterien sind fähig, anorganischen Kohlenstoff zu fixieren

(Autotrophie), und die meisten untersuchten Stämme sind obligat phototroph. Einige sind auch in der Lage, einfache Zucker aus dem umgebenden Medium aufzunehmen und als Kohlenstoffquelle zu verwenden (Photoheterotrophie), und manche Stämme können solche Zucker als Wachstumssubstrat nutzen (fakultative Chemoheterotrophie). Die Fähigkeiten zur anaeroben Gärung sowie zur Fixierung molekularen Stickstoffs sind weit verbreitet. Cyanobakterien besitzen circadiane Uhren, mit denen sie ihren lichtabhängigen Stoffwechsel dem Tag-Nacht-Rhythmus anpassen (Johnson *et al.*, 1996). Viele Cyanobakterien können gleitende und manche schwimmende Bewegungen ausführen, durch die sie sich in chemischen Gradienten (Chemotaxis) und Lichtgradienten (Phototaxis) positionieren können; sie haben jedoch keine Geißeln (Flagellen). Cyanobakterien gedeihen in nahezu allen Lebensräumen der Erde, in denen Licht zur Verfügung steht. Man findet sie weit verbreitet in unterschiedlichen terrestrischen, marinen und limnischen Habitaten, an die verschiedene Arten jeweils besondere Anpassungen entwickelt haben. Viele sind aus Symbiosen bekannt, und es gilt heute als sicher, daß die Plastiden, die Photosynthese betreibenden Organellen der Pflanzen, aus endosymbiontischen Vorläufern der Cyanobakterien hervorgegangen sind. Besonders erfolgreich sind Cyanobakterien unter Bedingungen, die für höhere Pflanzen abträglich sind, wie hohe oder niedrige Temperaturen oder die geringe Verfügbarkeit von Wasser, Licht oder Nährstoffen. Einige Arten tolerieren hohe Konzentrationen freien Sulfids durch besondere Anpassungen ihres Photosystems II, oder indem sie eine anoxygene Photosynthese betreiben und Schwefelwasserstoff anstelle von Wasser als Elektronendonator benutzen (Castenholz & Waterbury, 1989).

Die Morphologie von Cyanobakterien kann im Vergleich zu der anderer Prokaryonten sehr komplex sein. Es existiert eine Vielzahl lichtmikroskopisch unterscheidbarer einzelliger, filamentöser und koloniebildender Formen mit Zelldurchmessern von weniger als einem bis zu über 100 Mikrometern. Einige bilden spezialisierte Zellen wie stickstofffixierende Heterocysten, widerstandsfähige Akineten, bewegliche Hormogonien oder speziell geformte Zellen an den Enden der Filamente. Wegen ihrer Fähigkeit zur oxygenen Photosynthese und der morphologischen Ähnlichkeit mit eukaryontischen Grünalgen und anderen Mikroalgen wurden die Cyanobakterien traditionell als Pflanzen (Cyanophyten) angesehen und werden auch heute noch als Blaualgen (Cyanophyceae) bezeichnet. Auf der Grundlage der beobachteten morphologischen Diversität haben Botaniker mehr als 2.000 Arten und über 140 Gattungen von Cyanobakterien beschrieben. Die bis heute wichtigste Grundlage für ihre Systematik wurde von Geitler zusammengestellt (Geitler, 1932). Daneben existieren weitere, umfangreiche Abhandlungen (zum Beispiel: Bourrelly, 1979; Desikachary, 1959; Drouet, 1981; Elenkin, 1936/1938/1949; Frémy, 1929-1933), die sich zum Teil widersprechen und deren gleichzeitiger Gebrauch für nicht unerhebliche taxonomische Konfusion sorgt. Alle ausschließlich auf mikroskopischen Beobachtungen beruhenden Systeme zur Klassifizierung von Cyanobakterien haben gemeinsam, daß die Abgrenzungen der Taxa oftmals auf einzelnen morphologischen Merkmalen beruhen und häufig willkürlich und wenig natürlich erscheinen (Castenholz &

Waterbury, 1989; Whitton, 1992). In den frühen siebziger Jahren begannen Stanier und Mitarbeiter damit, eine neue Taxonomie für Cyanobakterien auf der Grundlage von solchen Organismen zu entwickeln, die in axenischen, klonalen Kulturen experimentell untersucht werden können (Rippka *et al.*, 1979; Stanier *et al.*, 1971). Dies ermöglicht die Berücksichtigung von chemischen, genetischen und physiologischen Eigenschaften der kultivierten Stämme als Merkmale für ihre Klassifizierung und entspricht im wesentlichen der Systematik anderer Prokaryonten. Gegenwärtig wird versucht, einen Kompromiß zwischen der bakteriologischen und der botanischen Klassifizierung zu finden (Anagnostidis & Komárek, 1985; Castenholz & Waterbury, 1989), und die gültige Beschreibung neuer Taxa ist nach beiden internationalen Nomenklatur-Codes möglich. Soweit passende botanische Namen zuvor veröffentlicht wurden, werden sie zur Benennung von Kulturen verwendet, die dann als Typstämme dienen (Rippka & Herdman, 1992). Aufgrund der geringen Zahl der berücksichtigten kultivierten Cyanobakterien sind alle bisher beschriebenen Taxa noch immer weitgehend morphologisch definiert (Castenholz, 1992; Castenholz & Waterbury, 1989; Rippka & Herdman, 1992). Sie wurden in fünf Gruppen eingeteilt (Rippka *et al.*, 1979), denen in Anlehnung an die traditionelle Klassifizierung der Rang von Ordnungen zugesprochen wurde (Castenholz & Waterbury, 1989):

- | | |
|-----------------------------|--|
| I. <i>Chroococcales</i> | einzellig, Vermehrung durch einfache Zellteilung |
| II. <i>Pleurocapsales</i> | einzellig, Vermehrung durch vielfache Teilungen innerhalb einzelner Zellen möglich |
| III. <i>Oscillatoriales</i> | filamentös, unverzweigte Trichome, keine Heterocysten |
| IV. <i>Nostocales</i> | filamentös, unverzweigte Trichome, Heterocystenbildung möglich |
| V. <i>Stigonematales</i> | filamentös, Bildung von verzweigten Trichomen und Heterocysten möglich |

Die Chlorophyll *b* produzierenden "Prochlorophyten" wurden in die Ordnung *Prochlorales* gestellt (Lewin, 1989).

Schon seit langer Zeit wird für alle Organismen eine Klassifizierung auf der Grundlage ihrer phylogenetischen Beziehungen angestrebt. Das wichtigste Instrument zur Erforschung der Phylogenie von Prokaryonten ist zur Zeit die Analyse der für ribosomale RNA (rRNA) codierenden Gene (Woese, 1992). Auf der Grundlage der Überlegung, daß aus einem Lebewesen extrahierte Makromoleküle seine evolutionäre Entwicklungsgeschichte dokumentieren (Zuckerkindl & Pauling, 1965), werden Unterschiede zwischen Sequenzen der rRNA-Gene aus verschiedenen Organismen für die Konstruktion von phylogenetischen Stammbäumen herangezogen (Woese, 1987). rRNA-Gene sind für diese Untersuchungen besonders geeignet, da sie in allen Organismen vorkommen und ihre Funktion durch einen konstanten, das heißt von den Umweltbedingungen weitgehend unabhängigen Selektionsdruck in hohem Maße konserviert ist. Sequenzveränderungen entstehen dadurch mit gleichmäßiger

Geschwindigkeit und langsam genug, um die gesamte lange Zeit der bakteriellen Evolution widerspiegeln zu können (Woese, 1987). Durch methodische Fortschritte ist die Analyse der Gene für 16S-rRNA in der Mikrobiologie zu einem Standardwerkzeug geworden, das die phänotypische Klassifizierung und Identifizierung unterstützt. Bis heute wurden bereits etwa 10.000 Sequenzen von 16S-rRNA-Genen in speziellen Datenbanken abgelegt (Ludwig *et al.*, 1998). Nahezu 200 dieser Sequenzen sind cyanobakteriellen Ursprungs, von denen allerdings mehr als die Hälfte zu unvollständig sind, um für stabile phylogenetische Rekonstruktionen benutzt werden zu können (Stackebrandt & Rainey, 1995).

Ein Stammbaum der Cyanobakterien auf der Grundlage der heute verfügbaren Informationen über 16S-rRNA-Gene ist in Abbildung 1 dargestellt. Er zeigt einen frühen, einmaligen Ursprung aller Plastiden aus der Radiation der Cyanobakterien (Bhattacharya & Medlin, 1995) und eine Reihe getrennter cyanobakterieller Entwicklungslinien. Viele dieser Entwicklungslinien werden zur Zeit nur durch einzelne Sequenzen vertreten, und der begrenzte Umfang der Daten läßt eine Berechnung der tiefen Verzweigungspositionen nicht zu (Turner, 1997). Heterocysten produzierende Cyanobakterien bilden eine monophyletische Gruppe, während alle anderen auf Morphologie basierenden übergeordneten Gruppierungen (Ordnungen, siehe oben) so wie auch die "Prochlorophyten" als polyphyletisch angezeigt werden. Einige Gattungen sind phylogenetisch ebenfalls nicht kohärent, zum Beispiel *Synechococcus* und *Oscillatoria*. Die Analyse der 16S-rRNA-Gene macht deutlich, daß weder die traditionelle botanische noch die derzeitige bakteriologische Systematik der Phylogenie der Cyanobakterien entspricht und daß beide Systeme die evolutionäre Vielfalt dieser Organismen unterschätzen. Zusätzlich muß berücksichtigt werden, daß der in Abbildung 1 gezeigte Stammbaum nahezu ausschließlich auf Sequenzen aus Laborkulturen beruht und daß bisher aber nur ein geringer Teil der in der Natur existierenden Cyanobakterien erfolgreich kultiviert werden konnte (Castenholz, 1992).

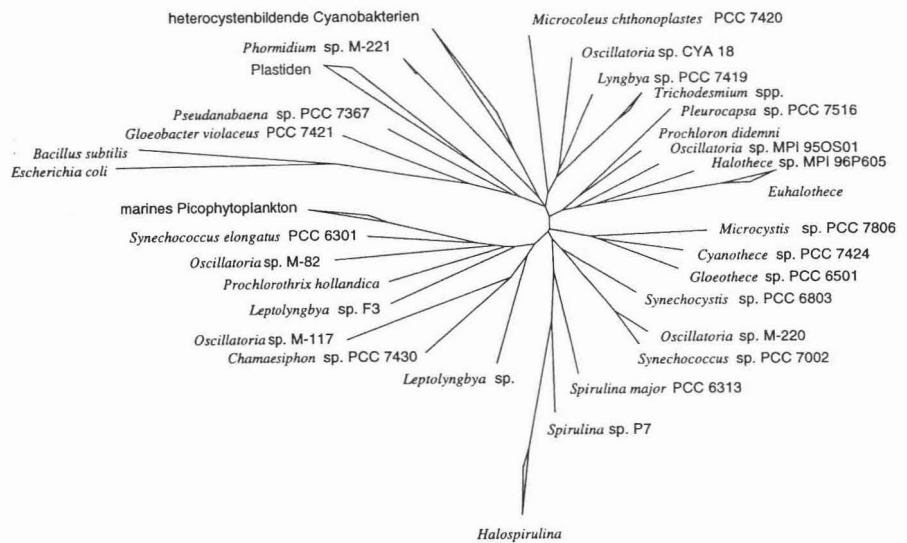


Abbildung 1. Die Phylogenie der Cyanobakterien und Plastiden. Die Bakterien *Escherichia coli* und *Bacillus subtilis* bilden die Wurzel des gezeigten Stammbaums.

B. Ergebnisse und Diskussion

1. Spezifische Amplifizierung der 16S-rRNA-Gene aus Cyanobakterien

Zur Erforschung der Ökologie von Cyanobakterien ist es wünschenswert, die aus Kulturen gewonnenen Erkenntnisse zu ihrer Ökophysiologie auf Organismen in ihrer natürlichen Umgebung zu übertragen (Castenholz & Waterbury, 1989). Dazu werden verlässliche Identifizierungsmethoden benötigt. Auf der Grundlage von publizierten Nukleinsäuresequenzen wurde daher eine PCR (Polymerase-Kettenreaktion, englisch: polymerase chain reaction) entwickelt, die die spezifische Amplifizierung von Abschnitten der 16S-rRNA-Gene aus Cyanobakterien ermöglicht. Damit konnten diese Gen-Segmente aus allen getesteten Cyanobakterienkulturen sowie aus cyanobakteriellen Flechtensymbionten und den Plastiden von kultivierten Diatomeen isoliert werden. Wurde DNA aus anderen Eubakterien oder Archaeobakterien als Vorlage für die PCR eingesetzt, wurden keine Amplifikationsprodukte erzeugt. Die erzeugten PCR-Produkte konnten durch Denaturierungsgradienten-Gelelektrophorese (DGGE) sequenzabhängig aufgetrennt werden. PCR-Produkte aus unialgalen, nicht-axenischen Kulturen konnten direkt sequenziert werden. Damit steht nun eine Methode zur Verfügung, die die schnelle und phylogenetisch aussagefähige Identifizierung von Cyanobakterien ermöglicht, ohne daß dazu Reinkulturen oder molekulare Klonierungsschritte erforderlich sind. Sammlungen von Isolaten können damit sehr schnell nach einzigartigen Stämmen durchsucht werden und mit der Zusammensetzung natürlicher Lebensgemeinschaften verglichen werden (Publikation 1; Nübel *et al.*, 1997).

2. Beiträge zur Systematik halotoleranter Cyanobakterien

Insgesamt 24 Kulturen von Cyanobakterien zweier morphologischer Gruppen wurden hinsichtlich ihrer Morphologie, photosynthetischen Pigmente, Thermotoleranz, Halotoleranz und Phylogenie untersucht. Dadurch konnten erstmalig zwei phylogenetisch kohärente Gruppen von Cyanobakterien auf der Grundlage ihrer Physiologie definiert werden. Drei der einzelligen Cyanobakterien aus hypersalinen Lebensräumen stammten aus öffentlichen Kultursammlungen. Ein Stamm wurde uns von Y. Cohen überlassen. Alle anderen wurden von Ferran Garcia-Pichel isoliert. Diese Isolate wiesen eine bemerkenswerte morphologische Diversität auf, und einige variierten auch stark in Abhängigkeit von den Kulturbedingungen. Alle waren in der Lage, bei Salinitäten zwischen 6 und 15% mit nahezu optimaler Rate zu wachsen. Sie gehören damit zu den halotolerantesten aller bisher beschriebenen Cyanobakterien. Eine phylogenetische Analyse auf Grundlage der 16S-rRNA-Gene ordnete alle Stämme in eine monophyletische Gruppe, wobei eines der neuen Isolate sehr tief abzweigt. Der Gruppe der zwölf deutlich

miteinander verwandten Stämme wurde der Name "*Euhalothece*-Cluster" gegeben (Publikation 2; Garcia-Pichel *et al.*, 1998). Elf weitere untersuchte Stämme, die nach der derzeitigen Klassifizierung in die Cyanobakterien-Gattung *Spirulina* gehören, stammten entweder aus öffentlichen Kultursammlungen oder wurden von R. W. Castenholz, E. Clavero oder J. Rethmeyer zur Verfügung gestellt. Die meisten waren marinen oder hypersalinen Ursprungs. Einer der Stämme war aus einer sulfidischen, geothermalen Quelle isoliert worden. Diese Cyanobakterien waren morphologisch untereinander sehr ähnlich, wiesen jedoch deutlich unterschiedliche Halo- und Thermotoleranzen auf, die mit den Umweltbedingungen in den Standorten ihrer Herkunft korrelierten. Die Analyse der 16S-rRNA-Gene zeigte eine große genetische Divergenz dieser Stämme an. Drei der Isolate stammten aus hypersalinen Solen, tolerierten Salinitäten von mindestens 16% und Temperaturen von mindestens 38°C, und wurden durch die phylogenetische Analyse in eine enge monophyletische Gruppe gestellt. Folglich soll die Reklassifizierung dieser Cyanobakterien in eine neue Gattung, *Halospirulina* gen. nov., vorgeschlagen werden (Publikation 3; Nübel *et al.*, in Vorbereitung). Alle untersuchten, stark halotoleranten Stämme waren gleichzeitig auch in der Lage, bei niedrigen Salinitäten zu wachsen und können daher als extrem euryhalin bezeichnet werden.

3. Untersuchungen zur Zusammensetzung mattenbildender Lebensgemeinschaften entlang eines Salinitätsgradienten

Die phylogenetische Diversität von Cyanobakterien und Diatomeen in hypersalinen mikrobiellen Matten und ihre Verteilung entlang eines Salinitätsgradienten wurden untersucht und konnten mit den Halotoleranzen nahe verwandter, kultivierter Stämme verglichen werden. Dazu wurden 16S-rRNA-Gensegmente mit Hilfe der entwickelten spezifischen PCR direkt aus Umwelt-DNA isoliert und durch Denaturierungsgradienten-Gelelektrophorese sequenzabhängig aufgetrennt. Durch Sequenzanalysen der aus DGGE-Banden extrahierten DNA konnte gezeigt werden, daß die Mehrheit der in den Matten lebenden Cyanobakterien und Diatomeen phylogenetischen Gruppen kultivierter Isolate zugeordnet werden kann, für die zuvor jeweils recht einheitliche Ansprüche an die Salinität festgestellt worden waren. Die anhand der Fluoreszenz-Intensitäten von DGGE-Banden abgeschätzten relativen Häufigkeiten der amplifizierten 16S-rRNA-Gensegmente aus diesen phylogenetischen Gruppen stimmten im allgemeinen mit mikroskopischen Zählungen der entsprechenden Morphotypen überein. Diatomeen machten in allen untersuchten Matten einen geringen Anteil der Zellen aus. *Microcoleus chthonoplastes* und sehr nahe verwandte Cyanobakterien dominierten in den phototrophen Lebensgemeinschaften bis zu einer Salinität von 11%. Bei einer Salinität von 14% waren die häufigsten Cyanobakterien Verwandte von sehr halotoleranten kultivierten Isolaten wie den Gruppen *Euhalothece* und *Halospirulina*. Während diese Organismen zuvor in Laborkulturen ihre Fähigkeit demonstriert hatten, über ein sehr breites Salinitätsspektrum mit nahezu maximalen

Raten zu wachsen, ist ihr Vorkommen im natürlichen Habitat offensichtlich auf die höchsten Salinitäten beschränkt (Publikation 4; Nübel *et al.*, in Vorbereitung).

4. Bewertung mikrobieller Diversität als quantifizierbare ökologische Größe

Zusammenhänge zwischen der Diversität und der Funktion von ökologischen Systemen werden schon seit längerer Zeit kontrovers diskutiert, empirische Untersuchungen zum Thema sind jedoch selten (Schulze & Mooney, 1993). Während sich mikrobielle Lebensgemeinschaften für derartige Untersuchungen anbieten, da ihre experimentelle Manipulation und die Messung von funktionellen Parametern wie der Produktivität verhältnismäßig einfach durchgeführt werden können, bereitet die quantitative Ermittlung der Diversität von Mikroorganismen erhebliche Schwierigkeiten (Klug & Tiedje, 1993; O'Donnell *et al.*, 1995).

In der vorliegenden Arbeit wird die Quantifizierung der Diversität von Cyanobakterien und Diatomeen in acht mikrobiellen Matten beschrieben. Dazu wurde die morphologische Diversität dieser Organismen mikroskopisch untersucht, die Diversität der Carotinoide konnte nach Extraktion aus Mattenproben und Analyse durch HPLC ermittelt werden und die Diversität der 16S-rRNA-Gene wurde nach Extraktion der DNA, PCR-Amplifizierung und nachfolgender sequenzabhängiger Auftrennung durch DGGE abgeschätzt. Die Übereinstimmung der mit den verschiedenen Methoden erzielten Ergebnisse zeigte, daß eine quantitative Erhebung der relativen Diversität dieser Organismen prinzipiell möglich ist (Publikation 5; Nübel *et al.*, 1999). Daraufhin wurde gefunden, daß die Lebensgemeinschaften mit der höheren Diversität auf Schwankungen der Salinität mit relativ geringeren Änderungen ihrer Produktivität reagierten, also eine höhere funktionelle Stabilität aufwiesen (Publikation 6; Garcia-Pichel *et al.*, in Vorbereitung).

Die Gesamtzahl der in der untersuchten Saline und ihrer Umgebung vorhandenen Morphotypen und 16S-rRNA-Gene von Cyanobakterien und Diatomeen wurde geschätzt, indem die mit zunehmender Probenanzahl kumulativ wachsende detektierte Diversität extrapoliert wurde. Dabei stellte sich heraus, daß die kumulative Zahl der unterschiedlichen rRNA-Gene die Zahl der Morphotypen um mehr als das doppelte übertraf. Dieses Ergebnis lässt vermuten, daß ein Teil der scheinbar weit verbreiteten Morphotypen tatsächlich eine größere Zahl von unterschiedlichen Genotypen verbirgt (Publikation 7; Nübel *et al.*, im Druck).

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Teil 2: Publikationen

A. Erläuterungen zu den Publikationen

Diese Dissertation beruht auf den aufgelisteten Publikationen. Die Erläuterungen zeigen meine Beiträge zu den einzelnen Arbeiten auf.

1. PCR primers to amplify 16S rRNA genes from cyanobacteria

U. Nübel, F. Garcia-Pichel, G. Muyzer

Vorgabe des Themas durch F. Garcia-Pichel und G. Muyzer. Eigenständige Entwicklung des Konzepts. Durchführung aller Versuche. Erstellung des Manuskripts unter redaktioneller Mitwirkung von F. Garcia-Pichel und G. Muyzer

2. The phylogeny of unicellular, extremely halotolerant cyanobacteria

F. Garcia-Pichel, U. Nübel, G. Muyzer

Thema und Konzept von F. Garcia-Pichel. Durchführung der molekularbiologischen Versuche und der phylogenetischen Analysen. Erstellung der Manuskriptteile zu Methoden, Ergebnissen und Diskussion dieser Untersuchungen unter redaktioneller Mitwirkung von F. Garcia-Pichel und G. Muyzer.

3. The halotolerance and phylogeny of cyanobacteria with tightly coiled trichomes (*Spirulina* spp. Turpin)

U. Nübel, F. Garcia-Pichel, G. Muyzer

Eigenständige Entwicklung des Konzepts und Durchführung aller Versuche. Erstellung des Manuskripts unter redaktioneller Mitwirkung von F. Garcia-Pichel.

4. Matching molecular diversity and ecophysiology of benthic cyanobacteria and diatoms in communities along a salinity gradient

U. Nübel, F. Garcia-Pichel, E. Clavero, G. Muyzer

Eigenständige Entwicklung des Konzepts und Durchführung aller Versuche. Daten zur Halotoleranz der Diatomeen von E. Clavero. Erstellung des Manuskripts unter redaktioneller Mitwirkung von F. Garcia-Pichel.

5. Quantifying microbial diversity: morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats

U. Nübel, F. Garcia-Pichel, M. Kühl, G. Muyzer

Vorgabe von Thema und Konzept durch F. Garcia-Pichel. Durchführung der molekularbiologischen Versuche mit technischer Unterstützung durch C. Wawer. Durchführung der mikroskopischen Analysen. Erstellung des Manuskripts unter redaktioneller Mitwirkung von F. Garcia-Pichel, M. Kühl und G. Muyzer.

6. Diversity and functional stability in microbial mats

F. Garcia-Pichel, U. Nübel, M. Kühl, G. Muyzer

Thema und Konzept von F. Garcia-Pichel. Beteiligung an den Versuchen zur Produktivität und redaktionelle Mitwirkung am Manuskript.

7. Spatial scale and the diversity of benthic cyanobacteria and diatoms in a salina

U. Nübel, F. Garcia-Pichel, M. Kühl, G. Muyzer

Eigenständige Entwicklung des Konzepts und Durchführung aller Versuche mit technischer Unterstützung durch C. Wawer. Erstellung des Manuskripts unter redaktioneller Mitwirkung von F. Garcia-Pichel.

B. Publikationen

1

PCR primers to amplify 16S rRNA genes from cyanobacteria

Ulrich Nübel, Ferran Garcia-Pichel, and Gerard Muyzer

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Abstract

We developed and tested a set of oligonucleotide primers for the specific amplification of 16S rRNA gene segments from cyanobacteria and plastids by polymerase chain reaction (PCR). PCR products were recovered from all cultures of cyanobacteria and diatoms checked and none from other bacteria and archaea. Gene segments selectively retrieved from cyanobacteria and diatoms in uni-algal but non-axenic cultures, and from cyanobionts in lichens could be directly sequenced. In the context of growing sequence databases this procedure allows a rapid and phylogenetically meaningful identification without the need for pure cultures or molecular cloning. We demonstrate the use of this specific PCR in combination with denaturing gradient gel electrophoresis to probe the diversity of oxygenic phototrophic microorganisms in cultures, lichens, and complex microbial communities.

Introduction, Results, and Discussion

To understand the ecology of cyanobacteria it is desirable to match isolated strains and their counterparts in nature. Only then can physiological data gained from culture studies begin to be confidently extrapolated to natural conditions (Castenholz & Waterbury, 1989; Garcia-Pichel *et al.*, 1996). Inadequate culture conditions leading to the loss of various morphological characteristics (Castenholz & Waterbury, 1989), our inability to grow certain organisms in the laboratory (Ferris *et al.*, 1996; Ward *et al.*, 1997), and misidentifications of strains held in culture collections (Garcia-Pichel *et al.*, 1996; Wilmotte, 1995), make it difficult in many cases to apply taxonomic assignments based on cultures to field populations. Both classification systems for the cyanobacteria, the bacteriological approach (Castenholz & Waterbury, 1989; Lewin, 1989; Rippka *et al.*, 1979) as well as the traditional botanical approach (Anagnostidis & Komárek, 1985; Geitler, 1932), rely primarily on morphological characteristics of cells and colonies and do not necessarily lead to the identification of phylogenetically coherent taxa (Castenholz, 1992; Wilmotte & Golubic, 1991). At all taxonomic levels above species the sequence analysis of genes encoding small subunit ribosomal RNA (16S rRNA) is currently the most promising approach towards a phylogenetic classification of the cyanobacteria (Wilmotte, 1995). Furthermore, the comparative analysis of 16S rRNA gene sequences provides new means to investigate the discrepancy between strain collections and natural communities (Ferris *et al.*, 1996; Weller *et al.*, 1991). Sequences of 16S rRNA genes are independent from cultivation or growth conditions and using the polymerase chain reaction (PCR) they can be retrieved from small amounts of DNA extracted from laboratory cultures or natural

environments (Giovannoni, 1991). Several different approaches have been described to extend the analysis of 16S rRNA from cyanobacteria beyond that performed on axenic cultures. These include antibiotic treatments to suppress heterotrophic bacteria in non-axenic cultures (Wilmotte *et al.*, 1992), physical cleaning of cyanobacteria by micromanipulation (Garcia-Pichel *et al.*, 1996), and molecular cloning followed by screening for the plasmid inserts of interest (Nelissen *et al.*, 1996; Weller *et al.*, 1991).

On the basis of published 16S rRNA sequences we developed a PCR procedure for the selective retrieval of cyanobacterial rRNA gene fragments from a variety of natural and artificial settings. The combination of this procedure with denaturing gradient gel electrophoresis (DGGE), a technique for sequence dependent separation of DNA molecules (Lerman *et al.*, 1984; Muyzer *et al.*, 1993), proved to be useful to visualize the diversity of cyanobacterial 16S rRNA genes in environmental samples, to detect the uniqueness of isolated strains, and to assign PCR products derived from cultures to populations in the field. PCR products containing a single homogeneous population of DNA molecules are recognized as single bands after DGGE and can be directly sequenced yielding information about approximately 700 nucleotides of the 16S rRNA genes. Sequence data therefore can be generated from cyanobacterial cultures containing heterotrophic bacteria without the need for time-consuming molecular cloning procedures. This allows a rapid survey of a collection of strains for genetic diversity.

Specificity of PCR. Primer design was based on an alignment of all 16S rRNA sequences from cyanobacteria available from the Ribosome Database Project (RDP) (Maidak *et al.*, 1997) and GenBank (Benson *et al.*, 1997). Primer sequences and target regions within the 16S rRNA gene are listed in Table 1. The forward primers CYA106F or CYA358F were used alternatively, whereas the reverse primer, referred to hereafter as CYA781R, is an equimolar mixture of CYA781R(a) and CYA781R(b). 40-nucleotide GC-rich sequences at the 5'-end of the forward primers improved the detection of sequence variations in the amplified DNA fragments by subsequent DGGE (Sheffield *et al.*, 1989). The primers were synthesized commercially (Biometra, Göttingen, Germany).

TABLE 1. Primer sequences and target sites

Primer	Sequence (5' to 3')	Target site ^a
CYA 106 F ^b	CGG ACG GGT GAG TAA CGC GTG A	106-127
CYA 359 F ^b	GGG GAA TYT TCC GCA ATG GG ^c	359-378
CYA 781 R (a) ^d	GAC TAC TGG GGT ATC TAA TCC CAT T	781-805
CYA 781 R (b) ^d	GAC TAC AGG GGT ATC TAA TCC CTT T	781-805

The R (reverse) and F (forward) designations refer to primer orientation in relation to the rRNA. A 40-nucleotide GC-rich sequence (5'-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3') is attached to the 5'-end of the forward primers.

^a *Escherichia coli* numbering of 16S rRNA nucleotides (7).

^b Forward primers CYA106F or CYA359F were used in alternative reactions.

^c Y, a C/T nucleotide degeneracy.

^d The reverse primer CYA781R was an equimolar mixture of CYA781R(a) and (b).

Primer specificities with reference to published sequences were checked using the CHECK_PROBE program supported by the RDP and the BLAST program (Altschul *et al.*, 1990) at the National Center for Biotechnology Information (NCBI) (Washington, D. C.). The primers CYA359F and CYA781R match virtually all 143 16S rRNA sequences from cyanobacteria currently deposited in public databases (February 1997). There is evidence that the apparent mismatches in the few exceptions correspond to sequencing errors because of either one or more of the following reasons: the non-matching nucleotides (i) would disrupt the secondary structures of the corresponding RNA molecules, (ii) affect positions considered to be invariant among prokaryotic small-subunit ribosomal RNA sequences (Van de Peer *et al.*, 1996), (iii) simply are contradictory to sequences determined for the same strains by other researchers. In contrast, CYA106F has one to three mismatches to 16S rRNA genes from many strains of cyanobacteria affiliated to various phylogenetic groups. Therefore this primer is not recommended for the analysis of microbial community composition. However, in this study it was successfully used in combination with CYA781R to specifically generate amplification products from cyanobacteria and diatom plastids from (axenic and non-axenic) uni-algal cultures (Table 2).

Plastids are believed to be an early offshoot of the cyanobacterial evolutionary line (Nelissen *et al.*, 1995). Probably after a single primary endosymbiotic event a near-simultaneous radiation of the ancestors of recent cyanelles, rhodoplasts and chloroplasts occurred, and, subsequently, other plastids evolved through multiple secondary endosymbioses (Bhattacharya & Medlin, 1995). Published 16S rRNA sequences from cyanelles match primers CYA106F, CYA359F, and CYA781F. Among the other two main plastid lineages 16S rRNA sequences exist which have one or more mismatches to the cyanobacterial primers. On the basis of the limited dataset currently available a more detailed evaluation of primer specificity for phylogenetic groups of

plastids is not yet possible. Therefore, the applicability of the PCR reported here for the analysis of eukaryotic phototrophs must await the advent of new 16S rRNA sequences from plastids.

TABLE 2. Strains used in this study

Strain	Taxonomy/phylogeny ^a	Source ^b	Axenic	Amplification ^c
Cyanobacteria				
<i>Cyanothece</i> PCC 7418	<i>Chroococcales</i>	PCC	+	+
<i>Cyanothece</i> PCC 7424	<i>Chroococcales</i>	PCC	+	+
<i>Synechococcus leopoliensis</i> SAG 1402-1	<i>Chroococcales</i>	SAG	+	+
<i>Dactylococcopsis</i> PCC 8305	<i>Chroococcales</i>	PCC	+	+
<i>Cyanothece</i> MPI 95 AH 10	<i>Chroococcales</i>	MPI	+	+
<i>Cyanothece</i> MPI 95 AH 13	<i>Chroococcales</i>	MPI	-	+
<i>Cyanothece</i> MPI 96 P4 02	<i>Chroococcales</i>	MPI	-	+
<i>Aphanothece halophytica</i> ATCC 43922	<i>Chroococcales</i>	ATCC	-	+
<i>Synechococcus</i> C 1	<i>Chroococcales</i>	Y. C.	+	+
<i>Gloeoecapsa</i> C-90-Cal-G. (2)	<i>Chroococcales</i>	MPI	-	+
<i>Gloeothece</i> sp. HW-91-Gth. (2)	<i>Chroococcales</i>	MPI	-	+
<i>Gloeothece rupestris</i> SAG 36.87	<i>Chroococcales</i>	SAG	+	+
<i>Lyngbya</i> PCC 7419	<i>Oscillatoriales</i>	PCC	+	+
<i>Microcoleus chthonoplastes</i> MPI-NDN-1	<i>Oscillatoriales</i>	MPI	-	+
" <i>Microcoleus</i> " 10 mfx (PCC 9452)	<i>Oscillatoriales</i>	MPI	-	+
<i>Geitlerinema</i> SAG 3192	<i>Oscillatoriales</i>	SAG	-	+
<i>Oscillatoria</i> MPI 95 OS 01	<i>Oscillatoriales</i>	MPI	-	+
" <i>Oscillatoria limnetica</i> "	<i>Oscillatoriales</i>	Y. C.	-	+
<i>Spirulina</i> MPI 95 SS 01	<i>Oscillatoriales</i>	MPI	-	+
<i>Spirulina</i> MPI 95 SL 01	<i>Oscillatoriales</i>	MPI	-	+
<i>Scytonema</i> B-77-Scy jav.	<i>Nostocales</i>	MPI	-	+
<i>Calothrix</i> Y-89-Cpwt.03	<i>Nostocales</i>	MPI	-	+
<i>Aphanizomenon flos-aquae</i> CCAP 1401/1	<i>Nostocales</i>	P. K. H.	-	+
Plastids				
<i>Amphora coffeaformis</i> MPI 96 P2 01	<i>Bacillariophyceae</i> (diatom)	MPI	-	+
<i>Nitzschia</i> sp. MPI 96 P2 05	<i>Bacillariophyceae</i> (diatom)	MPI	-	+
Other bacteria				
<i>Paracoccus denitrificans</i> DSM 65	Proteobacteria, α -subdivision	DSMZ	+	-
<i>Agrobacterium tumefaciens</i> DSM 30205	Proteobacteria, α -subdivision	DSMZ	+	-
<i>Comamonas testosteroni</i> DSM 50244	Proteobacteria, β -subdivision	DSMZ	+	-
<i>Escherichia coli</i> DSM 489	Proteobacteria, γ -subdivision	DSMZ	+	-
<i>Thiomicrospira</i> sp. MA 2-6	Proteobacteria, γ -subdivision	H. J.	+	-
<i>Desulfobacterium vacuolatum</i>	Proteobacteria, δ -subdivision	DSMZ	+	-
<i>Clostridium acetobutylicum</i> DSM 792	gram positive, low G+C	DSMZ	+	-
<i>Paenibacillus polymyxa</i> DSM 36	gram positive, low G+C	DSMZ	+	-
<i>Pectinatus frisingensis</i> DSM 6306	gram positive, low G+C	DSMZ	+	-
<i>Rhodococcus rhodochrous</i> DSM 43241	gram positive, high G+C	DSMZ	+	-
<i>Cytophaga johnsonae</i> DSM 2064	Cytophaga group	DSMZ	+	-
Archaea				
<i>Sulfolobus shibatae</i> DSM 5389	<i>Sulfolobales</i>	DSMZ	+	-

^ataxonomic affiliations of cyanobacteria *sensu* Castenholz and Waterbury 1989 (Castenholz & Waterbury, 1989).

^bculture collections: ATCC, American Type Culture Collection, Rockville, Maryland. CCAP, Culture Collection of Algae and Protozoa, Ambleside, UK. DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. H. J., H. Jannasch, Woods Hole Oceanographic Institution, Massachusetts. MPI, Max Planck Institute for Marine Microbiology, Bremen, Germany. P. K. H., P. K. Hayes, University of Bristol, UK. PCC, Pasteur Culture Collection, Paris, France. SAG, Sammlung von Algenkulturen, Göttingen, Germany. Y. C., Y. Cohen, University of Jerusalem, Israel.

^camplification products yielded applying both primer combinations CYA106F/CYA781R and CYA359F/CYA781R.

All primers presented here match some 16S rRNA sequences from bacteria not affiliated to the phylum of cyanobacteria. However, none of these sequences has less than two mismatches total to CYA359F and CYA781R. Thus, the combined use of both primers results in a PCR highly specific for cyanobacteria. CYA359F matches the 16S rRNA sequences from a number of Gram-positive bacteria with a low G+C content of the genomic DNA, namely *Heliobacterium* spp., several anaerobic halophiles, and relatives of *Desulfotomaculum* and *Syntrophomonas*. Information about the target site for reverse priming is also available for all these strains; most of them have four, some have two mismatches to CYA781R. An example for the latter is *Pectinatus frisingensis* DSM 6306^T, which we included in the experimental study of PCR specificity for this reason (Table 2). It could be safely discriminated against when PCR conditions were used as described below. On the other hand, CYA781R is complementary to the 16S rRNAs from several Crenarchaeota affiliated to the order *Sulfolobales*. However, these rRNA sequences have either ten or seven mismatches to CYA359F. *Sulfolobus shibatae* DSM 5389^T (seven mismatches to CYA359F) has been included in the experimental study of PCR specificity (Table 2). The alternative forward primer CYA106F matches a number of published 16S rRNA sequences from prokaryotes with various phylogenetic affiliations outside the phylum of the cyanobacteria, not all of which contain information about the target site for reverse priming. Therefore, and in addition to the reasons discussed above, its applicability is limited, and it should not be used for studies on environmental nucleic acids of unknown compositions. Yet, it has been included in the present report because it proved useful in the generation of sequence data from uni-algal cultures. In these cases the amplification specificity was checked by DGGE to investigate the sequence homogeneity of PCR products prior to sequence analysis. Despite its limitations CYA106F might be preferred because compared to CYA359F its use generates longer amplification products (approximately 700 basepairs instead of 450), therefore enabling the determination of more informative sequence data.

PCR amplifications were performed with a Techne Cyclogene Temperature Cycler (Techne, Cambridge, UK). 50 pmol of each primer, 25 nmol of each deoxynucleoside triphosphate, 200 µg of bovine serum albumine (Romanowski *et al.*, 1993), 10 µl of 10× PCR buffer (100 mM Tris-HCl [pH 9.0], 15 mM MgCl₂, 500 mM KCl, 1% Triton X-100, 0.1% [wt/vol] gelatin), and 10 ng of template DNA were combined with H₂O to a volume of 100 µl in a 0.5-ml test tube and overlaid with two drops of mineral oil (Sigma Chemicals Co., Ltd.). To minimize nonspecific annealing of the primers to nontarget DNA, 0.5 U of SuperTaq DNA polymerase (HT Biotechnology, Ltd., Cambridge, UK) were added to the reaction mixture after the initial denaturation step (5 min at 94°C), at a temperature of 80°C. 35 incubation cycles followed, each consisting of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The annealing temperature for PCR was optimized empirically by performing PCRs with a non-degenerate reverse primer [50

pmol/100 μ l of CYA781R(a) instead of an equimolar mixture of CYA781R(a) and CYA781R(b)] and containing amplification products of the complete 16S rRNA genes (Muyzer *et al.*, 1995) from various cyanobacteria with known sequences as templates. Using an annealing temperature of 60°C no amplification product was generated from templates with two mismatches to the reverse primer as determined by agarose gel electrophoresis. Applying the degenerate reverse primer in combination with either one of the forward primers, gene fragments from chromosomal DNAs were amplified from all cyanobacteria checked, spanning a broad range of cyanobacterial phylogeny as known today, whereas other bacteria and archaea (Table 2) were discriminated against.

Non-axenic cultures. The purification of cyanobacteria can be a difficult and time-consuming procedure, and often they are cultivated more easily when accompanied by heterotrophic bacteria (Castenholz & Waterbury, 1989; Fitzsimons & Smith, 1984). Therefore some strains growing as uni-cyanobacterial but non-axenic cultures have been included in the bacteriological classification system (Castenholz & Waterbury, 1989). If conventional primers are used, targeting on highly conserved regions of 16S rRNA genes, PCR yields a heterogeneous mixture of amplification products from such cultures (Fig 1C, lane 6), the direct sequence analysis of which is not possible. Prior to sequence determination different DNA molecules need to be separated by cloning, and the clone library has to be screened for the plasmid inserts of interest by multiple partial sequencing reactions (Nelissen *et al.*, 1996). In contrast, the PCR reported here has been used to amplify cyanobacterial 16S rRNA gene fragments exclusively (nucleotide positions 106 to 805; *E. coli* numbering system; (Brosius *et al.*, 1981) and thereby retrieve them from impure cultures. 20 to 100 μ g of cells from cultures of cyanobacteria were lysed by three sequential freezing (in liquid N₂) and thawing (at 65°C) steps followed by incubation for 20 min at 50°C in 5 ml of TESC buffer (100 mM Tris-HCl [pH 8], 100 mM EDTA, 1.5 M NaCl, 1% (wt/vol) hexadecylmethylammonium bromide) containing Proteinase K at 100 μ g/ml and 1% (wt/vol) sodium dodecyl sulfate (SDS). Chromosomal DNAs were extracted applying phenol, chloroform, and isoamylalcohol (Wilson, 1990) before they were used as templates in PCR. DGGE was performed as described previously (Muyzer *et al.*, 1996) with the following modifications: polyacrylamide gels of 1 mm thickness with a denaturant gradient from 20% to 60% were used, and electrophoresis was run in 1 \times TAE for 3.5h at 200V. DGGE analysis detects sequence differences among the PCR products and therefore allows the identification of unique strains in a collection, which then may be selected for more detailed studies. Furthermore, interoperon sequence heterogeneities of 16S rRNA genes within single genomes potentially leading to ambiguities in the sequence data can be detected (Nübel *et al.*, 1996). In this study the amplification products confirmed by DGGE

to contain a single sequence were sequenced directly. After purification by using the QIAquick PCR purification kit (Diagen, Düsseldorf, Germany) PCR products were used as templates in sequencing reactions applying the Applied Biosystems PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit supplied with AmpliTaq DNA polymerase. Sequences of both DNA strands were determined by using the same primers as for amplification. Products of sequencing reactions were analysed by using an Applied Biosystems 377 DNA sequencer. Many fragments were analysed yielding cyanobacterial sequences in all cases. Sequence data generated for the non-axenic strains "*Oscillatoria limnetica*", *Geitlerinema* PCC 9452 ("*Microcoleus*" 10 mfx), and *Gloeotheca rupestris* SAG 36.87, and for the plastids from *Amphora coffeaformis* MPI 96P201 and *Nitzschia* sp. MPI 96P205 were deposited in GenBank. Whereas the use of complete 16S rRNA gene sequences is recommended for reliable phylogeny reconstruction, information about shorter gene segments usually is sufficient for the identification of bacteria (Stackebrandt & Rainey, 1995).

Symbiotic cyanobacteria in lichens. Cyanobacteria belonging to the morphologically defined genera *Calothrix*, *Chroococcidiopsis*, *Chroococcus*, *Cyanosarcina*, *Dichothrix*, *Entophysalis*, *Gloeocapsa*, *Hyella*, *Hyphomorpha*, *Myxosarcina*, *Nostoc*, *Scytonema*, and *Stigonema* are all found in lichens as primary or secondary phototrophic symbionts (Buedel, 1992). However, with some exceptions, the identification of the cyanobacterial photobionts in intact lichen thalli is impossible because their morphology is modified by interactions with the fungal hyphae and because only some stages of the life-cycle may be present. Thus, cultivation is a necessary, although not necessarily sufficient, requirement for positive identification (Friedl & Buedel, 1996). We applied our methodology for selective amplification of 16S rRNA gene segments to whole thalli of the cyanobacterial lichens *Collema cf. coccophorum* and *Peltula lingulata*. After cleaning the thalli surface by flushing with distilled water under a dissecting microscope, small pieces (approximately 2 mm³) were transferred to 10 µl of TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA) in 1.5 ml microcentrifuge tubes and gently ground with a pipette tip to release the cyanobacterial cells into suspension. A DNA extraction step was not necessary, and the suspensions (1 µl) could be directly used as templates for PCR. DGGE analysis of the PCR products obtained (Fig. 1D) showed that only one sequence was amplified from each lichen and that they were different. These results are consistent with the expected identity of the cyanobionts in *C. cf. coccophorum* (*Nostoc* sp.) and *P. lingulata* (*Chroococcidiopsis* sp.). The combination of selective 16S rRNA gene amplification, DGGE, and sequence analysis should provide a cultivation-independent, powerful tool for the reliable identification of cyanobacterial symbionts at large.

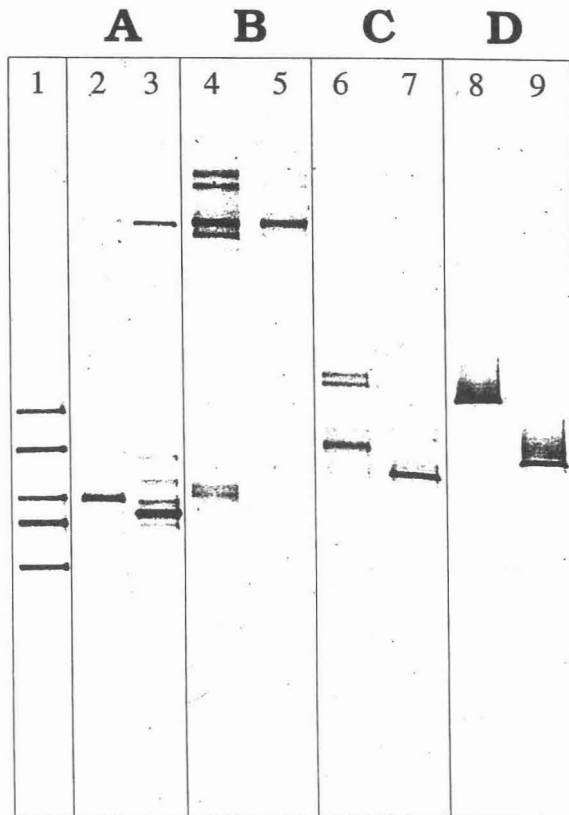


FIGURE 1. Composite figure of ethidium-bromide stained DGGE separation patterns of PCR-amplified segments of 16S rRNA genes. A mixture of PCR products derived from five cyanobacterial strains was applied on each gel as a standard to enable comparisons (lane 1, top to bottom: *Scytonema* B-77-Scyjav., *Synechococcus leopoliensis* SAG 1402-1, *Microcoleus chthonoplastes* MPI-NDN-1, *Geitlerinema* PCC 9452 [*Microcoleus* 10 mfx], *Cyanothece* PCC 7418). Primers CYA359F and CYA781R were used for amplification. A: PCR products derived from microbial mats P4 (lane 2) and NC20 (lane 3), which had been sampled from environments of different salinities (9 ‰ and 5-30 ‰, respectively). Primers CYA359F and CYA781R were used for amplification. B: PCR products derived from marine plankton, which had been sampled from a seawater mesocosm after incubation for 65 (lane 4) and 281 h (lane 5). Primers CYA359F and CYA781R were used for amplification. C: PCR products derived from a non-axenic culture of *Gloeotheca rupestris* SAG 36.87. The use of primers complementary to gene stretches highly conserved among bacteria (Teske *et al.*, 1996) yields amplification products heterogenous in sequence (lane 6), whereas the primers CYA106F and CYA781R enable the selective amplification of the cyanobacterial gene segment (lane 7). D: PCR products derived from photobionts from the lichens *Collema cf. coccophorum* (lane 8) and *Peltula lingulata* (lane 9). Primers CYA106F and CYA781R were used for amplification.

Oxygenic phototrophs in complex microbial communities. Benthic microbial mats growing in evaporation ponds of the Exportadora de Sal saltworks in Guerrero Negro, Baja California Sur, Mexico, and marine plankton samples from a mesocosm study in the Western Mediterranean were investigated. Mat core samples (diameter 25 mm) were frozen on site, transported to the laboratory in liquid nitrogen, and stored at -70°C until further use. Prior to the extraction of nucleic acids the photosynthetically active layer as determined by oxygen microelectrode measurements (Revsbech & Jørgensen, 1986) (data not shown) was aseptically cut from mat cores (300 mg) and homogenized in a Dounce tissue homogenizer (Novodirect, Kehl). Cell lysis was performed as described above and controlled by light-microscopic observation. Mesocosm water samples containing 2×10^8 bacterial cells were filtered through Durapore GVWP 02500 filters (pore size $0.22 \mu\text{m}$; filter diameter 25 mm; Millipore). Nucleic acids were extracted from microorganisms retained on the filters applying the protocol described by Teske *et al.* (1996).

As shown in figures 1A and 1B, DGGE analysis of PCR amplification products visualizes the genetic diversity of cyanobacteria and plastids as reflected in 16S rRNA gene sequences. Similarities, differences, and successions in space and time of the composition of oxygenic phototrophic microbial communities can be observed by comparing different PCR products. More information can be obtained from these band patterns by sequence analysis of PCR products from single bands after elution from the gel and reamplification (Ferris *et al.*, 1996; Muyzer *et al.*, 1996). DNA bands may also be identified by hybridization analysis using suitable nucleic acid probes if available (Muyzer *et al.*, 1996). PCR products derived from cultivated strains may be assigned to bands of the complex pattern visible at the same height in the DGGE gel; although equal mobility of DNA molecules does not prove their sequence identity this information can support comparisons of cultures and field samples. For example compare gel lanes 1 and 2 in Figure 1: the most intense band in the pattern derived from the microbial mat P4 (lane 2) corresponds to the band derived from the cultured strain MPI-NDN-1 (lane 1). This result is consistent with the microscopic observation of filaments morphotypically corresponding to *Microcoleus chthonoplastes* (Garcia-Pichel *et al.*, 1996), which dominate this mat.

Concluding remarks. In general, rRNA genes are considered to be more conserved in function and structure than protein-coding genes and thus the genetic diversity measured might not sufficiently reflect the physiological diversity of the respective organisms (Fox *et al.*, 1992; Ward *et al.*, 1994). On the other hand sequence heterogeneities due to multiple copies of *rrn* operons within single genomes of bacteria may complicate the interpretation of sequence data or DGGE band patterns particularly when retrieved from natural microbial communities. The detection of slightly different 16S rRNA gene sequences is not sufficient to prove the presence

of different bacterial populations in an environmental sample (Nübel *et al.*, 1996). The *rpoC1* gene, encoding the γ subunit of the RNA polymerase, has been described as an alternative target to be analysed to reveal cyanobacterial phylogeny (Bergsland & Haselkorn, 1991) and community structure (Palenik, 1994). However, the amount of sequence data available for these genes is rather limited, whereas the determination of 16S rRNA gene sequences is a routine procedure in prokaryotic taxonomy today, resulting in large and steadily growing databases, which improve the robustness of phylogeny reconstructions, identification results, and primer specificity evaluations. Other molecular biological approaches which have been described for the identification of cyanobacteria are applicable exclusively to axenic cultures. These include multiplex RAPD analysis (Neilan, 1995) and the sequence analysis of internal transcribed spacer regions of ribosomal RNA operons (Wilmotte, 1995). Some other approaches apply only to certain groups of cyanobacteria. The latter include the analysis of genes encoding phycocyanin (Neilan *et al.*, 1995; published primers were reported to yield no PCR products with some of the strains checked) or the nitrogenase (Ben-Porath & Zehr, 1994) and the detection of a repetitive DNA sequence in toxin-producing heterocystous cyanobacteria (Rouhiainen *et al.*, 1995). In part reflecting the wide application of PCR in the field of microbial ecology several potential pitfalls of this technique have been described, such as the formation of chimera molecules (Liesack *et al.*, 1991) or the amplification of template molecules with differential efficiencies in spite of identical priming sites (Reysenbach *et al.*, 1992; Suzuki & Giovannoni, 1996). It has to be considered that the primers used in microbial ecology always are designed on the basis of limited sets of data; future research might reveal sequences from target organisms which do not contain the signatures necessary for efficient amplification. However, the approach described here is a powerful tool to investigate the phylogenetic diversity of cyanobacteria and its ecological significance. It should prove to be especially useful to unravel the connections between cyanobacterial populations observed in nature, molecular sequence data, and physiology.

Nucleotide sequence accession numbers. Accession numbers U96442 to U96446 were assigned to the 16S rRNA gene sequences determined for *Geitlerinema* PCC 9452 ("*Microcoleus*" sp. 10 mfx), "*Oscillatoria limnetica*", *Gloeotheca rupestris* SAG 36.87, and the plastids from *Amphora coffeaformis* MPI 96P201 and *Nitzschia* sp. MPI 96P205.

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The phylogeny of unicellular, extremely halotolerant cyanobacteria

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Abstract

We examined the morphology, physiology, and 16S rRNA gene sequences of three culture collection strains and of ten novel isolates of unicellular cyanobacteria from hypersaline environments. The strains were morphologically diverse, with average cell widths ranging from 2.8 to 10.3 μm . There were single-celled, colonial, and baeocyte-forming strains. However, morphological traits were markedly variable with culture conditions. In contrast, all strains displayed extreme halotolerance (growing close to optimally at above 15% salinity); all were obligately marine, euryhaline, and moderately thermophilic; and all shared a suite of chemotaxonomic markers including phycobilins, carotenoids, and mycosporine-like amino acids. 16S rRNA gene sequence analysis indicated that the strains were related to each other. Sequence similarity analysis placed the strains in a monophyletic cluster (which we named the Halothece cluster) apart from all cultured or uncultured, not extremely halotolerant cyanobacteria whose 16S rRNA gene sequences are available in public nucleotide sequence databases. This represents the first case in which a phylogenetically coherent group of cyanobacteria can be defined on the basis of physiology. The Halothece cluster contained two sub-clusters that may be divergent at the generic level, one encompassing 12 strains (spanning 5% 16S rRNA gene sequence divergence and named the Euhalothece subcluster), and a single deep-branching isolate. Phenotypic characterization of the isolates, including morphological, physiological, and chemotaxonomic traits, did not distinguish these subclusters and only weakly suggested the existence of two separate clades, one encompassing strains of small cell size (cell width $< 5 \mu\text{m}$) and another one encompassing strains of larger cell size.

Introduction

Cyanobacteria are prominent primary producers in hypersaline environments, especially in the benthos (Javor, 1989). These environments harbor a large array of unicellular and filamentous forms, although the morphological diversity at salinities above 13% is restricted. At high salinities, unicellular forms become comparatively important: hypersaline waters are usually dominated by unicellular cyanobacteria, and the most halotolerant cyanobacteria in culture are unicellular (Berland *et al.*, 1989; Brock, 1976; Dor & Hornhoff, 1985; Javor, 1989; MacKay *et al.*, 1984; Walsby *et al.*, 1983; Yopp *et al.*, 1978).

In bacteriology it is common to describe new species or genera on the basis of halophily alone. This approach is based implicitly on an evolutionary standpoint that regards it unlikely that halophilic or extremely halotolerant strains are closely related to their freshwater or even marine morphological counterparts, given that the range of adaptations needed for growth at high salinities is large [e.g., see (Galinski, 1995)] and requires long evolutionary divergence. In the cyanobacteria, however, morphological distinctions form the basis of taxonomic differentiation. The concomitant use of the bacteriological and botanical taxonomic approaches and the lack of a sound taxonomic treatment has resulted in long-lasting controversies about the proper treatment of unicellular halotolerant cyanobacteria [see (Roussomoustakaki & Anagnostidis, 1991) for a comprehensive review]. Most studies on cultured strains and most descriptions of field populations assign these cyanobacteria to the botanical species *Aphanothece halophytica* (Hof & Frémy, 1933) or to the genus *Cyanothece sensu* Rippka *et al.* (Rippka *et al.*, 1979) depending on the preferences of the authors. More rarely, they have been placed under the epithet *Coccochloris elabens* (Kao *et al.*, 1973) or assigned to the genus *Synechococcus* (Imhoff *et al.*, 1978). At least in one case, clear-cut morphological traits allowed the description of a new species placed within the genus *Dactylococcopsis* (Walsby *et al.*, 1983).

On the other hand, newer methods of phylogenetic inference such as 16S rRNA (gene) sequence analysis have shown that morphological characters may or may not result in a phylogenetically reliable taxonomy among cyanobacteria (reviewed by Wilmotte [1994]). Probably the simpler the morphology with which one deals, the more uncertain the systematics become (Garcia-Pichel *et al.*, 1996). The thermophilic unicellular cyanobacteria, which can be assigned in principle to the single morphospecies *Synechococcus lividus*, for example, have been shown to encompass a polyphyletic, diverse array of cyanobacterial sequences dissimilar among themselves (Ward *et al.*, 1994) and only distantly related to, for example, the marine *Synechococcus/Prochlorococcus* cluster. Thus, the true biodiversity within the extremely halotolerant unicellular cyanobacteria remains unknown.

In this contribution, we analyze 13 strains of unicellular cyanobacteria isolated from hypersaline environments. Three strains correspond to well-known standard strains from culture collections, while the rest are novel isolates that encompass a variety of morphologies. We present results on their morphology and on their growth responses to salt and temperature, a phylogenetic reconstruction analysis based on 16S rRNA gene sequences, and a comparison of chemotaxonomic markers such as phycobiliproteins, carotenoids, and mycosporine-like amino acids in an attempt to clarify their systematics.

Materials and methods

Strains and strain histories. The denomination of the strains used is given together with the taxonomic assignment under which they can be found in the respective collection (PCC: Pasteur Culture Collection of Cyanobacteria; ATCC: American Type Culture Collection; MPI: Max-Planck-Institute for Marine Microbiology). Relevant information on origin, status, and original taxonomic assignments is also included.

PCC 7418, *Cyanothece*. Axenic. Isolated by Y. Cohen from the plankton of Solar Lake, Sinai, Egypt (Cohen *et al.*, 1975) as *Aphanothece halophytica*. Subject of various physiological studies (Belkin & Padan, 1978; Garlick *et al.*, 1977; MacKay *et al.*, 1984; Moore *et al.*, 1987).

PCC 8305, *Dactylococcopsis*. Axenic. Isolated by A. E. Walsby from the plankton of Solar Lake, Sinai, Egypt (Walsby *et al.*, 1983) as *Dactylococcopsis salina* sp. nov.

ATCC 43922, *Aphanothece halophytica*. Unicyanobacterial. Yopp's strain. Isolated from the mud bottom of solar evaporation ponds of the Leslie Salt Co., San Francisco, California, USA, as *Aphanothece halophytica* (Yopp *et al.*, 1978). Subject of many physiological studies (Miller *et al.*, 1976; Ritter & Yopp, 1993; Sibley & Yopp, 1987; Tindall *et al.*, 1978; Tindall *et al.*, 1977; Yopp *et al.*, 1979).

MPI 95AH10, *Cyanothece*. Axenic. Isolated during this work from benthic gypsum crusts in solar evaporation ponds in Eilat, Israel, by dilution to extinction and plating. Deposited in the Pasteur Culture Collection as PCC 9712.

MPI 95AH11. Unicyanobacterial. Isolated during this work from benthic gypsum crusts in solar evaporation ponds in Eilat, Israel, by dilution to extinction and plating.

MPI 95AH13. Unicyanobacterial. Isolated during this work from benthic gypsum crusts in solar evaporation ponds in Eilat, Israel, by direct plating.

MPI 96P402. Axenic. Isolated during this work from benthic cyanobacterial mats in the solar evaporation ponds of Exportadora de Sal, Guerrero Negro, Baja California Sur, Mexico, by spray-plating.

MPI 96P605. Unicyanobacterial. Isolated during this work from benthic cyanobacterial mats in the solar evaporation ponds of Exportadora de Sal, Guerrero Negro, Baja California Sur, Mexico, by direct plating (spray-plating).

MPI 96AL03. Unicyanobacterial. Isolated during this work from benthic gypsum crusts in the solar evaporation ponds of Salinas del Cabo de Gata, Almeria, Spain, by dilution to extinction and plating.

MPI 96AL06. Unicyanobacterial. Isolated during this work from benthic gypsum crusts in the solar evaporation ponds of Salinas del Cabo de Gata, Almeria, Spain, by dilution to extinction and plating.

SYN CI P22. *Synechococcus*. Axenic. Isolated by Y. Cohen from benthic microbial mats on Christmas Island. Kept under the given designation at our institute.

MPI 96N303. Unicyanobacterial. Isolated during this work from a hypersaline pool in an upper tidal channel, Laguna Ojo de Liebre, Guerrero Negro, Baja California Sur, Mexico, by direct plating.

MPI 96N304. Unicyanobacterial. Isolated during this work from a hypersaline pool in an upper tidal channel, Laguna Ojo de Liebre, Guerrero Negro, Baja California Sur, Mexico, by direct plating.

Growth media, isolation, and culture. Freshwater medium was BG11 (Rippka, 1988). Seawater and hypersaline medium were prepared by dissolving appropriate amounts of commercial seawater salts mixture 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, hereafter designated PES/2. The mixture was acidified with HCl to pH 3 and was bubbled overnight with air in order to drive excess CO₂ out of the 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. Solid media were prepared using agarose to 1% (w/v) before autoclaving. Petri dishes were sealed with parafilm to prevent evaporation. Several procedures were employed simultaneously for isolation. Samples from hypersaline environments containing unicellular cyanobacteria were either plated directly on hypersaline [12% (w/v) total salts or higher] PES/2 1.5% (w/v) agar plates or were dispersed in a small amount of sterile seawater brine to a fine suspension that was then used to inoculate dilution series in PES/2 hypersaline medium. High-dilution tubes showing growth were plated thereafter. Similar suspensions were used to feed a mouth-operated artist's spray from which agar plates were inoculated by passing them briefly before the spray. Strains were considered clonal after single-colony picking at least twice. The final strains chosen for the study were selected so as to span a range of different morphotypes.

Measurements of instantaneous growth rate (μ). Cells were grown in thermostated incubators at the desired temperature and received 50–60 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ of white light from fluorescent tubes during 12 h daily. Growth rate measurements were carried out in deep Petri dishes filled with liquid medium by noninvasively monitoring the increase of bulk phycobilin/chlorophyll *a* fluorescence in the cultures with respect to time. For each strain, the correspondence between fluorescence estimates of biomass and direct biomass measurements (as dry weight) was checked at least once ($R^2 > 0.87$; data not shown). Fluorescence was measured with the aid of a fluorimeter especially designed for use with cultures that do not form homogeneous suspensions (Karsten *et al.*, 1996); the measurements were always taken during the same period of the light/dark cycle only on diluted cultures in order to avoid the progressive decrease in growth rate that sets in with self-shading and the strong decrease in fluorescence per cell typical of nutrient-limited cultures. Typically, growth was followed in triplicate cultures for

each condition during periods of 1–4 weeks, so that at least four doublings were reached in each culture; growth was exponential. Under some conditions, growth was steady but very slow. In these cases (strain MPI 95AH13 at 3% salt, 38°C; strain MPI 95AH10 at 3% salt; strain PCC 8305 at 20% salt, 25°C), growth was followed for a maximum period of 7 weeks and correspond to 1–1.5 doublings; both exponential and arithmetic growth models fit the data. Linear regression analysis of the ln-transformed fluorescence values yielded the single estimates of μ (with $R^2 > 0.83$) in all cases. The average value of the three independent μ estimates (and the standard deviation) is presented here.

Determination of carotenoid and mycosporine-like complement. Carotenoid complement was determined by HPLC separation and on-line ultraviolet/visible spectroscopy. Vacuum-dried samples were extracted in acetone. Carotenoids were identified by spectroscopic matching and by cochromatography with authentic primary or secondary standards. Details on extraction, chromatographic conditions for separation, identification, and quantification, as well as a list and sources of standards have been published elsewhere (Karsten & Garcia-Pichel, 1996). Mycosporine-like amino acid compounds (MAAs) were also determined by HPLC coupled to on-line UV spectroscopy after extraction in warm, aqueous 20% (v/v) methanol according to Garcia-Pichel and Castenholz (Garcia-Pichel *et al.*, 1993). Cultures were grown either under visible light only or under visible light supplemented with 4 h of UV-B radiation (0.6–0.8 W m⁻²) per day to check for UV-inducibility of MAA synthesis. Identification of particular MAAs was achieved by spectroscopic matching and co-chromatography with a set of primary standards [a generous gift from D. Karentz (University of San Francisco, Calif., USA) and J. Dunlap (University of Sydney, Australia)].

Miscellaneous procedures. Phycobiliproteins were extracted after breaking the cells by osmotic downshift in a 30 mM citrate buffer (pH 5) without added salts. Extracts were clarified by precipitation with streptomycin sulfate [1% (w/v) final concentration], and the presence or absence of typical absorption or fluorescence corresponding to either phycoerythrin, phycoerythrocyanin, or phycocyanin was determined. Cell morphometry was measured either on projections of photomicrographs or directly on the screen of a video microscope for 50–100 cells per treatment.

PCR amplification and cloning of 16S rRNA genes. Cells were harvested from 0.5 ml of hypersaline cultures by centrifugation and were suspended in 50 μ l TE buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. These suspensions could be used directly as templates

for amplification of 16S rRNA genes by polymerase chain reaction (PCR) using a Techne Cyclogene Temperature Cycler (Techne, Cambridge, UK). Nearly complete genes were amplified by using primers 8F (Buchholz-Cleven *et al.* 1997) and 1528R (5'-AAA GGA GGT GAT CCA-3'). Fifty picomoles of each primer, 25 nmol of each deoxynucleoside triphosphate, 200 µg of bovine serum albumin, 10 µl of 10 × PCR buffer [100 mM Tris-HCl (pH 9.0), 15 mM MgCl₂, 500 mM KCl, 1% (v/v) Triton X-100, and 0.1% (w/v) gelatin], and 1 µl cell suspension were combined with H₂O to a volume of 100 µl in a 0.5-ml test tube and were overlaid with two drops of mineral oil (Sigma). To minimize nonspecific annealing of the primers to nontarget DNA, 0.5 U of SuperTaq DNA polymerase (HT Biotechnology, Cambridge, UK) was added to the reaction mixture at a temperature of 80°C after the initial denaturation step (5 min at 94°C). Thirtyfive incubation cycles followed, each consisting of 1 min at 94°C, 1 min at 46°C, and 3 min at 72°C. A final incubation for 10 min at 72°C allowed extension of incomplete products. Amplification products were analyzed by electrophoresis in 1.5% (w/v) agarose gels and were stored at -20°C until they were used.

The primers 8F and 1528R are complementary to stretches in 16S rRNA genes highly conserved among bacteria. Therefore, their use on unicyanobacterial cultures that contain heterotrophic bacteria yields a heterogeneous mixture of amplification products (Nübel *et al.* 1997). To determine almost complete sequences of 16S rRNA genes from such cultures, PCR products were purified applying the QIAquick PCR Purification Kit and were cloned applying the pGEM-T plasmid vector system and competent *Escherichia coli* JM109 cells (Promega, Heidelberg, Germany) in accordance with the manufacturers' instructions. Plasmid DNA was prepared from 20 to 30 clones using the Wizard Plus Minipreps Kit (Promega). To screen for cyanobacterial 16S rRNA gene inserts, each plasmid DNA sample (1 ng) was used as template in a PCR reaction using the primers CYA359F and CYA781R. These primers enable specific amplification of 16S rRNA gene sequences from cyanobacteria (Nübel *et al.*, 1997) and thus allowed the detection of plasmids containing the inserts of interest. The PCR conditions used were as described above except that 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C were performed. Subsequently, full-length cyanobacterial 16S rRNA genes from chosen plasmid inserts were amplified using the primers 8F and 1528R. To avoid errors due to PCR artefacts or operon micro-heterogeneities, PCR products derived from ten different plasmids were mixed and processed for sequencing. However, for most of the unicyanobacterial nonaxenic strains, only partial sequences (approximately 560 nucleotides) were determined. Using the primers CYA106F and CYA781R, 16S rRNA gene fragments were specifically retrieved by PCR from cyanobacteria in such cultures (35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C). PCR products were checked for sequence homogeneity by

denaturing gradient gel electrophoresis analysis prior to purification and sequence analysis (Nübel *et al.*, 1997).

Sequence analysis. PCR products were purified by applying the QIAquick PCR Purification Kit (Qiagen, Düsseldorf, Germany) and were subsequently used as templates in sequencing reactions with the Applied Biosystems PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit supplied with AmpliTaq DNA polymerase. Both DNA strands of the amplification products of complete 16S rRNA genes were sequenced using the primers 8F, 1099F, 1175R (Buchholz-Cleven *et al.*, 1996), CYA359F, CYA781R (Nübel *et al.*, 1997), 341R (5'-CTG CTG CCT CCC GTA GG-3'), and 1528R. PCR products generated by using the primers CYA106F and CYA781R were sequenced using the same primers as for amplification (Nübel *et al.*, 1997). Primer designations refer to 5'-ends of the respective target sites in the 16S rRNA genes (*E. coli* numbering of 16S rRNA nucleotides; Brosius *et al.*, 1981) and to forward (F) or reverse (R) orientation relative to that of the rRNA. Products of sequencing reactions were analyzed on an Applied Biosystems 377 DNA sequencer.

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 almost complete sequences (from nucleotide positions 49–1389 corresponding to the *E. coli* numbering). Alignment positions at which one or more sequences had gaps or ambiguities were omitted from the analysis. The maximum likelihood, maximum parsimony, and neighbor-joining methods as integrated in the ARB software package were applied. The latter calculation was based on a matrix of evolutionary distances determined by using the Jukes-Cantor equation and subject to bootstrap analysis (1,500 replicates). Partial sequences were integrated in the dendrogram according to the maximum parsimony criterion without allowing them to change the topology of the tree as established with complete sequences (see ARB manual).

Phenotypic similarity. Indices of phenotypic similarity were computed by pairwise comparisons of 13 binary (absence/presence) phenotypic traits for which at least one strain was different. Each matching trait added 1/13 to the index, so that completely matching strains were allotted a similarity of 1, and strain pairs with no matching trait received a value of 0. Five morphology-based traits were computed: (1) presence of large cells (width > 5 µm); (2) presence, at least under some conditions, of asymmetrical divisions to yield both large and

small cells (including baeocytes); (3) presence of fusiform cells; (4) ability to form colonies at least under specific conditions; and (5) presence of gas vesicles. In addition, five chemotaxonomic traits were included for which the presence of trace amounts was considered a negative: (6) presence of shinorine; (7) presence of an unidentified MAA, unidentified-360; (8) presence of zeaxanthin; (9) presence of myxoxanthophyll-1; (10) presence of myxoxanthophyll-2; and (11) presence of β -cryptoxanthine. The ability to grow in brine saturated with NaCl (12) and in medium of 1.5% salinity (13) were the two physiological traits used. Cluster analysis subjected to bootstrap was carried out on the data using the program PAUP (D. Swofford) test version 4.

Results

Morphology. Although all strains studied were unicellular, there were conspicuous morphological differences among them. The morphological characteristics of the isolates are shown in Table 1 together with plausible taxonomic assignments on the basis of morphology alone. Cell shapes ranged from coccoid or coccobacilloid to clearly bacilloid, in some cases fusiform (spindle-shaped), involuted, or irregular (see Fig.1). Some strains grew as colonies, while others did not. Colonies ranged from tight packages of irregular cells held within firm sheaths to groups of cells embedded in a more or less defined mucilage. The majority of strains divided in one apparent plane only. In most of these strains, cleavage occurred centrally, but at least three strains (PCC 8305, MPI 96AH13, and MPI 96AL03) could carry out asymmetric divisions resulting in one large and one (or two) small cell(s). Two of the strains, MPI 96N303 and MPI 96N304, divided in multiple planes but without apparent order, resulting in tightly packed colonies made up of cells of irregular shape. Both strains could apparently carry out multiple division without growth to form coccoid, nonmotile baeocytes (Fig.1).

Cell width was the least variant character among the strains, ranging along a continuum from 2.6 to ca. 10 μm . Cell length, however, varied over one order of magnitude, from 4 to 15 μm in most cases, and up to 82 μm or longer in strain PCC 8305. Except for the number of division planes, all morphological traits were dependent on growth conditions, particularly on salinity. For example, strain MPI 95AH13 formed cells smaller at high salinity than at low salinity, changing concomitantly in shape from involuted fusiform cells that often divided asymmetrically to bacilloid cells that divided symmetrically (see also Fig.2), i.e., adopted a typical *Cyanothece* appearance at high salinity. The involuted, asymmetrically dividing cells cannot correspond to an effect of adverse growth conditions since the cells grew optimally under these conditions (see Fig.3). Other strains such as PCC 7418, MPI 95AH10, MPI 95AH11, SYN CI P22, and ATCC 43922 showed the opposite trend, becoming longer and

Table 1 Morphological traits of the halotolerant strains studied. Unless otherwise stated, all traits reported correspond to cultures grown at 38°C, 7% salinity, and 50 µmol m⁻² s⁻¹ quantum irradiance [*B* bacilloid, *CB* coccobacilloid, *C* coccoid, *F* fusiform elliptical), and *I* irregular]

Strain	Cell			Division		Colo- nies	Baeo- cytes	Gas vesicles	Possible generic assignments, <i>sensu</i>	
	Width (µm)	Length (µm)	Shape	Plane(s)	Assymetry				Castenholz and Waterbury (1989)	Anagnostidis and Komárek (1986)
MPI 95AH10	3.1 (0.3)	4.2 (0.7)	B-CB	1	-	(+/-) ^a	-	-	<i>Cyanothece</i>	<i>Cyanothece</i> / <i>Aphanothece</i> ^b
MPI 95AH11	2.8 (0.5)	4.0 (1.0)	B-CB	1	-	(+/-) ^a	-	-	<i>Cyanothece</i>	<i>Cyanothece</i> / <i>Aphanothece</i> ^b
PCC 7418	5.0 (0.7)	7.4 (1.7)	B-CB	1	-	-	-	+	<i>Cyanothece</i>	<i>Cyanothece</i>
ATCC 43922	3.3 (0.5)	6.8 (3.3)	B-CB	1	-	-	-	-	<i>Cyanothece</i>	<i>Cyanothece</i>
SYN CI P22	2.6 (0.4)	4.5 (1.0)	B-CB	1	-	-	-	-	<i>Cyanothece</i>	<i>Cyanothece</i>
MPI 96AL06	3.5 (0.6)	4.5 (1.0)	C-CB	1	-	-	-	-	<i>Cyanothece</i>	<i>Cyanothece</i>
MPI 96P402	3.7 (0.6)	7.8 (2.4)	B-CB	1	-	+	-	-	<i>Cyanothece</i>	<i>Aphanothece</i>
MPI 96P605	6.5 (1.3)	9.9 (2.7)	CB	1	-	+	-	-	<i>Cyanothece</i>	<i>Aphanothece</i>
MPI 96AL03	5.8 (0.7)	11.6 (1.6)	CB-F	1	+	+	-	-	<i>Cyanothece</i> ^b (?)	<i>Aphanothece</i> ^b (?)
MPI 95AH13	10.3 (0.9)	15.9 (1.6)	F-CB	1	+	+	-	-	<i>Cyanothece</i> ^b (?)	<i>Aphanothece</i> ^b / <i>Rhabdoglea</i>
PCC 8305	6.1 (1.9)	82 (29)	F-B	1	+	-	-	+	(?)	<i>Myxobactron</i>
MPI 96N304	Variable	Variable	C-1	>2	-	+	+	-	<i>Chroococciopsis</i>	<i>Chroococciopsis</i>
MPI 96N303	Variable	Variable	C-1	>2	-	+	+	-	<i>Chroococciopsis</i>	<i>Chroococciopsis</i>

^aOnly at high salinity

^bMorphology (and assignment) vary with culture conditions

sometimes even pseudofilamentous at high salinity. Significant size differences also occurred with respect to incubation temperatures, cells usually being smaller and shorter at 25°C than at 38°C (Fig.2). The mucilaginous envelopes, if present, tended to be more diffuse at low salinities, resulting in less-defined colonies. In strain MPI 96N304, exposure to low salinities (3%) resulted in massive baeocyte production, which was otherwise not often seen at higher salinities. While it is well-known that a certain morphological plasticity occurs in many, but not all, cyanobacteria, the changes observed in many of these strains are remarkable. The instability of morphological traits among halotolerant *Cyanothece/Aphanothece halophytica* has been noted in the past in some of the present strains (ATCC 43922; Yopp *et al.*, 1978) and in other isolates (Berland *et al.*, 1989; Dor & Hornhoff, 1985). MacKay *et al.* (1984) have even reported that some strains can divide in more than one plane at high salinities. The range of morphological variance among the strains is certainly wider than the variations displayed by any one of them, so that some strains can be easily recognized under the microscope; but in most cases, the size ranges overlap and important features are not constitutively displayed.

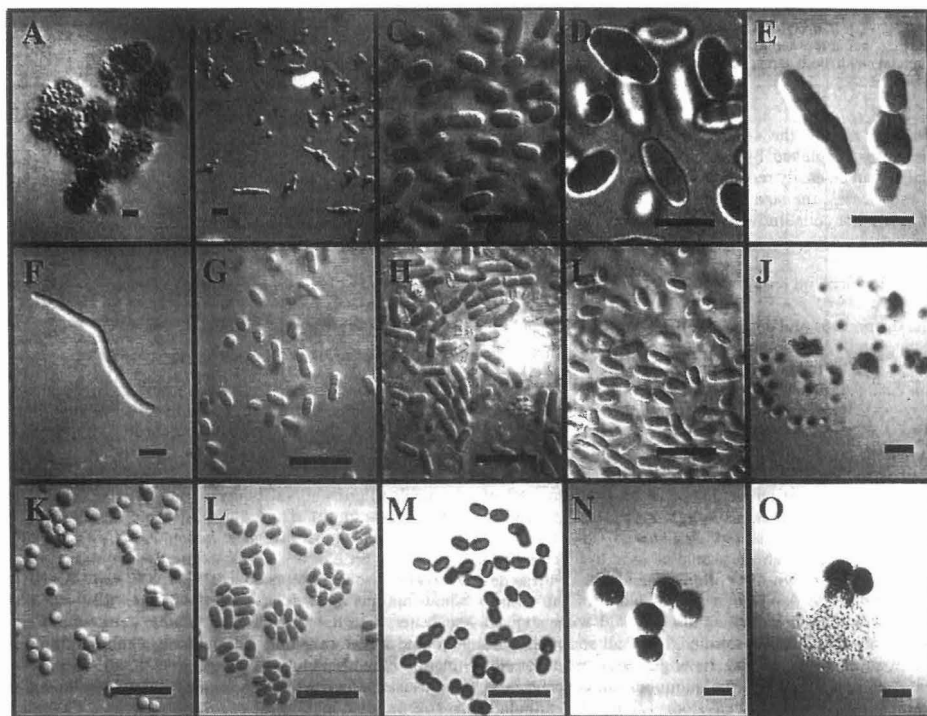


FIGURE 1. A–O Photomicrographs of unicellular cyanobacterial strains from hypersaline environments (all scale bars 20 μm). A–E Strain MPI 95AH13: A colonies grown at 3.3% salinity and 38°C; B diffluent colonies grown at 10% salinity and 38°C, with involuted cells; C *Cyanothece*-like cells grown at 20% salinity and 25°C; D *Dactylococcopsis*-like cells grown at 7% salinity; E close-up of asymmetrical cell division; F cell of strain PCC 8305; G strain MPI 95AH11 grown at 10% salinity and 38°C; H strain MPI 95AH11 grown at 20% salinity and 38°C; I strain MPI 96AL03 grown at 10% salinity and 38°C; J strain MPI 96AL03 grown at 3% salinity and 38°C, displaying bimodal cell-size distribution due to asymmetrical divisions; K strain MPI 96AL06 grown at 10% salinity and 38°C; L strain MPI 96P402 grown at 10% salinity and 38°C, showing typical *Aphanothece* morphology with groups of cell immersed in a common sheath; M strain MPI 96P402 grown at 1.5% salinity and 38°C, showing typical *Cyanothece* morphology; N strain MPI 96N304, displaying orthogonal planes of division in vegetative cells; and O strain MPI 96N303, showing a burst of baeocytes

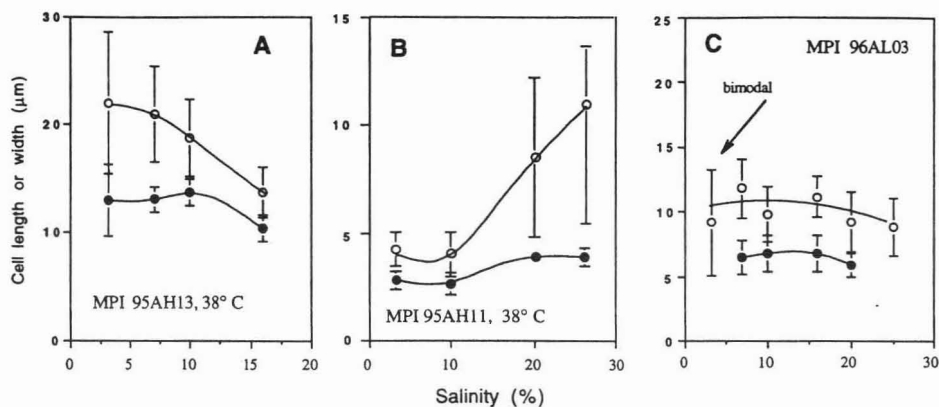
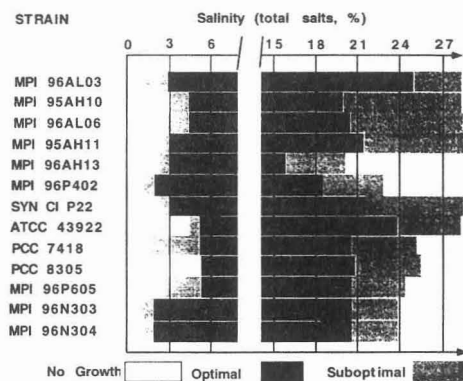


FIGURE 2. A–C Examples of the influence of environmental conditions on cell size. **A** Variation in cell length (o) and width (•) with salinity in strain MPI 95AH13. Cells became slightly thinner and significantly shorter with increasing salinity. **B** Variation in cell length (o) and cell width (•) with salinity in strain MPI 95AH11. Cells became slightly thicker and significantly longer at high salinities. **C** Variations in cell length in strain MPI 96AL03. Cells grown at 25°C (•) are significantly smaller than cells grown at 38°C (o). Salinity does not have significant effects on average cell size, but at low salinities the distribution of cell sizes becomes bimodal, with both larger and smaller cells (asymmetrical divisions).

Salt tolerance and requirements. The differences and commonalities with regard to salt tolerance and requirements were determined by recording the growth of triplicate cultures at 1.5, 3.2, 7, 10, 16, 20, and 25% salinity. Strains showing growth at 25% salinity were tested for growth in NaCl-saturated brine medium (29–34% salinity). Medium BG 11 was used to check the ability to grow under freshwater conditions in those strains that showed growth at 1.5% salinity. Experiments were carried out at an irradiance of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ of white light (12-h-light, 12-h-dark cycle) and at both 25 and 38°C, and progressed stepwise so that a culture grown at a certain salinity would be used to inoculate the next upshift or downshift culture. The results of these determinations are depicted in Fig.3. None of the strains could grow in freshwater medium, although some showed growth already at 1.5% salinity. Thus, all strains can be described as strictly marine, having a requirement for elevated salt concentration. The minimum salt concentration required for growth varied from 1.5 to 6%, although in many strains low salinities allowed only suboptimal growth. It has been reported that strain PCC

8305 is unable to grow in seawater salinities (Walsby *et al.*, 1983). Yopp *et al.* (1978) have reported that strain ATCC 43922 is unable to grow at NaCl concentrations of less than 1 M (ca. 5.8% salinity). Thus, if one accepts the convention that halophiles must not grow in standard seawater salinity, only strain ATCC 43922 was strictly halophilic. Alternatively, if one accepts that a halophile only needs to show optimal growth at salinities higher than standard seawater, then most of the strains were halophilic in nature and some were only halotolerant, although this distinction may ultimately be superfluous. In all cases, however, sustained growth was observed throughout the range 6–16% salinity, making all of these strains truly halotolerant. In several strains, growth was observed even in NaCl-saturated brine. It is also clear that, in comparison with other cyanobacteria, these strains rank at the top of the halotolerance list [see MacKay *et al.* (1984)], in this sense being extremely halotolerant cyanobacteria.

FIGURE 3. Ranges of salinity for growth in cyanobacterial isolates as determined by observation of triplicate, step-wise cultures. Approximate ranges of suboptimal growth indicate cultures in which growth was markedly slow. Note that most differences among strains occur at salinities below 6% or beyond 16%.



In order to determine salinity optima more accurately, we measured the growth rate of some of the strains. The results are presented in Fig.4. Differences among strains in salinity-dependent growth were significant, especially in the maximal absolute rates of growth and in the ranges of salinity tolerated. For example, strains PCC 7418 and MPI 95AH11 reached much higher growth rates than did strains MPI 95AH13 and MPI 96P402. Although they could not be quantitated, growth rates of strains MPI96N303 and MPI 96N304 were extremely low under all conditions. As indicated by the previous observations (Fig.3), a salinity optimum was not well-defined in most strains; rather, close-to-optimal rates formed a broad plateau across a large salinity range (roughly between 3–7 and 15–21%), indicating that very efficient acclimation to salinity occurs and that these strains can be classified as euryhaline. In some cases (strains MPI AL03, MPI 96AL06, MPI 95AH10, MPI 95AH11, ATCC 43922, and SYN CI P22), slow but steady growth was observed in NaCl-saturated brines.

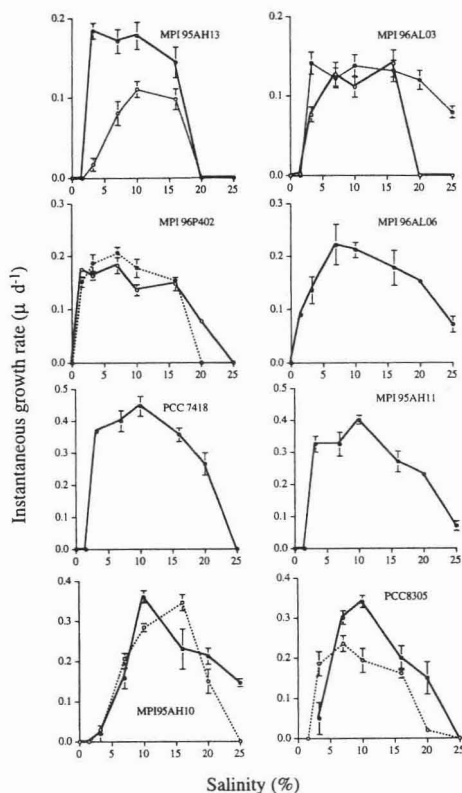


FIGURE 4. Growth rates versus salinity in several isolates. Strain denominations are indicated in each panel. *Solid lines* represent measurements at 38°C and *broken lines* show measurements at 25°C. Note different scales for each panel. All strains can be classified as euryhaline, but incubation temperature can influence growth rates significantly, especially at high salinities

Incubation temperature had, in all strains tested, a marked effect on the salt tolerance displayed. Incubation at 38°C resulted in growth at higher salinities than did incubation at 25°C in strains PCC 8305 and MPI 95AH11. The opposite was true for strain MPI 95AH13. Temperature effects of this kind had been previously noticed in extremely halotolerant unicellular cyanobacterial isolates (Berland *et al.*, 1989; Dor & Hornhoff, 1985; Tindall *et al.*, 1978) and in mixed populations thereof (Dor & Hornhoff, 1984). Dor & Hornhoff (1985) have noted that the same strain can be classified as euryhaline or stenohaline depending just on the incubation temperature.

The slow growth rate at very high salinities in some strains (i.e., strain MPI 95AH13 at 20%, and strain MPI 95AH10 at 25% and in NaCl-saturated brines) was at least partly due to a tendency to form compact mucilage constraining the dispersability of the cells in the cultures and resulting in the formation of large colonies. Under these conditions, we could not measure growth rates satisfactorily, and albeit growth was very slow, it was steady and non-zero. This condition was constitutive in strains MPI 96N303 and MPI 96N304, and satisfactory growth rates could not be measured.

In conclusion, this group of strains can be safely classified as strictly marine, euryhaline, and extremely halotolerant cyanobacteria. Although a physiological diversity exists among them regarding the minimal and maximal salinity tolerated, the establishment of clear distinctions, if they exist, between salinity optima may require an in-depth investigation of the effect of other parameters (e.g., temperature and light intensity) on salinity tolerance.

Temperature requirement for growth. Temperature ranges were determined by following growth at a salinity of 10% and an irradiance of $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ of white light (12-h-light, 12-h-dark cycle). Tested were 15, 20, 25, 30, 38, 45, and 50°C in a step-wise fashion so that each culture was subjected to a single-step shift in temperature. Under these conditions, the temperature range of growth was quite homogeneous among all isolates: no growth was observed at 15 or at 50°C ; suboptimal growth occurred at 20°C in most strains and at 45°C in all strains. Growth optima were 25°C in strain MPI 95AH13, and between 30 and 38°C in all others. Maximal temperatures for growth were previously determined to be 48.5°C in strain PCC 8305 (Walsby *et al.*, 1983) and 43°C in strain ATCC 43922 (Tindall *et al.*, 1978). Because of their ability to grow at 45°C or at slightly higher temperatures, all of these strains must be regarded as moderately thermophilic, a physiological capacity of adaptive value for halophilic organisms since, given the low specific heat of brines, sunlit hypersaline environments may easily reach elevated temperatures (Castenholz, 1969).

Complement of phycobiliproteins, carotenoids, and mycosporine-like amino acids. Phycocyanin and allophycocyanin were present in all strains, and both phycoerythrocyanin and phycoerythrin were conspicuously absent from all even when strains were grown under very low light conditions. Between 6 and 11 different carotenoids were detected in exponentially growing cultures. However, six carotenoids (Table 2) were the most abundant, and together they made up 94% of the total carotenoids in all cases. Either β -carotene or echinenone was the most abundant carotenoid in all strains, followed by a myxoxanthophyll (here, myxoxanthophyll I carrying the sugar L-fucose glycosidically bound to the chromophore

myxol). These three carotenoids were present in all strains. Many, but not all strains contained zeaxanthine, β -cryptoxanthine, and a second myxoxanthophyll (here, myxoxanthophyll 2 carrying L-chinovose bound to the myxol group). Carotenoids that were found in trace amounts included canthaxanthin, nostoxanthin, hydroxyechinenone, and several unidentified ones. Not one of the latter made up more than 1% (absorbance ratios) of the total carotenoid extract, and it is possible that some of them represent degradation products of the more common carotenoids. The composition of principal carotenoids in these strains is remarkably similar to that reported by Berland *et al.* (1989) in a halotolerant "*Aphanothece halophytica*" isolate.

TABLE 2. Carotenoids in exponentially growing cells of halotolerant unicellular cyanobacteria. Reported are the total number of carotenoids detected and the relative molar ratio of each of six major carotenoids with respect to β -carotene. In all cases, these six major carotenoids made up 95% or more of the total carotenoid content in the cells.

Strain	Total number	β -Carotene	Echinenone	Myxoxanthophyll 1 ^a	Zeaxanthin	β -Cryptoxanthin ^b	Myxoxanthophyll 2 ^a
MPI 95AH10	7	1	0.62	0.69	0.17	0.06	—
MPI 95AH11	7	1	1.06	0.68	0.26	0.06	—
PCC 7418	8	1	1.19	0.26	0.16	0.16	0.32
ATCC 43922	6	1	0.37	0.12	0.33	0.05	—
SYN CI P22	9	1	1.05	0.05	0.31	0.05	0.17
MPI 96AL06	9	1	0.72	0.14	0.16	0.12	0.16
MPI 96P402	11	1	1.12	1.50	0.04	—	—
MPI 96P605	9	1	0.13	Traces	0.34	0.02	0.17
MPI 96AL03	8	1	1	0.73	0.03	0.21	0.03
MPI 95 AH13	6	1	0.85	1.09	—	—	—
PCC 8305	8	1	0.79	0.45	—	—	—
MPI 96N304	8	1	0.52	0.76	0.18	0.09	0.08
MPI 96N303	9	1	0.74	0.24	0.18	0.06	0.08

^a Myxoxanthophyll 1 = myxol+L-fucose; myxoxanthophyll 2 =myxol + L-chinovose

^b Tentative identification on the basis of spectrum and HPLC retention only

The yellow-to-brown color typical of natural populations of unicellular cyanobacteria in hypersaline environments is due to the prominence of carotenoids in the cells. All strains studied here displayed this yellow coloration under either high light conditions or in cultures in the stationary phase of growth. This was due to a severe loss of phycobiliproteins and chlorophyll accompanied by an increase in carotenoids. While this phenomenon is typical of many cyanobacteria, the extent and speed with which the changes occurred were remarkable. In several "yellow" cultures analyzed, myxoxanthophylls and xanthins seemed to acquire relative prominence (data not presented).

Mycosporine-like amino acid derivatives (MAAs) are a family of ultraviolet-absorbing, water-soluble, low-molecular-weight compounds sharing a cyclohexene ring core substituted with one or two amino acid residues (Dunlap & Chalker, 1986; Favre-Bonvin *et al.*, 1976; Karentz *et*

al., 1991). They are common and diverse in cyanobacteria exposed to strong solar radiation (Garcia-Pichel & Castenholz, 1993), where there is evidence that they may function as ultraviolet sunscreens (Garcia-Pichel *et al.*, 1993). The MAA complement has been used as a chemotaxonomic marker for low-rank differentiation in cyanobacterial cultures (Karsten and Garcia-Pichel, 1996). Three MAAs were detected in the present strains; their occurrence and distribution is presented in Table 3. Only two MAAs were present in significant concentrations. Shinorine (Tsujino *et al.*, 1980) was the most common and abundant, and it has also been identified in other cyanobacteria (Karsten & Garcia-Pichel, 1996), R. Appel and F. Garcia-Pichel, unpublished work). A second MAA absorbing maximally at 360–365 nm (unidentified-360) was also present in some isolates. There are no previous reports of such an MAA in cyanobacterial strains, although a spectroscopically similar compound has been detected in field samples of hypersaline microbial mats (Oren *et al.*, 1995). A previously described MAA, palythene (Ito & Hirata, 1977), presents spectroscopic similarity to unidentified-360, but on the basis of HPLC cochromatography with authentic palythene, the two compounds are different.

TABLE 3. Distribution and cellular specific content of mycosporine-like amino acids (MAA) in halotolerant unicellular cyanobacterial strains. In some strains, synthesis of MAA was constitutive; in others, it was inducible by exposure to UV-B radiation. In all cases, exposure to UV-B increased their specific contents. Maximal cellular contents are given as mg of compound per g of dry cell mass.

Strain	Shinorine	Unidentified-360	Synthesis
MPI 95AH10	0.03	–	Inducible
MPI 95AH11	0.05	–	Inducible
MPI 95AH13 ^a	0.63	0.04	Constitutive
MPI 96AL03 ^a	0.15	–	Constitutive
MPI 96AL06	0.03	–	Inducible
MPI 96P402	0.55	–	Constitutive
MPI 96P605	Traces	–	
MPI 96N303 ^a	0.70	0.03	Constitutive
MPI 96N304 ^a	0.63	0.03	Constitutive
SYN CI P22	0.91	–	Inducible
ATCC 43922	Traces	–	
PCC 7418	0.22	–	Inducible
PCC 8305 ^a	7.9	0.39	Constitutive

^aTraces (less than 0.01 mg per g dry cell mass) of mycosporine-gly also found in these strains

16S rRNA gene sequences and phylogeny. 16S rRNA gene sequences were deposited in the European Molecular Biology Laboratory (EMBL) database under accession nos. AJ000708–AJ000724. Similarities among (partial and complete) 16S rRNA gene sequences from extremely halotolerant unicellular cyanobacteria and other selected strains are presented in Table 4. Phylogenetic relationships of the strains investigated and other cyanobacteria calculated by applying the neighbor-joining method are shown in Fig.5. 16S rRNA gene sequences from most unicellular, extremely halotolerant cyanobacteria are 95.5% or more similar; some strains

have virtually identical sequences (i.e., strains MPI 96N303 and MPI 96N304, as well as strains MPI 95AH10, MPI 95AH11, and ATCC 43922). The sequences are 90.2–91.0% similar to the sequence from strain MPI 96P605, and 84.6–91.0% similar to sequences from other cyanobacteria. Calculations of similarity matrices and phylogenetic trees involving all partial and complete 16S rRNA sequences from cyanobacteria that are available from public databases did not detect any relative of the extremely halotolerant unicellular cyanobacteria closer than those presented in Table 4 (data not shown).

Phylogenetic reconstruction trees obtained by using the neighbor-joining, maximum likelihood, and maximum parsimony methods resulted in slightly different tree topologies (data not shown) involving phylogenetic relationships among some deeply branching lineages within the cyanobacterial radiation. However, all extremely halotolerant unicellular strains (including strain MPI 96P605) clustered together no matter which treeing method was applied.

Due to sequence variation among multiple copies of 16S rRNA genes (Nübel *et al.* (1996) and references therein) in the genome of strain MPI 95AH10, molecular cloning was necessary prior to sequence analysis of this axenic strain. A fraction of the genes contains an insertion of a single deoxyadenylate nucleotide between positions 197 and 198 (*E. coli* numbering).

Cluster analysis of phenotypic traits. A similarity matrix for 13 phenotypic traits is presented in Table 5. Phenotypic similarity indices were not always congruent with the sequence similarity of 16S rRNA genes. Phenotypic traits do not separate strain MPI 96P605 from the rest, as the sequence similarity did. The closeness of strains MPI 95AH10, MPI 96AH11, and ATCC 43922, and that of strains MPI 96N303 and MPI 96N304, however, can be supported by both types of analysis. Cluster analysis carried out on this restricted number of chemotaxonomic, morphological, and physiological traits (results not shown) indicated the presence of a clade encompassing strains of small cell size (roughly corresponding to the strains with high similarity in the upper left-hand corner of Table 5), but this grouping did not stand bootstrap analysis. High bootstrap values were found only for the grouping of strain MPI 95AH10 with strain MPI 95AH11 (85%), strain MPI 96N303 with strain MPI 96N304 (98%), and strain MPI 95AH13 with strain PCC 8305 (87%).

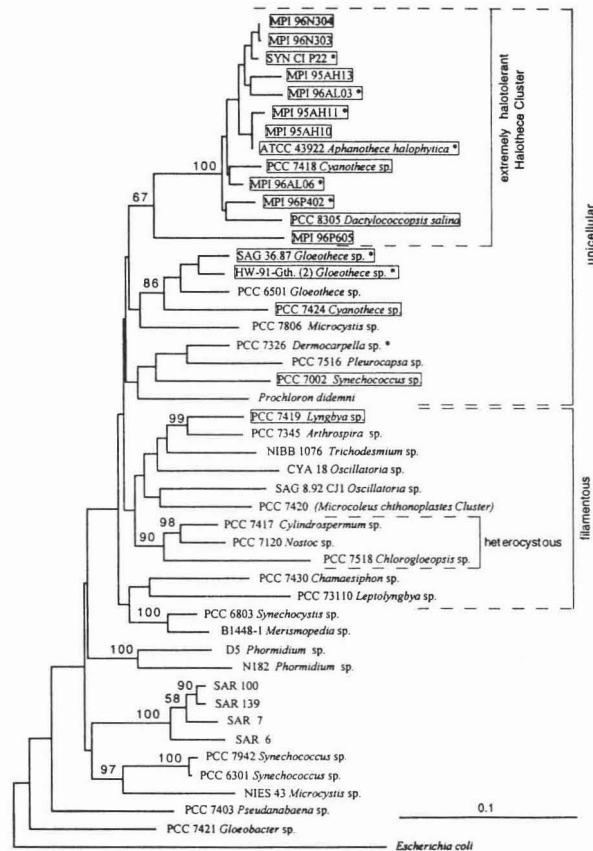


FIGURE 5. Distance tree based on 16S rRNA gene sequences containing at least 1,400 nucleotides. The 16S rRNA gene from *Escherichia coli* was used as an outgroup sequence. Evolutionary distances were determined by the Jukes-Cantor equation, and the tree was calculated with the neighbor-joining algorithm. Bootstrap values (%) from 1,500 replicates are shown for nodes attaining more than 50%. The phylogenetic positions of organisms represented by partial sequences (approximately 560 nucleotides; strains are indicated by asterisks) were reconstructed by applying the parsimony criteria without changing the overall tree topology (see text). No bootstrap values can be assigned to apparent clusters containing only partial sequences. Strains for which sequences were determined in this study are framed. The bar indicates 10% estimated sequence divergence. Sequences designated "SAR" are derived from noncultured bacterioplankton DNA from the Sargasso Sea (Giovanonni *et al.* 1990; Britschgi and Giovanonni 1991). Strain PCC 7420 represents the "*Microcoleus chthonoplastes* cluster" (Garcia-Pichel *et al.* 1996). Strain Gloeotheca HW-91-Gth. (2) is that of Garcia-Pichel and Castenholz (1991). All other entries are named after data-base labels regardless of taxonomic considerations. Indications on strain morphology on the right are for information only and are not necessarily indicative of phylogenetic coherence.

Table 5 Similarity index matrix for extremely halotolerant unicellular cyanobacteria calculated on the basis of 13 phenotypic traits

Strain	MPI 95AH10	MPI 95AH11	ATCC 43922	SYN CI P22	MPI 96AL06	MPI 96P402	PCC 7418	MPI 96P605	MPI 96AL03	MPI 95AH13	PCC 8305	MPI 96N303	MPI 96N304
MPI 95AH10	1												
MPI 95AH11	1	1											
ATCC 43922	0.85	0.85	1										
SYN CI P22	0.85	0.85	0.85	1									
MPI 96AL06	0.69	0.69	0.69	0.85	1								
MPI 96P402	0.77	0.77	0.62	0.62	0.77	1							
PCC 7418	0.54	0.54	0.54	0.77	0.69	0.46	1						
MPI 96P605	0.69	0.69	0.54	0.62	0.69	0.62	0.54	1					
MPI 96AL03	0.54	0.54	0.54	0.62	0.54	0.46	0.69	0.54	1				
MPI 95AH13	0.46	0.46	0.31	0.31	0.31	0.54	0.31	0.46	0.46	1			
PCC 8305	0.31	0.31	0.31	0.23	0.31	0.38	0.46	0.31	0.31	0.79	1		
MPI 96N303	0.54	0.54	0.39	0.54	0.69	0.62	0.54	0.69	0.69	0.56	0.46	1	
MPI 96N304	0.54	0.54	0.39	0.54	0.69	0.62	0.54	0.69	0.69	0.56	0.46	1	1

Discussion

The "Halothece" cluster. The results from our analysis indicate that the group of strains investigated constitute a monophyletic cluster within the cyanobacteria. The cluster can be defined simply and independently from molecular analyses on the grounds of basic morphology and physiology as encompassing unicellular, extremely halotolerant cyanobacteria. Thus, we refer to it as the "Halothece" cluster, from the Greek terms for salt (*Halo-*) and box (*thece*). Additionally, common features of strains pertaining to this cluster are: (1) growth at 45°C (moderate thermophiles), (2) the presence of β -carotene, echinenone, and L-fucose-myxol, (3) the ability to synthesize shinorine as the principal MAA, (4) the absence of phycoerythrin and phycoerythrocyanin as light-harvesting phycobiliproteins, and (5) formation of markedly pale cells (a combined result of low pigment contents and cytoplasm keratomization). Other known marine and freshwater unicellular cyanobacteria failed to show extreme halotolerance (no growth above 13% salinity) and did not cluster within Halothece (see Fig.5). Known filamentous halotolerant strains failed to show extreme halotolerance (close to optimal growth above 13% salinity) and did not cluster within Halothece in the phylogenetic analysis of 16S rRNA genes. It is interesting to note that analogous clustering of extremely halotolerant strains has been found in the purple sulfur bacteria of the family Ectothiorhodospiraceae (Imhoff & Siling, 1996).

The Halothece cluster as defined above includes strains that would be classified under different generic epithets according to both the botanical and the bacteriological systems [e.g., *Cyanothece Aphanothece* PCC 7418 and *Dactylococcopsis Myxobactron* PCC 8305; see Table 1]. But perhaps most surprisingly, it contains strains that would be traditionally classified under different orders according to Bergey's Manual (Castenholz & Waterbury, 1989): Pleurocapsales for strains MPI 96N303 and MPI 96N304, and Chroococcales for the rest. We have only visual, microscopy observations for the production of beaocytes in strains MPI 96N303 and MPI 96N304, and one should perhaps await more convincing evidence stemming from slide-cultures and/or electron microscopy. We have tested other marine pleurocapsalean strains (PCC 7326, PCC 7516, PCC 7319, and PCC 7325) for salinity tolerance and/or sequence analysis; none grew above 12% salinity or clustered within Halothece. In any event, a taxonomic treatment along the lines of morphological characters does not serve the systematics of extremely halotolerant cyanobacteria well. There is an obvious need for creating new taxa to accommodate the extremely halotolerant unicellular cyanobacteria and to separate them from their nonhalotolerant morphological analogues. The use of *Cyanothece* or *Aphanothece* for the extremely halotolerant strains should be avoided.

Diversity within the Halothece cluster. The phylogenetic diversity contained within the Halothece cluster, as judged by the 9% divergence of the 16S RNA gene sequences, is substantial. Such divergences correspond to suprageneric taxonomic divisions in bacteriology. However, most of the divergence stems from the distance imposed by strain MPI 96P605, while the other 12 strains cluster tightly within less than 5% sequence divergence, which corresponds to distances contained within many bacteriological genera [e.g., (Amann *et al.*, 1992; Ash *et al.*, 1993; Vandamme *et al.*, 1996; Wisotzkey *et al.*, 1992)]. Thus, the reconstructed phylogenetic group of Halothece consists, at least so far, of a tight subcluster (hereafter termed the "Euhalothece" subcluster, the true Halothece) and a deep-branching strain (MPI 96P605). The validity of this division must be regarded with caution. New isolates might span the gap between strain MPI 96P605 and Euhalothece, or alternatively, MPI 96P605 might represent a true cluster, with other closely associated strains yet to be cultured. A mere look at the proportions of 12:1 in the partition of the strains seems to support the latter hypothesis. We note that at present we do not have a single phenotypic trait that would set strain MPI 96P605 apart from the rest.

16S rRNA gene sequence similarities of less than 97.5% usually correspond to less than 70% DNA-DNA hybridization (Stackebrandt & Goebel, 1994), which is the borderline for segregating species in bacteriology (Wayne *et al.*, 1987). Application of this pragmatic approach would thus probably segregate, for example, strains PCC 8305 and PCC 7418 as two separate specific entities from the rest of strains in Euhalothece. However, our analysis of phenotypic data is not congruent with the phylogenetic structure of the Euhalothece subcluster that can be gained from 16S rRNA gene sequence analysis.

The cluster analysis of phenotypic similarity (within the Euhalothece subcluster) tends to separate two groups, one of which includes six strains sharing high phenotypic similarity indices and which encompasses the morphologically smallest, simplest strains (MPI 95AH10, MPI 95AH11, SYN CI P22, ATCCC 43922, MPI 96AL06, and MPI 96P402); this division, however, finds low statistical support. Although there seems to be a simple, practical coincidence of morphology and chemotaxonomy, one should resist the temptation of assigning previous specific descriptions to this group. Some strains fit the botanical description of "*Aphanothece halophytica*" (Hof & Frémy, 1933) in the sense in which it is usually applied; other strains more closely fit the descriptions of "*Aphanothece cohenii*" (Campbell & Golubic, 1985) or those typical of "*Cyanothece halobia*" (Roussomoustakaki & Anagnostidis, 1991); none of the specific descriptions, however, would include all. High phenotypic similarity is also found between strains PCC 8305 and MPI 96AH13, which, interestingly, may both well be included in the type-genus *Dactylococcopsis* (or *Myxobactron*) on the basis of morphology (spindle-shaped cells). These strains, however, neither share high similarity with strain MPI 96AL03 (perhaps also qualifying for the latter genus) nor attain particularly high 16S rRNA sequence similarity.

In conclusion, although the results seem to point to the presence of several taxonomically practical taxa within the Halothece cluster, a definitive assessment may require the analysis of more traits and perhaps of more isolates; furthermore, these taxa can possibly be tested for phylogenetic coherence only by alternative methods such as DNA/DNA hybridization.

Implications for the ecology and evolution of unicellular, halotolerant cyanobacteria. If the collection of strains in culture faithfully represents the biodiversity of extremely halotolerant unicellular cyanobacteria in nature, i.e., if no significant fraction of those present in natural strong brines has eluded cultivation efforts, then it must follow that extreme halotolerance evolved only once among unicellular cyanobacteria. The crucial evolutionary step in the process may have been the acquisition of an ability to synthesize quaternary ammonium compounds to function as osmolites. These are conspicuously absent from all but the most extremely halotolerant cyanobacteria (MacKay *et al.*, 1984), but they have been found in all strains within the Halothece cluster that have been analyzed [strains PCC 7418 (MacKay *et al.*, 1984), PCC 8305 (Moore *et al.*, 1987), and ATCC 43922 (Sibley & Yopp, 1987)]. One might speculate that gaining access to the potential niche of strongly saline waters may have enabled the common ancestor in the Halothece cluster to undergo an evolutionary radiation by optimizing adaptations for particular conditions of hypersaline environments (benthic vs. planktonic, encrusting vs. sessile, etc.), which we see now in the physiological and morphological diversity that the cluster contains.

Alternatively, it might be possible that cultivation techniques strongly selected for specific strains (although in many cases blind enrichments were avoided) so that the natural diversity of unicellular, extremely halotolerant cyanobacteria is not represented by the strains in the Halotheca cluster. We are currently working on molecular ecological approaches to answer this question.

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3

**The halotolerance and phylogeny of cyanobacteria with tightly
coiled trichomes (*Spirulina* spp. Turpin)**

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Manuscript in preparation

Abstract

We investigated 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. Morphologically all strains were very similar and could be assigned to the genus *Spirulina* (*Euspirulina sensu* Geitler) according to traditional classification. However, the isolates showed significantly different requirements for salinity and temperature which are in accordance with their respective environmental origins. The genetic divergence among the strains investigated was large. Our 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 investigated originated from hypersaline waters, were similar with respect to their high halotolerance, broad euryhalinity, and elevated temperature tolerance, and by phylogenetic analyses were placed in a tight monophyletic cluster apart from all other cyanobacteria. Thus, we propose the reclassification of highly halotolerant cyanobacteria with tightly coiled trichomes in the new genus *Halospirulina* gen. nov.

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 favorable 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 & Komarek, 1988; Castenholz, 1989 a; 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 & Komarek, 1988; Castenholz, 1989; Tomaselli *et al.*, 1996). Depending on trichome diameter and coil shape,

cyanobacteria of the genus *Spirulina* are commonly assigned to one of a few species, most frequently to *S. subsalsa* Oersted, *S. labyrinthiformis* Gomont, and *S. major* Kützing, regardless of their habitat of origin. Consequently, they are traditionally considered cosmopolitan microorganisms with remarkable capabilities to acclimate 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 & Süling, 1996; Imhoff *et al.*, 1998; Overmann & Tuschak, 1997). In a recent study, 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, doubts 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 contents of guanosine and cytosine 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. On the basis of these data a phylogenetic pattern emerges which in part is supported by phenotypic characteristics. We propose the reclassification at the generic level of those halotolerant, euryhaline cyanobacteria with helical trichomes from hypersaline waters.

Material and methods

Cyanobacterial strains, strain histories, and cultivation. Clonal strains of cyanobacteria used for this study are listed in Table 1. Information is included about the respective origins of strains, possible morphology-based classification, and affiliated literature. Freshwater medium was BG11 (Rippka *et al.*, 1979) modified by decreasing the content of NaNO_3 to 0.75 g/l. Seawater and hypersaline medium were prepared by dissolving appropriate amounts of commercial seawater salts mixture (Wiegandt GmbH, Krefeld, Germany) 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 in order to drive excess CO_2 out of the 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).

Growth rate measurements. All strains were grown in deep Petri dishes filled with liquid media of various salinities. Strain CCC Snake-P.Y85 was incubated at 38°C receiving 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of white light from fluorescent tubes during 12 h daily. All other cultures were incubated at 25°C receiving 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of constant white light from fluorescent tubes. Growth rates were measured by noninvasively monitoring the increase with time of bulk phycobilin/chlorophyll *a* fluorescence in the cultures using a fluorimeter especially 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 weight) was checked ($R^2 \geq 0.8$; data not shown). Growth was followed in triplicate cultures during periods of one to four weeks, so that four to five doublings during exponential growth could be monitored. Linear regression analysis of the ln-transformed 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 maximally 43 days. Strain CCC Snake-P.Y85 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 $\mu\text{mol photons/m}^2\text{s}$ of white light. Temperatures tested were 4, 10, 15, 20, 25, 35, 40, 45, and 50°C in a stepwise fashion so that each culture was subjected to a single-step shift in temperature.

TABLE 1. Strains used in this study and of additional cyanobacteria assigned to the genus *Spirulina*.

strain ^a	possible classification ^c	origin			references
		site of isolation	salinity	isolator ^d	
MPI S3	<i>S. subsalsa</i>	Mediterranean Sea, Spain	10%	EC	this work
MPI 95SS01	<i>S. subsalsa</i>	Pacific Ocean, salina Guerrero Negro, Mexico	16%	FGP	this work
MPI 95SL01	<i>S. labyrinthiformis</i>	Pacific Ocean, salina Guerrero Negro, Mexico	16%	FGP	this work
MPI S1	<i>S. labyrinthiformis</i>	Mediterranean Sea, Spain	10%	EC	this work
MPI S2	<i>S. subsalsa</i>	Mediterranean Sea, Spain	5.7%	EC	this work
MPI S4	<i>S. subsalsa</i>	Mediterranean Sea, Spain	3.7%	EC	this work
P7 (SAG 59.90, CCAP 1475/7)	<i>S. subsalsa</i>	Mediterranean Sea, harbour of Calvi, Corsica, France	3.5%	AW	(Wilmotte, 1991), (Wilmotte <i>et al.</i> , 1997)
UBMM Bo 89	<i>S. subsalsa</i>	Baltic Sea, Boiensdorf, Germany	1.5%	JR	(Rethmeier, 1995)
UBMM Hi 45	<i>S. major</i>	Baltic Sea, Hiddensee, Germany	1.0%	JR	(Rethmeier, 1995)
PCC 6313 (ATCC 29542)	<i>S. major</i>	Pacific Ocean, Berkeley, USA	"brackish"	MMA	(Rippka <i>et al.</i> , 1979), (Rippka & Herdman, 1992)
CCC Snake P.Y85	<i>S. labyrinthiformis</i>	"Snake Pit" (hot spring), Yellowstone National Park, USA	2 mS ^e	RWC	(Castenholz, 1977), personal communication
NIVA CYA 163 ^b	<i>S. subsalsa</i>	Atlantic Ocean, Oslofjord, Drøbak, Norway ^f	?	RS	(Aakermann <i>et al.</i> , 1992), (Rudi <i>et al.</i> , 1997)
NIVA CYA 164 ^b	<i>S. subsalsa</i>	Atlantic Ocean, Oslofjord, Drøbak, Norway ^f	?	RS	(Aakermann, <i>et al.</i> , 1992), (Rudi <i>et al.</i> , 1997)
M-223 ^b	<i>S. subsalsa</i>	?	?	?	(Ishida <i>et al.</i> , 1997)
SAG B256.80	" <i>S. laxissima</i> "	Lake Nakuru (natron lake), Kenya	?	EH	(Schlösser, 1994)

^a Culture collections: ATCC, American Type Culture Collection, Rockville, Md., USA; CCAP, Culture Collection of Algae and Protozoa, Ambleside, United Kingdom; CCC, R. W. Castenholz, University of Eugene, Oregon, 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 brackets indicate related strains.

^b Strains not available during this study.

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

^d Researchers that isolated the cyanobacterial strains are abbreviated with their initials: M. M. Allen, R. W. Castenholz, E. Clavero, F. Garcia-Pichel, E. Hegewald, J. Rethmeier, R. Skulberg, A. Wilmotte.

^e Conductivity.

^f In contrast, a seawater aquarium is quoted as site of isolation in the NIVA catalogue of strains (Skulberg, 1990).

? Information not available.

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.Y85 and 25°C for all others. Carotenoid complement was determined by HPLC separation and on-line ultraviolet/visible spectroscopy. Approximately 50 mg of cells (wet weight) were extracted in acetone. 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 elsewhere (Karsten & Garcia-Pichel, 1996). Phycobiliproteins were released from approximately 50 mg of cells (wet weight) into 20 mM sodium acetate, pH 5.5 (Tandeau de Marsac & Houmard, 1988), after breaking the cells by repeatedly freezing and thawing them (45°C / liquid nitrogen) 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] and 1 mM EDTA) were directly applied as templates for polymerase chain reactions (PCR). To determine almost complete sequences of 16S rRNA genes from cyanobacteria in unialgal but nonaxenic 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, Heidelberg, Germany). Full-length cyanobacterial 16S rRNA genes from plasmid inserts were reamplified using the same primers as before. To avoid errors due to PCR artefacts or operon microheterogeneities, PCR products derived from ten different plasmids were mixed and processed for sequencing. For some strains only partial sequences (approximately 560 nucleotides) 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 determined 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 76 almost complete sequences (from nucleotide positions 45-1455 corresponding to the *E. coli* numbering). 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 neighbor-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 (1,000 replicates). In the dendrogram presented partial sequences 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 (Figure 1). Thus, they fit the description of the genus *Spirulina sensu* Castenholz (Castenholz, 1989) and *sensu* Anagnostidis and Komarek (Anagnostidis & Komarek, 1988), and the "section" *Euspirulina* of the genus *Spirulina sensu* Geitler (Geitler, 1932). Coiling is clockwise or counter-clockwise depending on the respective strains. For all strains, motility was observed as apparent rotation along the helix axis in the sense of coiling. In PCC 6313 and UBMM Hi 45, trichome coils are open whereas in all other strains coils are closed (Figure 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 unfavorable 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).

TABLE 2. Microscopic observations.

strain	helix shape	trichome width ^a (μm)	helix width ^a (μm)	sense of coiling ^b	cross walls visibility	cell color	motility
MPI S3	closed	2.5	5.0	ccw	-	blue-green	+
MPI 95SS01	closed	3	6	ccw	-	blue-green	+
MPI 95SL01	closed	1.5	4	ccw	-	blue-green	+
MPI S1	closed	1.0	2.0	cw	-	blue-green	+
MPI S2	closed	1.7	3.0	cw	-	blue-green	+
MPI S4	closed	1.5	3.0	cw	-	blue-green	+
P7	closed	1.6	2.7	cw	-	blue-green	+
UBMM Bo89	closed	1.7	3.0	cw	-	brown	+
UBMM Hi 45	open	1.1	3.0	ccw	-	blue-green	+
PCC 6313	open	2.0	4.5	ccw	-	blue-green	+
CCC Snake P.Y85	closed	1.5	3.0	cw	-	blue-green	+
SAG B256.80	no helix	1.5	-	-	+	blue-green	-

^a Means of at least 20 trichomes measured.

^b cw, clockwise; ccw, counter-clockwise.

Trichomes of strain SAG B256.80, designated "*Spirulina laxissima*" in (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 (Figure 1). Motility was not observed. This strain differed from all other strains investigated in this study by maintaining buoyancy and growing homogeneously suspended 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 the sense of Geitler (Geitler, 1932) is questionable, who had assigned this morphospecies to his "section" *Euspirulina*, encompassing helically coiled cyanobacteria with crosswalls that are invisible in living specimens.

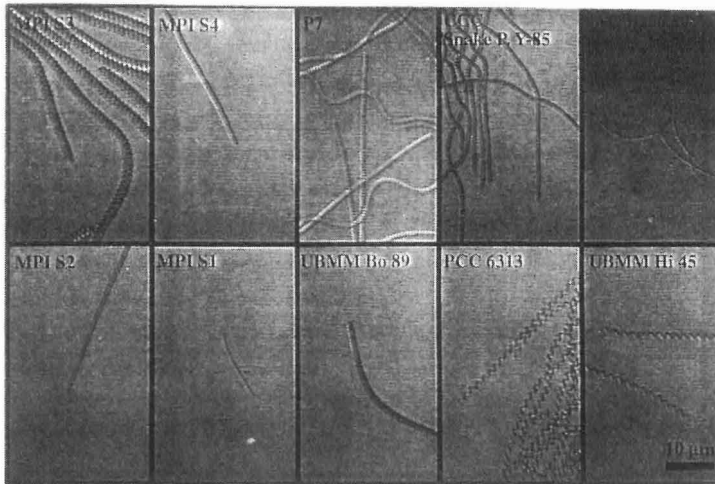


FIGURE 1. Photomicrographs of cyanobacterial isolates. All organisms are shown at the same magnification.

Salt requirements. The dependence of growth rates of the strains studied on salinity is illustrated in Figure 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 they had been isolated from: 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 are somewhat variable with respect to salinity optima and tolerances.

Typically they grow 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 are remarkable in that they do not show a distinct and narrow salinity optimum, but are 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 showed growth at salinities from 1.6% (MPI S3) or 3.2% (MPI 95SL01, MPI 95SS01) to 16%. In strain MPI 95SL01 elevated temperature (38°C) lead to 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 are extremely euryhaline and are 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).

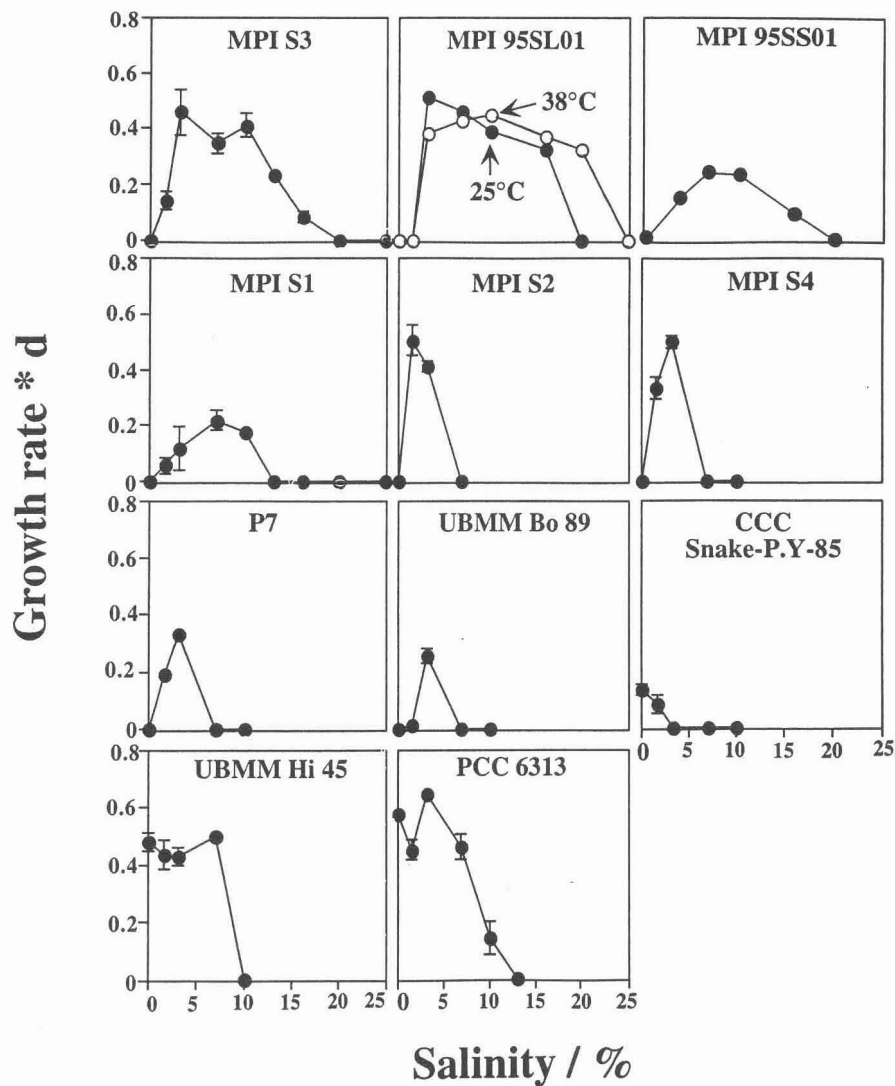


FIGURE 2. Growth rates versus salinity in the cyanobacterial isolates investigated. All measurements have been performed at 25°C, except with strains CCC Snake P. Y-85 (38°C) and MPI 95SL01 (25°C and 38°C).

Temperature requirements. The cyanobacterial strains analysed showed markedly different temperature requirements (Figure 3). Strain CCC Snake P. Y-85 had been isolated from a 50°C freshwater hot spring (named "Snake Pit") in Yellowstone National Park, Wyoming, USA (Castenholz, 1977), (Castenholz, personal communication) and must be termed thermophilic. In our experiments it showed growth between 35°C and 45°C and did not grow at 50°C. However, its upper temperature limit previously had been determined to be 51°C (Castenholz, personal communication). Natural populations of this organism showed maximum photosynthesis rates at 45°C (Castenholz, 1977). The strains that had shown the highest halotolerance (MPI S3, MPI 95SS01, MPI 95SL01) tolerated 40°C or 38°C, respectively, and did not grow at 15°C and below. Thus, they displayed a slightly elevated temperature requirement compared to the marine strains, most of which tolerated 10°C to 35°C. Similarly, this characteristic previously 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).

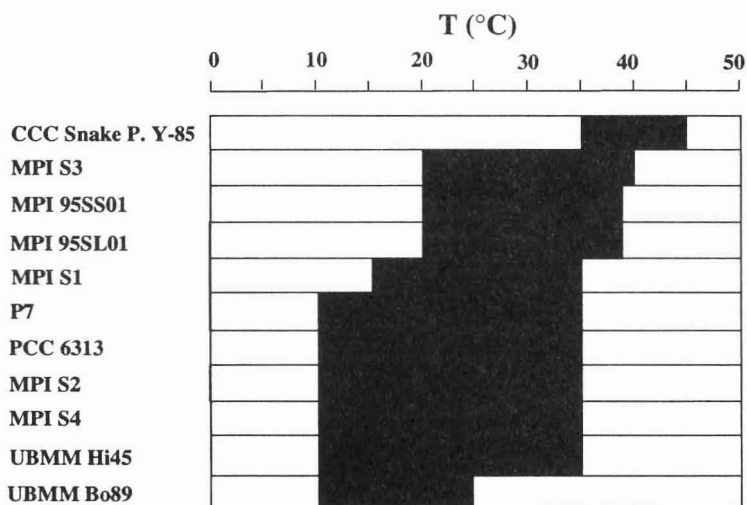


FIGURE 3. Temperature ranges for growth of the cyanobacterial isolates investigated.

Pigment compositions. All strains analysed contained phycocyanin. In addition, phycoerythrin was detected in strains P7 and UBMM Bo 89 (Table 4). The latter strain had a brownish to black appearance when observed in white light. The ability for chromatic

adaptation was not checked during this work, however, it previously 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). Phycoerythrocyanin was not present in any strain.

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

Carotenoid	Retention time / min	Absorption maxima ^a	E / mM ⁻¹ *cm ⁻¹
Aphanzophyll	1.37	(452) 476 506	69.2 ^c
Myxoxanthophyll ^b	3.51	(455) 478 509	69.2
Isozeaxanthin	6.04	(430) 454 481	83.2
unidentified carotenoid 1	6.08	480	143.9 ^c
Zeaxanthin	7.64	(430) 454 481	83.2
Canthaxanthin	8.45	478	143.9
unidentified carotenoid 2	8.51	(430) 448 478	83.2 ^c
unidentified carotenoid 3	9.71	467	143.9 ^c
unidentified carotenoid 4	14.91	(435) 453 480	83.2 ^c
Echinenone	15.11	458 (480)	75.3
β-Carotene	19.58	(435) 453 480	125.3

^a Shoulders are given in parenthesis

^b L-chinovose-myxol

^c extinction coefficients unknown and arbitrarily assigned on the basis of spectral resemblance to known carotenoids

With respect to their carotenoid contents cyanobacteria with *Spirulina*-like morphology are rather diverse (Tables 3 and 4). The total number of carotenoids in the different strains varied from 3 to 9. β-carotene was found in all strains and echinenone in most. Two of the most halotolerant strains (MPI S3, MPI 95SS01) contained the same types of carotenoids in similar ratios, however, MPI 95SL01 produced aphanzophyll and an unidentified carotenoid with an absorption maximum at 480 nm instead of myxoxanthophyll (L-chinovose-myxol) 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 two phycoerythrin

producing strains P7 and UBMM Bo 89 were different from each other with respect to their carotenoids. Strain NIVA CYA 163, identified as *Spirulina subsalsa*, previously had been reported to contain considerable amounts of astaxanthin (Aakermann *et al.*, 1992) which we did not detect in any of our strains. Thus, from the data currently available, no obvious correlation of carotenoid composition and any other trait of these cyanobacteria emerges.

TABLE 4. Pigment composition in cyanobacterial strains with *Spirulina*-like morphology. Reported are the presence or absence of phycoerythrin, the total number of carotenoids detected, and the relative molar ratio of each of the major carotenoids with respect to β -Carotene.

Strain	Phycobiliprotein*			Carotenoids total number of carotenoids	Myxoxantho- phyll	Iso- zeaxanthin	unidentified carotenoid 1	Zeaxanthin	Cantha- xanthin	unidentified carotenoid 2	unidentified carotenoid 3	unidentified carotenoid 4	Astaxanthin	Lichonene	β -Carotene
	PC	PE	PEC												
MPI S3	+	-	-	7	0.50	-	-	-	0.59	-	-	-	-	-	0.50
MPI 95SSD1	+	-	-	5	0.18	-	-	-	1.86	-	-	-	-	-	0.73
MPI 95SLJ1	+	-	-	5	2.05	-	1.05	-	-	-	-	-	-	-	0.81
MPI S1	+	-	-	6	1.05	-	-	-	1.00	-	0.14	-	-	-	0.95
MPI S2	+	-	-	5	0.38	2.12	-	-	-	0.46	-	-	-	-	-
MPI S4	+	-	-	3	-	1.42	-	-	-	-	-	-	-	-	-
P7	+	+	-	6	-	1.36	-	0.36	-	0.59	-	1.41	-	-	-
UBMM B-89	+	+	-	3	-	0.49	-	-	-	0.12	-	-	-	-	-
UBMM H45	+	-	-	4	-	-	-	-	2.57	-	0.29	-	-	-	2.36
PCC 6313	+	-	-	6	0.81	-	-	-	1.24	-	0.14	-	-	-	1.14
CCC Snake P. Y. 85	+	-	-	5	0.33	-	-	-	1.22	-	0.11	-	-	-	0.41
NIVA CYA 163 [†]	?	?	?	9	0.09	-	-	1.25	-	-	-	-	0.56	-	0.03
NIVA CYA 164 [†]	?	?	?	8	0.02	-	-	0.44	-	-	-	-	-	-	0.02

* PC, phycoerythrin; PE, phycoerythrin; PEC, phycoerythroxanthin.
[†] The composition of phycoerythrin in NIVA CYA 163 and 164, however, the latter has been reported to display red trichome color (Ackermaun *et al.* 1992), thus, most likely contains phycoerythrin.

16S rRNA gene sequences and phylogeny. 16S rRNA gene sequences were deposited in the European Molecular Biology Laboratory (EMBL) database under accession numbers Y18789-Y18799. Similarities among (partial and almost complete) 16S rRNA gene sequences from cyanobacteria and other selected bacteria are presented in Table 5. A tree based on maximum likelihood computation is illustrated in Figure 4. Since phylogeny reconstruction applying the maximum likelihood method is computationally very expensive, bootstrap values were determined based on maximum parsimony (1,000 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, MPI 95SS01, MPII 95SL01) are 98.6% or more similar to each other, 7.7% or more different from all other cyanobacteria, and consistently cluster together in reconstructed phylogenetic trees regardless of the calculation methods applied and supported by results of bootstrap analysis. The second cluster encompasses six sequences from organisms of marine origin (P7, MPI S4, MPI S2, UBMM Bo 89, NIVA-CYA 163, NIVA-CYA 164) that are 95.5% or more similar to each other. The third cluster contains the 16S rRNA gene sequences from the two strains with openly coiled trichomes (PCC 6313, UBMM Hi 45), which in the stretch analysed (165-747, *E. coli* numbering) differ 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 basis of the Maximum Parsimony algorithm or on 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.*, 1998). It must be noted here, however, that neither biological material nor illustrations of strains M-223 and NIVA-CYA 163/164 were available to us, and therefore tight trichome coiling in these strains was not confirmed during this work, but is assumed on basis of published assignments of the species epithet "*subsalsa*." (Ishida *et al.*, 1997; Skulberg, 1990). Strain SAG B256.80 ("*Spirulina laxissima*"), is not particularly closely related to any other strain included in this study.

TABLE 5. 16S rRNA gene sequence similarities (uncorrected) among cyanobacteria with *Spirulina* morphology and other selected bacteria.

Organism	% similarity to:																		
	MPI 95SS01	PCC 6313	P7	MPI S4	CCC Snake P Y-85	M-223	PCC 7345	PCC 9712	PCC 6301	PCC 7421	<i>E. coli</i>	MPI 95SL01	MPI S1	MPI S2	Hi 45	Bo 89	NIVA- CYA 163	NIVA- CYA 164	SAG H256.80
complete sequences*:																			
MPI S3	98.6	91.3	90.6	90.8	90.7	91.8	89.5	89.2	86.4	85.3	76.6	98.9	91.4	89.8	89.2	89.9	90.6	90.5	86.8
MPI 95SS01		91.6	91.3	91.3	91.0	92.3	89.8	89.6	86.6	85.9	76.6	99.2	91.4	89.8	89.0	90.1	91.2	91.2	87.0
<i>Spirulina major</i> PCC 6313			92.0	92.3	91.8	92.4	91.5	90.6	90.5	88.5	77.0	88.7	89.9	89.9	99.8	90.0	92.2	92.2	88.6
P7 (SAG 59.90)				99.4	92.7	93.0	90.0	89.5	87.3	86.1	76.8	91.0	92.6	98.0	89.7	95.5	97.5	97.5	87.1
MPI S4					93.2	93.0	90.5	89.7	87.8	86.2	77.3	90.4	91.9	99.3	89.9	96.2	97.9	97.9	87.0
CCC Snake P.Y-85						93.9	92.2	90.6	89.3	86.8	77.3	91.0	95.3	90.5	90.8	91.1	92.9	92.9	87.9
M-223							90.7	90.3	87.8	87.0	76.6	91.0	91.0	88.4	89.5	89.0	89.9	89.9	87.9
<i>Arthrospira platensis</i> PCC 7345								89.2	89.8	88.5	77.0	90.8	90.5	90.5	90.2	90.8	93.1	93.1	88.0
<i>Euhalothece</i> sp. PCC 9712 (MPI 95AH10)									88.1	86.9	76.2	87.9	88.0	87.5	88.1	86.7	88.1	88.0	88.2
<i>Synechococcus elongatus</i> PCC 6301										87.8	77.8	85.7	85.4	85.2	90.0	85.9	85.3	85.2	88.2
<i>Gloeobacter violaceus</i> PCC 7421											78.1	84.6	84.3	84.3	87.7	84.5	85.5	85.5	86.6
<i>Escherichia coli</i>												74.5	75.8	76.0	75.6	76.2	75.7	75.8	78.4
partial sequences*:																			
MPI 95SL01													91.4	90.1	89.2	90.5	91.8	91.8	87.1
MPI S1														91.6	90.4	91.8	91.3	91.3	88.5
MPI S2															89.9	96.4	98.1	98.1	86.4
Hi 45																90.2	91.0	91.0	89.0
Bo 89																	98.1	98.1	85.9
NIVA-CYA 163																		100	88.0
NIVA-CYA 164																			88.0

*(Almost) complete sequences run from nucleotides 32 to 1489 at least, partial sequences determined in this study run from nucleotides 170 to 746 at least, partial sequences from NIVA cyanobacterial strains have been determined by Rudi and coworkers (Rudi et al. 1997) from nucleotides 346 to 845 (*E. coli* numbering).

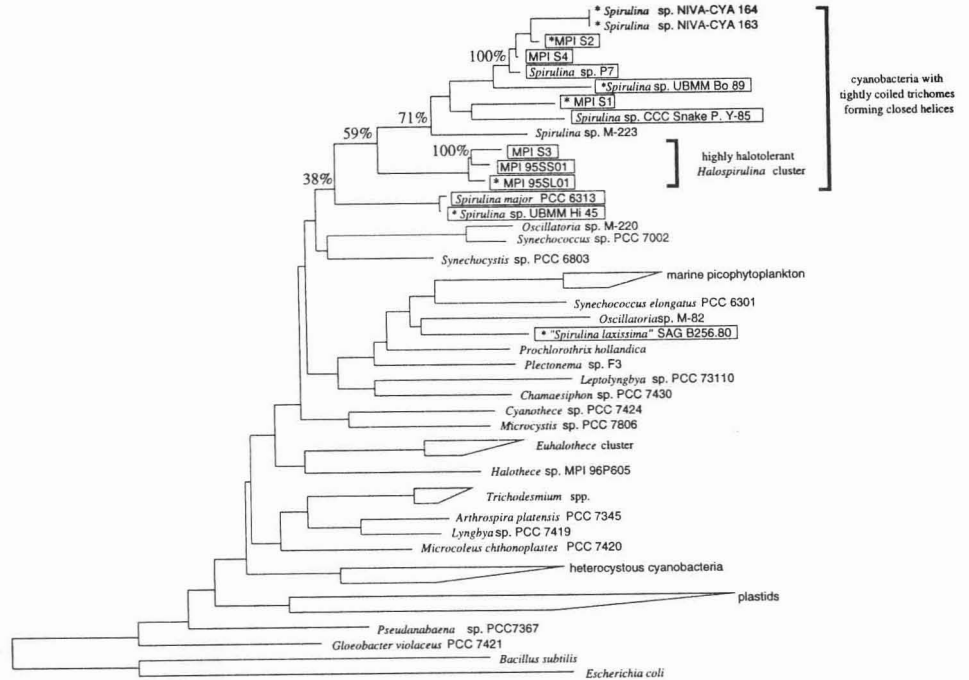


FIGURE 4. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences at least containing the nucleotides from 45 to 1455 (corresponding to the *E. coli* numbering (Brosius *et al.*, 1981). 16S rRNA gene sequences from *Escherichia coli* and *Bacillus subtilis* were used as outgroup sequences. Bootstrap values have been determined on basis of maximum parsimony calculations (1,000 replicates). The phylogenetic affiliations of organisms represented by partial sequences (strains are indicated by asterisks; partial sequences contain at least the nucleotides 170-746 or, in the case of strains NIVA CYA 163 and 164, nucleotides 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.

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 (Figure 4). The genetic divergence among these strains 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; Table 5), suggesting that *Spirulina* morphology is an early and stable development in the evolution of cyanobacteria. This is in accordance with microfossils resembling these recent cyanobacteria which date back 850 million years (Schopf, 1996). Strains PCC 6313 and UBMM Hi 45, representing cyanobacteria that would be identified as *S. major* according to morphology-based taxonomy because they are coiled more openly (Figure 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 it is suggested by the majority of phylogenetic trees calculated, bootstrap analysis failed to support this hypothesis. The short internodal branch in the phylogenetic tree (Figure 4) indicates that the separation of this group from other cyanobacteria is based on information provided by only 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 independent from their geographical origin. Phylogenetic analyses placed these three strains within a tight cluster clearly distinct from other cyanobacteria including the extremely halotolerant, unicellular cyanobacteria (Figure 4; Garcia-Pichel *et al.*, 1998). Growth in hypersaline environments requires a number of adaptations, with acquiring the ability to accumulate quaternary ammonium compounds as osmolites probably being 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 from freshwater and marine environments can be understood in terms of a separate evolutionary development based on the ecophysiological capability to exploit extreme environmental niches. Similar results were recently reported for unicellular cyanobacteria (Garcia-Pichel *et al.*, 1998) and phototrophic Bacteria of the families *Chromatiaceae* (Imhoff *et al.*, 1998) and *Ectothiorhodospiraceae* (Imhoff & Stiling, 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). This is supported by the large differences in G+C content of genomic DNA [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 maximally 10.4% (our calculation), and the DNA compositions of which span 38 to 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, 1989 b; Castenholz, 1989 c; Komárek & Anagnostidis, 1989) with a total of 80 different genera (Anagnostidis & Komárek, 1990; Komárek & Anagnostidis, 1989). This disproportion 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 microorganisms, 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 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 (García-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, are only distantly affiliated to their morphological counterparts from freshwater and moderate marine habitats, and are clearly distinct from all other cyanobacteria from which the 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 phylogenetic 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 trichome diameters (1.5 to 3.0 µm), have different carotenoids, and their 16S rRNA gene sequences differ by maximally 1.4%. According to traditional cyanobacterial taxonomy these strains would be classified as separate species based on trichome or helix diameter alone (Table 1; Anagnostidis & Golubic, 1966; Geitler, 1932). However, the high 16S rRNA gene sequence similarity indicates that additional data would be required to justify such a distinction (Stackebrandt & Goebel, 1994).

Strains PCC 6313 and UBMM Hi 45 have openly coiled trichomes and thus are morphologically similar to each other but distinct from all other strains. Interestingly, these strains were the only ones included in this study that were euryhaline in a sense 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 separated and deeply branching from the cluster of cyanobacteria with tightly coiled trichomes in which single helix turns touch each other. If strain PCC 6313 is accepted as the type strain for *Spirulina major* at the species and genus level as previously proposed (Rippka and 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 intended here and will require future studies. Additional physiological and genetic characters need to be investigated and the suitability of helix tightness as 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).

Description of *Halospirulina* gen. nov. Ha.lo.spi.ru.li'na. Gr. n. *halos* salt; L. n. *spira* a coil; L. n. *linea* a line; M. L. fem. n. *Halospirulina* salt tolerating coiled filament.

Halotolerant, euryhaline cyanobacteria with trichomes coiled into a tight helix, able to grow at salinities between 3 and 16‰ 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. They are found in sunlit hypersaline environments and tolerate temperatures for growth of at least 38°C.

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4

**Matching molecular diversity and ecophysiology
of benthic cyanobacteria and diatoms in communities along a
salinity gradient**

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Manuscript in preparation

Abstract

The phylogenetic diversity of oxygenic phototrophic microorganisms in hypersaline microbial mats and their distribution along a salinity gradient were investigated and compared to the halotolerances of closely related cultivated strains. Segments of 16S rRNA genes from cyanobacteria and diatom plastids were retrieved from mat samples by DNA extraction and PCR, and subsequently analysed by denaturing gradient gel electrophoresis (DGGE). Sequence analyses of DNA from individual DGGE bands suggested that the majority of these organisms was related to cultivated strains at levels which previously had been demonstrated to correlate with characteristic salinity responses. Proportional abundances of amplified 16S rRNA gene segments from phylogenetic groupings of cyanobacteria and diatoms were estimated by image analysis of DGGE gels, and generally were found to correspond to abundances of the respective morphotypes determined by microscopic analyses. The results indicated that diatoms accounted for low proportions of cells throughout, that the cyanobacterium *Microcoleus chthonoplastes* and close relatives dominated the communities up to a salinity of 11%, and that at a salinity of 14% the most abundant cyanobacteria were related to highly halotolerant cultivated cyanobacteria, such as the recently established phylogenetic clusters of *Euhalothece* and *Halospirulina*. While these organisms in cultures previously had demonstrated their ability to grow with close to optimal rates over a wide range of salinities, their occurrence in the field was restricted to the highest salinities investigated.

Introduction

It is a grand challenge for microbial ecologists to understand the distribution of microorganisms in nature in relation to their physiological capabilities. While the physiology of microscopically small organisms can be studied in great detail on strains growing in laboratory cultures, it has become increasingly apparent during the last decade that only a minority of the microorganisms occurring in nature is represented by cultivated isolates. During the last decade this insight has been promoted largely by the great advances achieved in the detection of extant microbial diversity through the culture-independent analyses of nucleic acids, particularly of small-subunit ribosomal RNAs and the respective genes (for reviews see Amann *et al.*, 1995; Embley & Stackebrandt, 1997; Pace, 1997; Ward *et al.*, 1998). It is a common observation that 16S rRNA gene sequences directly retrieved from environmental samples rarely match those of cultivated strains or are only very distantly related to these (e. g., Barns *et al.*, 1994; Ferris *et al.*, 1996; Giovannoni *et al.*, 1990; Hugenholtz *et al.*, 1998; Ward *et al.*, 1990). Under such circumstances, it is not possible to infer the organisms' ecologically relevant phenotypic properties from their phylogenetic relationships. Consequently, the distributions of nucleotide sequence clusters along environmental gradients generally are poorly understood in terms of the putative ecological niches of the underlying microorganisms (Field *et al.*, 1997; Massana *et al.*, 1997; Ruff-Roberts *et al.*, 1994; Ward *et al.*, 1998). In contrast, after previous isolation of representative cyanobacteria (Moore *et al.*, 1998; Urbach *et al.*, 1998), recently the depth distribution of genetically different oceanic picophytoplankton populations could in part be explained by their differential adaptations to light intensity and spectral composition (Ferris & Palenik, 1998).

We have attempted to unravel relationships between the ecology and diversity of benthic cyanobacteria and diatoms in hypersaline environments. In evaporation ponds of the saltern in Guerrero Negro, Baja California, Mexico, the primary production of these microorganisms is the basis for the existence of microbial mat ecosystems at salinities from approximately 6% to 16% (for reviews and detailed descriptions of these sites see Des Marais, 1995; Javor, 1989). Despite the considerable interest among biogeochemists and microbiologists in these systems, until recently only a limited number of cyanobacterial and diatom isolates from hypersaline environments has been characterized physiologically and phylogenetically. During the last years, however, this situation has changed to some extent. A significant number of clonal cultures has been recovered from microbial mats and characterized with respect to their phylogeny, morphology, and physiology, the latter particularly concerning salinity responses (Garcia-Pichel *et al.*, 1998; Garcia-Pichel *et al.*, 1996; Karsten, 1996; Clavero *et al.*, in preparation; Nübel *et al.*, submitted). It remains to be demonstrated if the isolates now available adequately represent the organisms flourishing in hypersaline mat ecosystems and if the physiological data gained from laboratory cultures can be confidently extrapolated to field

conditions. Since the comparative analysis of 16S rRNA gene sequences may offer the means to examine the congruences or discrepancies between strain collections and natural microbial communities, we recently developed a polymerase chain reaction (PCR) protocol to retrieve 16S rRNA gene segments from cyanobacteria and from plastids of eukaryotic phototrophs from environmental samples (Nübel *et al.*, 1997). Here, we report on the application of this tool in combination with denaturing gradient gel electrophoresis (DGGE) to investigate the distribution of 16S rRNA gene sequences from oxygenic phototrophic microorganisms in microbial mats along a gradient of salinity. Data on the abundance of specific morphotypes generally supported the results obtained. Comparisons to studies on cultivated clonal strains unveiled a general pattern of adaptations of cyanobacteria and diatoms to life in hypersaline environments.

Material and methods

Sampling. Sampling sites were located in evaporation ponds of the saltern in Guerrero Negro, Baja California Sur, Mexico (Des Marais, 1995; Javor, 1989). Microbial mats were sampled in April 1996 (mats P2, P4, and P6) and April 1997 (P3/4 and P5). Mat designations correspond to designations of evaporation ponds 2 to 6, along which a gradient of salinity is maintained which ranges from 5.5% to 14% total salt concentration (Nübel *et al.*, in press). Macroscopic characteristics of the mats investigated and field conditions have been described previously (Nübel *et al.*, 1999). For microscopic and molecular biological analyses triplicate cores 10 to 20 cm apart were taken as samples from each mat. For light microscopy the mat samples were fixed in 5% formaldehyde and stored at 4°C. For nucleic acid extractions the mat samples were frozen on site, transported to the laboratory in liquid nitrogen, and stored at -80°C until processed. Subsequent analyses were restricted to the photic zones of each of the mats, defined by the maximum depths where gross photosynthesis could be detected with oxygen microelectrodes in a field laboratory (Garcia-Pichel *et al.*, 1999).

Microscopy. The layers corresponding to the photic zones were cut from formaldehyde-fixed mat samples with scalpel blades. Pieces representing approximately 0.5- by 0.5-mm mat surface area were placed on glass slides and chopped and stirred to achieve even distribution. Randomly chosen phase-contrast microscopic fields were investigated at 400-fold magnification and 2,000 to 3,000 cells of cyanobacteria and diatoms per sample were counted to assure representative results (Nübel *et al.*, 1999). Green non-sulfur bacteria (*Chloroflexaceae*) were distinguished from cyanobacteria by their lack of visible fluorescence and omitted from the analyses.

Molecular biological techniques. DNA extraction, PCR, and denaturing gradient gel electrophoresis (DGGE) have been described previously (Nübel *et al.*, 1997; Nübel *et al.*, 1999). Briefly, the layers corresponding to photic zones were aseptically cut from mat cores

(representing approximately 60 mm² of mat surface) and homogenized in Dounce tissue homogenizers (Novodirect, Kehl, Germany). The suspensions were repeatedly frozen and thawed and subsequently incubated in the presence of sodium dodecyl sulfate and proteinase K. Cell lysis was controlled microscopically. DNA was extracted by applying hexadecyltrimethylammonium bromide, phenol, chloroform, and isoamyl alcohol and precipitated by the addition of isopropyl alcohol. The oligonucleotide primers CYA359F and CYA781R were applied to selectively amplify 16S rRNA gene segments from cyanobacteria and plastids (Nübel *et al.*, 1997). The numbers in the primer designations refer to the 5' ends of target signature sites in 16S rRNA genes (*Escherichia coli* nucleotide numbering (Brosius *et al.*, 1981)). A 40-nucleotide GC-rich sequence was attached to the 5'-end of the primer CYA359F to improve the detection of sequence variation in amplified DNA fragments by subsequent DGGE. As templates for amplifications, 10 ng of DNAs extracted from mat samples was added to each 100- μ l reaction mixture. 500 ng of amplified DNA was applied to denaturing-gradient gels. For DGGE, polyacrylamide gels with a denaturant gradient from 20 to 60% were used and electrophoreses were run for 3.5h at 200V and 60°C. Ethidium bromide stained gels were irradiated with UV light and photographed with a digital image gel documentation system (Cybertech, Berlin, Germany). The intensities of gel band fluorescences were measured on digital images by applying the gel-plotting macro implemented in the NIH-image software package version 1.62 (National Institutes of Health, Bethesda, Md.). Fluorescence values of individual bands were transformed into amounts of DNA by comparison to a DNA mass calibration standard for DGGE (Fig. 1; Nübel *et al.*, 1999). To determine nucleotide sequences of DNA molecules forming individual bands in DGGE, the bands were cut from the gels and incubated for three to twenty days in 50 μ l TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) at 4°C for elution of DNA. 1 μ l of these solutions were applied as templates in PCRs applying the primers CYA359F and CYA781R. The resulting reamplification products were checked by DGGE analysis for correspondence to the bands of interest, purified by applying the QIAquick PCR purification kit (Diagen, Düsseldorf, Germany), and sequenced by applying the Applied Biosystems PRISM Dye Terminator Cycle Sequencing Ready reaction kit and 377 DNA sequencer. For the majority of bands sequences of both DNA strands were determined by using the same primers as those used for amplification, however, while omitting the GC-clamp from primer CYA359F.

Phylogeny reconstruction. Cyanobacterial and plastid 16S rRNA gene sequences available from GenBank 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>. A phylogenetic tree was constructed on the basis of almost complete sequences (from nucleotide positions 45-1455 corresponding to the *E. coli* numbering) by applying the maximum likelihood method as integrated in the ARB software package. Alignment positions at

which one or more sequences had gaps or ambiguities were omitted from the analyses. Partial sequences (including those representing DGGE bands) were integrated in the dendrogram 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

16S rRNA gene sequences retrieved from microbial mats. Total nucleic acids were extracted from mat samples prior to the retrieval of 16S rRNA gene segments from cyanobacteria and diatom plastids by PCR by applying phylum-specific primers (Nübel *et al.*, 1997). Sequence-dependent separation of the amplification products by DGGE resulted in band patterns shown in Figure 1. Fluorescence measurements and comparisons to a DNA mass calibration standard (Figure 1) enabled the calculation of the amounts of DNA in individual gel bands. DNA could be eluted, reamplified, and sequenced (indicated in Figure 1) from 29 out of 42 bands present. Bands for which we were unable to obtain reliable sequences, each accounted

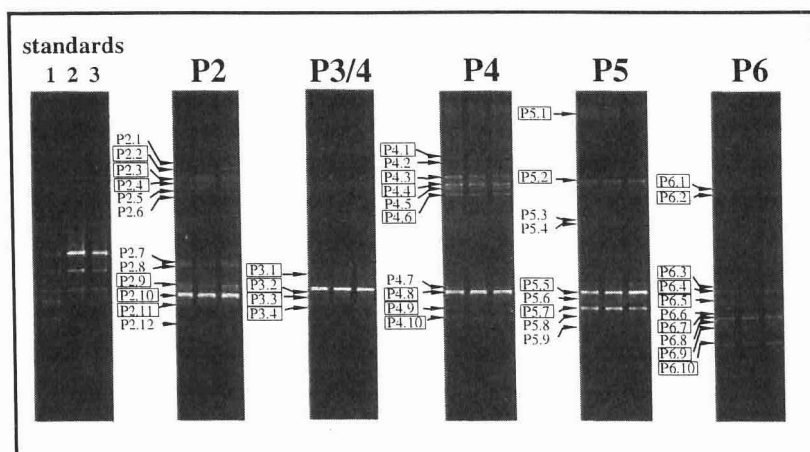


FIGURE 1. Composite figure of ethidium bromide-stained DGGE separation patterns of PCR-amplified segments of 16S rRNA genes. Results derived from triplicate sampling cores are shown for each of the five microbial mats investigated. Arrows indicate the positions and designations of the DNA bands included in the subsequent analyses. Framed designations indicate bands from which nucleotide sequences could be retrieved. Mixtures of PCR products derived from five cyanobacterial strains were applied to each gel as standards (in lanes 1 to 3 [top to bottom] are *Scytonema* sp. strain B-77-Scy.jav., *Synechococcus elongatus* SAG 1402-1, *Microcoleus chthonoplastes* MPI-NDN-1, *Geitlerinema* sp. strain PCC 9452 [*Microcoleus* sp. strain 10 mfx], and *Euhalothece* sp. strain PCC 7418). The standard in lane 1 allows gel-to-gel comparisons. The DNA mass calibration standard in lanes 2 and 3 enables the transformation of measured band fluorescence values into amounts of DNA (in lane 2, the amounts of DNA in individual bands are [top to bottom] 528, 176, 59, 20, and 7 ng; in lane 3, half of the amount of the standard in lane 2 was applied).

for 0.5% to 4.4% (mean, 1.9%) of the total PCR products visible as fluorescent bands after DGGE. Besides the generally low amounts of DNA forming those bands, potential reasons for difficulties with PCR reamplifications and subsequent sequence analyses might have been insufficient separation of sequence-different DNA molecules by DGGE (e. g., bands P4.7, P6.6, P6.8). Similarly, the reamplification of DNA from bands P2.7 and P2.8 resulted in PCR products migrating in subsequent DGGE at the position of band P2.10 (not shown), a problem which is possibly caused by the reamplification of incompletely focused predominant sequence populations visible as "background smear" in the primary DGGE gel (Figure 1). We did not observe, however, reamplification products forming patterns of bands in DGGE that would be consistent with the interpretation as being the result of the generation of heteroduplex DNA molecules during PCR (Ferris & Ward, 1997).

Nucleotide sequences obtained in this manner had a length of 247 to 381 nucleotides (mean, 335 nucleotides), the latter corresponding to the complete gene segment amplified. These partial 16S rRNA gene sequences were sufficient in all but five cases to identify very close relatives among cultivated cyanobacteria and diatoms and could be robustly integrated in the phylogenetic tree (Figure 2). In all microbial mats except in that from evaporation pond 2, 16S rRNA gene segments were found that were identical in sequence to those from several *Microcoleus chthonoplastes* strains that recently had been isolated from this and various other habitats (Garcia-Pichel *et al.*, 1996). From the community P2, two 16S rRNA gene sequences were retrieved that differed from the above sequences at single nucleotide positions each. A number of sequences were detected that are similar or identical, respectively (95.3-100%), to those from cultivated cyanobacteria of the recently described *Euhalothece* cluster (Garcia-Pichel *et al.*, 1998). Other sequences retrieved were highly similar (98.3-99.7%) to those from cultivated cyanobacteria of the genera *Halospirulina* (Nübel *et al.*, in preparation) and *Lyngbya* (strain MPI FGP-A was isolated from a microbial mat in the same area), or from the strain "*Oscillatoria limnetica*" (originating from hypersaline Solar Lake, Egypt (Cohen *et al.*, 1975)). Several sequences were obviously derived from diatom plastids. Only five sequences could not be placed in close affiliation to any oxygenic phototrophs for which 16S rRNA gene sequences have been determined to date. One (from band P3/4.4) seemed loosely affiliated to the marine filamentous cyanobacterium *Oscillatoria* sp. strain CJ1, others were deeply branching within the cyanobacterial radiation. Two of these as yet unidentifiable sequences (from bands P4.9, P5.7), however, significantly clustered together, which could be confirmed by calculating phylogenetic trees with various data subsets (not shown). In mat P5, PCR product derived from organisms of this cluster amounted to 28% of the total amplified DNA visible in DGGE bands. In all other mats, however, the relative contribution of unidentified sequences (including unsequenced bands) to the total was only minor, and 87% or more of the total PCR products could be traced unequivocally to cyanobacteria and diatoms that are closely related to known, cultivated strains. Although 16S rRNA gene sequences not perfectly matching genes from isolates were detected, our data suggest, in contrast to impressions left by previous studies (e. g., on hot spring mats),

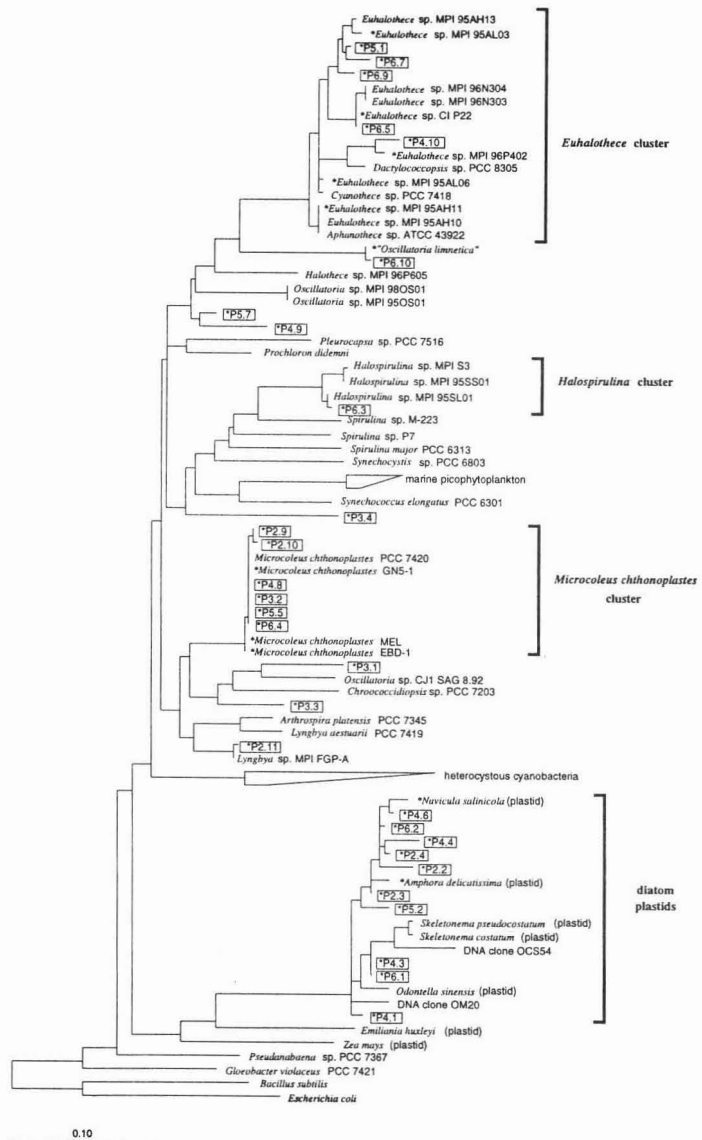


FIGURE 2. Maximum likelihood phylogenetic tree based on 16S rRNA gene sequences at least containing the nucleotides from 45 to 1455 (corresponding to the *E. coli* numbering [Brosius *et al.* 1981]). 16S rRNA gene sequences from *Escherichia coli* and *Bacillus subtilis* were used as outgroup sequences. The phylogenetic affiliations of organisms represented by partial sequences (indicated by asterisks) were reconstructed by applying the parsimony criteria without changing the overall tree topology (see ARB manual; Ludwig *et al.* 1998). Nucleotide sequences derived from DGGE bands are indicated by band designations (compare Figure 1) which are framed.

that the cultures currently available represent well the phylogenetic diversity of oxygenic phototrophs present in these hypersaline environments.

The distribution of 16S rRNA genes and morphotypes among mats. Compared to other microorganisms, cyanobacteria and diatoms display relatively complex cell and colony morphologies. This is the basis for their traditional classification (Anagnostidis & Komárek, 1985; Castenholz & Waterbury, 1989; Round *et al.*, 1990). The reliability of morphological criteria for the identification of phylogenetically and ecologically coherent taxa, however, may to a large extent depend on the particular organisms of interest and the habitats investigated. From the data available for cyanobacteria, it appears that the simpler the morphology the more uncertain predictions of phylogenetic placement based on morphology become (Ferris *et al.*, 1996; Garcia-Pichel *et al.*, 1996; Nübel *et al.*, submitted). For well studied groups, however, microscopic observations may yield valuable information about the distributions of these organisms in nature. For example, filamentous cyanobacteria prevalent in many microbial mats the world over and microscopically identifiable as *Microcoleus chthonoplastes* were demonstrated to have virtually identical 16S rRNA gene sequences (Garcia-Pichel *et al.*, 1996). In contrast, unicellular cyanobacteria and those with tightly, helically coiled trichomes turned out to be phylogenetically very diverse. However, in both these cases, basic morphology together with the ability to thrive at high salt concentrations defined tight monophyletic clusters of organisms. We termed the cluster *Euhalothece* (Garcia-Pichel *et al.*, 1998) and proposed the genus *Halospirulina* (formerly classified as *Spirulina* on the basis of morphology alone; Nübel *et al.*, submitted), respectively. Diatoms form a phylogenetically coherent group and are easily discernable from cyanobacteria by light microscopy.

On the basis of these considerations we can compare light-microscopic observations to analyses of 16S rRNA genes from microbial mats. Figure 3 illustrates, in relation to ambient salinities, the proportional abundances of morphological and phylogenetic groupings in the different mat samples based on cell counts and amounts of DNA measured in DGGE bands, respectively. Some technical limitations inherent to these quantifications such as potential PCR amplification biases and overestimations of abundances of large cells cannot be excluded here with certainty and have been discussed previously (Nübel *et al.*, 1999) and. It should be noted, however, that DGGE analysis is more suitable to quantitatively analyse the composition of PCR products than the more widely applied molecular cloning followed by subsequent random picking and screening of transformants. While in the latter approach usually less than a few hundred clones can be analysed, microscopic investigations of the (low diversity) oxygenic phototrophic mat communities studied here revealed that at least 2,000 to 3,000 individuals (cells) needed to be included in an analyses to enable stable estimates of proportional abundances (Nübel *et al.*, 1999). DGGE band patterns in each of the gel lanes in Figure 1 are composed of approximately 10^9 DNA molecules, and thus can be expected to adequately reflect proportional abundances of sequence-different amplification products (and, presumably, genes). A more serious problem

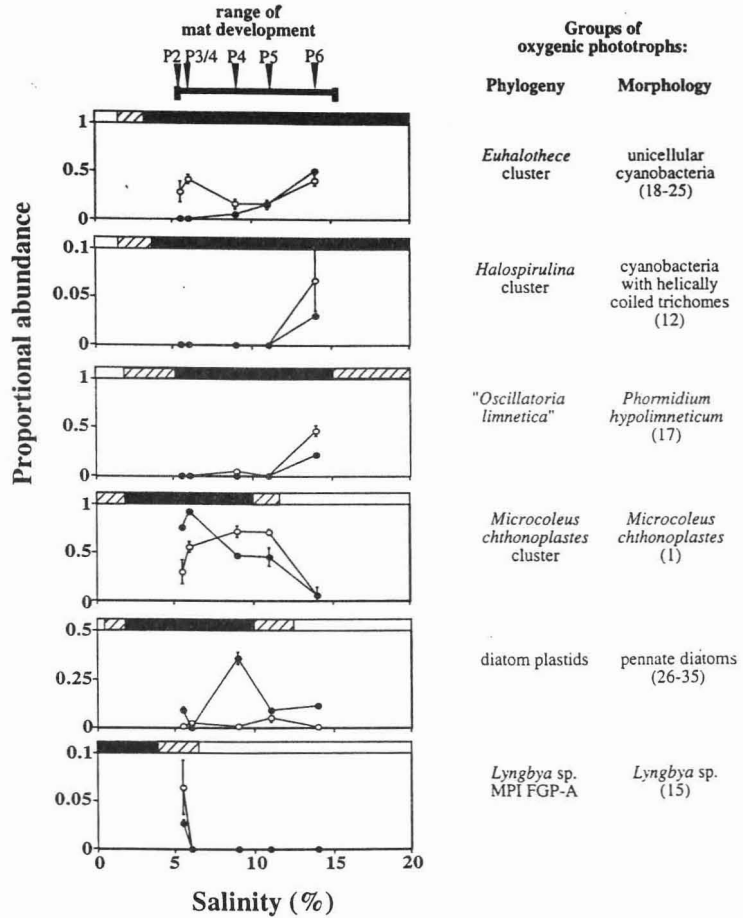


FIGURE 3. Proportional abundances of various groups of oxygenic phototrophs in microbial mats in relation to ambient salinity; note the different scales on the ordinate axes of the different panels. Means and standard errors from triplicate analyses are shown based on cell counts (open circles) and amounts of DNA measured in DGGE bands (closed circles), respectively. Bars on top of each panel illustrate the salinity tolerances for growth of cultivated strains of the phylogenetic groups investigated. Black bars indicate growth with at least half-maximal rates (optimal salinity ranges), hatched bars indicate growth with less than half-maximal rates (suboptimal salinity ranges). Phylogeny- or morphology-based definitions of groups of organisms investigated are given in the two columns on the right. Numbers in brackets correspond to morphotype illustrations in (Nübel *et al.* 1999). For taxon designations see text. In addition, the range of salinities is indicated within which microbial mat development occurs in the saltern system studied.

for the comparison of cell numbers to gene abundances might be the varying number of 16S rRNA genes per cell depending on the organisms' specific identities. Microalgal plastids have been reported to contain up to 650 copies of small circular genomes (Ersland *et al.*, 1981) while prokaryotes possess only 1 to 15 copies of rRNA operons. Possibly, these differences in gene copy numbers caused the proportional abundances of plastid derived PCR products to account for up to 36% of the total, whereas in terms of cell numbers diatoms were comparatively rare creatures with proportional abundances of maximally 4.7% and usually around or below 1% (Figure 3). Despite the potential drawbacks, abundance estimates based on PCR and cell counts for the various groups of cyanobacteria were rather congruent, particularly for those mat communities originating from higher ambient salinities. Both approaches indicated that *Microcoleus chthonoplastes* was the dominant inhabitant of all mats except in pond 6, where the total salt concentration reached 14%. *Euhalothece*-related 16S rRNA gene sequences were found in mats from ponds 4, 5, and 6, with increasing abundance accompanying increasing salinity. These abundance estimates coincided with cell counts of unicellular cyanobacteria, however, a second abundance maximum for rod-shaped and spherical cyanobacteria was microscopically determined at low salinity in mats P2 and P3/4. Most likely those organisms are marine forms and not related to the highly halotolerant *Euhalothece* cluster. Phylogenetic analyses suggest that these simplest cell shapes evolved independently several times within the cyanobacterial radiation (Turner, 1997) and the correlation of unicellular morphology and phylogenetic affiliation to *Euhalothece* will hold only in high-salinity habitats (Garcia-Pichel *et al.*, 1998). *Halospirulina*-related sequences were found in P6 exclusively, coinciding with the detection of sheathless, helically coiled filaments, which is the characteristic appearance of these cyanobacteria (Nübel *et al.*, in preparation). Similarly, the only sequence derived from an organism related to the isolate "*Oscillatoria limnetica*" [*Phormidium hypolimneticum sensu* (Campbell, 1985)] was detected at 14% salinity; filaments morphologically resembling this strain were abundant in this mat while rare or undetectable in all other samples. Sequences related to *Lyngbya* strains (*sensu* Castenholz [1989]) were found in mat P2 exclusively. This distribution is consistent with the microscopic detection of *Lyngbya*-like filaments.

Discussion

The results presented suggest that the phylogenetic diversity of oxygenic phototrophic microorganisms dominating the variety of microbial mats investigated along the salinity gradient is well represented by cultivated cyanobacteria and diatoms whose ecophysiology is known. In the samples originating from hypersaline brines [above 7% total salts (Por, 1980)] we were able to identify sequences as being derived from organisms closely related to groups of isolated

strains. The exception was a single cyanobacterial lineage awaiting bacteriological examination. Within established lineages, those 16S rRNA gene sequences that do not perfectly, but only closely, match previously known gene sequences, indicate the extent by which the diversity in the mats may exceed the diversity currently cultivated. The sequence divergence detected amounted to maximally 4.7% in the *Euhalothece* cluster, 5.6% in the clade of diatom plastids, and less than 0.6% in the remaining clusters. However, in none of the clusters the gene segments directly retrieved from the mats increased the maximum sequence divergence as established on the basis of cultivated strains. Because rRNA genes are highly conserved in function and structure, the sequence divergence observed may still correspond to significant, yet unknown, phenotypic differences among the underlying organisms (Palys *et al.*, 1997). However, for the clusters involved in this study, a remarkable ecophysiological uniformity in their salinity tolerance has been described (Clavero *et al.*, in preparation; Garcia-Pichel *et al.*, 1998; Garcia-Pichel *et al.*, 1996; Karsten, 1996; Nübel *et al.*, in preparation), indicating that the adaptation to life in high-salinity waters is an ancient and conserved evolutionary development that probably has been acquired by several lineages independently (Garcia-Pichel *et al.*, 1998; Nübel *et al.*, in preparation). Therefore, the cultures studied most likely are representative of the physiological tolerances to salinity of the majority of mat community components. Isolates of *Microcoleus chthonoplastes* and diatoms with various generic and specific identities were somewhat variable regarding their halotolerances, corresponding to the habitats they had been isolated from. Members of both groups, however, commonly preferred marine salinity and grew only poorly or not at all at total salt concentrations higher than 10% (Figure 3; (Clavero *et al.*, in preparation; Karsten, 1996). Cultivated strains of extremely halotolerant unicellular cyanobacteria formed one or possibly two phylogenetic clusters (including the monophyletic *Euhalothece* cluster) as indicated by their 16S rRNA gene sequences. Similarly, highly halotolerant cyanobacteria with helically coiled trichomes (*Halospirulina* spp.) are closely related to each other and distinct from their morphological counterparts in freshwater and temperate marine habitats. In addition, isolates representing these two latter groups can be termed extremely euryhaline because they are able to grow with close to optimal rates over wide ranges of salinities (Figure 3; (Garcia-Pichel *et al.*, 1998; Nübel *et al.*, in preparation). The phylogeny and halotolerances of relatives of *Lyngbya* sp. and "*Oscillatoria limnetica*" still await systematic investigations. However, we tentatively include here some preliminary data on these cyanobacteria because we detected related gene sequences and the respective morphotypes in some of the mat samples.

In terms of salinity, the fundamental ecological niches of cyanobacteria and diatoms of the different groups strongly overlap (Figure 3). However, the actual distribution of these organisms in the natural environment is much more restricted than dictated by their specific physiological tolerances to salinity. The development of cyanobacterial mats at salinities below 6% is limited by competition with larger eukaryotic algae (Des Marais, 1995) and increased grazing pressure (Javor & Castenholz, 1984). Gypsum precipitation, starting at a salinity of

approximately 16%, may prevent nutrient recycling from sediments and thereby demarcate an upper salinity limit for microbial mat maintenance (Javor, 1989). Within this range the mat communities investigated differed in composition along the salinity gradient (Figure 3). Most remarkably, perhaps, the highly euryhaline cyanobacteria (*Euhalothece*, *Halospirulina*, and, tentatively included here, "*O. limnetica*") are abundant in the most saline brines only. This restriction is probably due to interactions with hostile organisms such as competitors, predators, or parasites, the occurrence of which may again correlate with salinity. *M. chthonoplastes* dominated the mats up to 11% total salt concentration, above which its proportional abundance strongly decreased, coinciding with the upper limit for growth of related cultivated strains (Karsten, 1996). The reasons for the superiority of *M. chthonoplastes* are still little understood, however. Ecological niches must be considered multidimensional, and organisms that occupy overlapping (fundamental or realized) niches along one dimension such as salinity can be expected to differ with respect to some other property, such as their adaptations to light or nutrient availability or resistances to hostile substances or neighbors. Complex interactions of cyanobacteria, invertebrate grazers, and environmental gradients have been described for microbial mats in hot springs (Wickstrom & Castenholz, 1985) and may as well be expected here. In any case, our results indicate that the (post-interactive) distributions of cyanobacteria in nature in comparison to their physiological tolerances may be rather restricted. These realized niches (Begon *et al.*, 1990) do not necessarily center around the respective salinity optima as it had been hypothesized previously (Golubic, 1980). Highly halotolerant cyanobacteria acclimate to elevated ambient salinity by synthesizing quaternary ammonium compounds that function as osmolytes, but they keep the physiological potential to thrive at lower salinities (MacKay *et al.*, 1984; Reed & Stewart, 1988). Possibly, such a pattern of adaptations and distributions is rather common among microorganisms that through evolution have gained the ability to tolerate hostile environmental conditions.

The remarkable match of cultivation-based and molecular biological samplings of microbial diversity reported here in part certainly is due to the restriction to the study of a single functional group of organisms. In addition, the morphologies of cyanobacteria and diatoms in the habitats investigated to some extent correlate with their phylogeny, and thus microscopic examinations aid in the identification of organisms yet to be cultivated. This finding, however, suggests that the commonly found discrepancies between the compositions of strain collections and natural microbial communities to a considerable extent may arise from researchers' inability or neglect to recognize unique and predominant microorganisms (Pinhassi *et al.*, 1997) as opposed to their apparent immodesty or "unculturability". However, to fully appreciate the diversity of microorganisms, characteristics other than the highly conserved 16S rRNA genes need to be analysed (Palys *et al.*, 1997; Ward, 1998). Future research is required towards a more comprehensive understanding of the complex interactions of multiple physical, chemical, and biological factors that determine the composition of these microbial mat communities.

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**Quantifying microbial diversity:
morphotypes, 16S rRNA genes, and carotenoids
of oxygenic phototrophs in microbial mats**

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Abstract

We quantified the diversity of oxygenic phototrophic microorganisms present in eight hypersaline microbial mats on the basis of three cultivation-independent approaches. Morphological diversity was studied by microscopy. The diversity of carotenoids was examined by extraction from mat samples and HPLC analysis. The diversity of 16S rRNA genes from oxygenic phototrophic microorganisms was investigated by extraction of total DNA from mat samples, amplification of 16S rRNA gene segments from cyanobacteria and plastids of eukaryotic algae by phylum-specific PCR, and sequence-dependent separation of amplification products by DGGE. A numerical approach was introduced to correct for crowding the results of chromatographic and electrophoretic analyses. Diversity estimates typically varied up to two-fold among mats. The congruence of richness estimates and Shannon-Weaver indices based on numbers and proportional abundances of unique morphotypes, 16S rRNA genes, and carotenoids unveiled the underlying diversity of oxygenic phototrophic microorganisms in the eight mat communities studied.

Due to their physiological diversity, microorganisms play major roles in the cycling of chemical elements within the biosphere, but this relevance for environmental processes is only fragmentarily reflected in our current knowledge about microbial diversity (O'Donnell *et al.*, 1995; Pace, 1997) because the small size and morphological simplicity of microorganisms have hampered the study of their diversity. While microbial physiology and genetics can be investigated in great detail on cultivated isolates, the majority of microorganisms so far has resisted cultivation efforts (Pace, 1997). From most habitats studied, with only few exceptions (Pinhassi *et al.*, 1997), less than 1% of the microorganisms observed by microscopy have been brought into culture (Amann *et al.*, 1995). It is then clear, that present isolation procedures will fail to investigate microbial diversity extant in natural environments (Amann *et al.*, 1995; Embley & Stackebrandt, 1997). Molecular biological techniques, and particularly the study of small subunit ribosomal RNAs and the respective genes, have provided new insights into the phylogenetic diversity of microorganisms (Woese, 1987). Microbial nucleic acids extracted directly from environmental samples are amenable for comparative analyses of nucleotide sequences (Giovannoni *et al.*, 1990; Pace *et al.*, 1986; Ward *et al.*, 1990). Numerous publications based on this approach have reported the exploration of uncultivated microbial diversity in the last decade (Embley & Stackebrandt, 1997; Pace, 1997). However, our understanding of forces that shape and sustain microbial diversity in the environment and of the impact that microbial diversity may have on ecosystem processes is as yet very limited (Klug & Tiedje, 1993; O'Donnell *et al.*, 1995). Theoretically, empirical investigations of such interdependencies should lead to considerable progress in the field of microbial ecology, but such investigations depend unavoidably on the evaluation of biodiversity in quantitative terms. This quantification has not yet been achieved on the basis of the new molecular methodologies (O'Donnell *et al.*, 1995), but it is in principle possible and probably desirable (Garcia-Pichel *et al.*, in press).

The quantification of diversity requires the grouping of individual elements into non-overlapping classes according to a differentiating criterion (Kolasa & Biesiadka, 1984). If the study is to be restricted to certain organisms, which usually will be the case, individuals to be excluded from the analysis need to be identified as such. Ecological diversity is considered a function of the number of different classes (richness) and the relative distribution of individual elements among these classes (evenness) (Begon *et al.*, 1990; Washington, 1984). Various indices have been proposed as measures of diversity that incorporate both aspects, richness and evenness (Ludwig & Reynolds, 1988). The Shannon-Weaver index is the most common diversity index used by ecologists (Washington, 1984); it weights individual classes by their relative abundances. It can be understood as an estimator of the degree of uncertainty attached to the identity of any individual randomly selected from a community, which increases with richness as well as

with evenness (Legendre & Legendre, 1982). Optimally, individual elements in a class should be uniform with respect to their ecology. However, functional diversity, the actual ecologically relevant parameter, cannot be directly determined, and some deviation from this ideal must be expected when single criteria are used as basis for diversity determinations. Exposed to environmental selection, ecological units are also evolutionary units (Palys *et al.*, 1997; Ward, 1998), and the use of evolutionarily coherent entities as classes for diversity estimates is desirable. For practical reasons identification procedures should be as little time-consuming as possible, since often large numbers of organisms need to be investigated. Ecologists studying macroscopic plants and animals commonly use taxonomic species as classes for grouping individual organisms, and assess species richness and species diversity accordingly (Begon *et al.*, 1990). The delineation of species on the basis of morphologies is the most common practice (Claridge *et al.*, 1997), but it does not necessarily result in evolutionarily and ecologically coherent entities, particularly when applied to microorganisms. The determination of prokaryotic species richness and diversity in nature is impracticable because the current bacteriological species concept applies exclusively to organisms in pure cultures (Wayne *et al.*, 1987). The value of available species concepts for the quantification of diversity probably will depend on the group of microorganisms considered, and on the habitats to be studied; it may be necessary to substitute species by some other appropriate units of biodiversity (Klug & Tiedje, 1993).

We studied microbial mats from hypersaline waters in evaporation ponds of the saltern in Guerrero Negro, Baja California, Mexico, as well as from salt marshes in its proximity (Des Marais, 1995; Javor, 1989). The biomass of these benthic laminated ecosystems is almost exclusively composed of microorganisms, most of which are prokaryotes. We focused our investigations on communities of oxygenic phototrophs, whose activities are the basis for the existence of these mats. These microorganisms, namely cyanobacteria, diatoms, and, to a small proportion, green microalgae, traditionally have been classified on the basis of their morphologies (Anagnostidis & Komárek, 1985; Castenholz & Waterbury, 1989; Round *et al.*, 1990). Diversity studies on microalgae have been reported in the past that were restricted to morphotype analyses alone [e. g., Tinberg 1979)]. However, the phycological systems of classification do not necessarily reflect evolutionary relationships (Wilmutte, 1995; Medlin *et al.*, 1996; Pinevich *et al.*, 1997) and may underestimate diversity (Ferris *et al.*, 1996; Wood & Leatham, 1992). Therefore, we investigated the diversity of oxygenic phototrophic microorganisms by applying three cultivation-independent approaches in parallel. Classes for grouping the respective elements analysed were defined by unique cell and colony morphologies, ribosomal RNA gene sequences, and carotenoid molecule structures. Cells and molecules from organisms other than oxygenic phototrophs were excluded from the analyses. The relative

morphological complexity of oxygenic phototrophic microorganisms and their content of unique pigments allowed us to evaluate the diversity reflected in ribosomal RNA genes. The congruence of results obtained for eight different mat communities demonstrated that microbial diversity can be meaningfully quantified. Numbers and proportional abundances of unique morphotypes, carotenoids, and 16S rRNA genes could describe the diversity of oxygenic phototrophic microorganisms.

Materials and Methods

Sampling. Microbial mats were sampled during the second to fourth weeks of April of 1996 (mats P2, P4, P6, NC2, NC3) and 1997 (mats P3/4, P5, NC52). Sampling sites were located in evaporation ponds of the saltern in Guerrero Negro, Baja California Sur, Mexico and in the salt marsh of the Ojo de Liebre Lagoon. Detailed descriptions of these sites can be found elsewhere (Des Marais, 1995; Javor, 1989). Characteristics of the microbial mats investigated are summarized in Table 1. Measurements of photosynthesis were performed in a field laboratory with microsensor techniques (Revsbech & Jørgensen, 1986). Besides giving information about the thickness of the photic zone, based on the maximum depth where gross photosynthesis was detectable, we do not address the results of these supplementary studies in this report, but see (Garcia-Pichel, in press b). Three, 10 to 20 cm distant cores were sampled from each mat. For light-microscopy, mat samples (core diameter, 4 mm) were fixed in 5% formaldehyde (wt/vol) and stored at 4°C. For extractions of nucleic acids and carotenoids, mat samples (core diameter, 25 mm) were frozen on site, transported to the laboratory in liquid nitrogen, and stored at -80°C until processed.

TABLE 1. Characteristics of the microbial mats studied

Mat	Field conditions			Photic zone characteristics	
	Salinity (%) ^a	Water depth (m)	Origin	Thickness (mm)	Texture
P2	6	0.5	Evaporation pond	2.0	Soft
P3/4	6	0.5	Evaporation pond	1.5	Cohesive
P4	9	1.0	Evaporation pond	3.0	Cohesive, rubbery
P5	11	1.0	Evaporation pond	3.0	Cohesive, rubbery
P6	14	1.0	Evaporation pond	4.8	Gelatinous, translucent
NC2	5-8	0.2-0.5	Tidal channel	1.5	Soft
NC52	5-11	0.2-0.5	Tidal channel	1.6	Soft
NC3	6-21	0.3-0.6	Tidal channel	1.7	Very cohesive, rubbery, compact

^a Measured in the field site at time of collection. Salinities in evaporation ponds typically vary by less than 5% per year. The salinities and water depths in tidal channels vary with tidal cycles.

Microscopy and morphotype quantification. The layers corresponding to photic zones were cut from formaldehyde-fixed mat samples with scalpel blades, and sectioned vertically into subcores of approximately 0.5 × 0.5 mm mat surface area. These pieces were placed on glass slides in one drop of water and chopped and stirred to achieve even distributions. Sketches of morphotypes were prepared for illustration (Figure 1). Of each

subcore, 25 to 40 randomly chosen phase-contrast microscopic fields were photographed at 400-fold magnification. Counts and size measurements were performed on projections of the resulting slides, exclusively taking into account focused cells of oxygenic phototrophs. Diatom valves without plastids were omitted from the analyses. For unicellular organisms cell numbers were counted. For filamentous organisms total filament lengths were divided by the respective mean cell lengths to calculate cell numbers. Final cell numbers or total filament lengths were converted into biovolumes with geometric formulae (Garcia-Pichel *et al.*, 1994). Rounded cells were considered spheres, rod-shaped and filamentous organisms were considered cylinders. Naviculoid and nitzschoid diatom cells (Figure 1, morphotypes 26 to 30, 32, 33) were considered flat, elongated cylinders or prisms with elliptical or rhombic surface areas (valve view), respectively.

DNA extraction. The layers corresponding to photic zones were aseptically cut from mat cores (100 to 400 mg, representing approximately 60 mm² of mat surface) and homogenized in Dounce tissue homogenizers (Novodirect, Kehl, Germany). Cell lysis and DNA extraction were performed as described previously (Nübel *et al.*, 1997). Briefly, the suspensions were repeatedly frozen and thawed, and subsequently incubated in the presence of sodium dodecyl sulfate and proteinase K. Cell lysis was controlled microscopically. DNA was extracted by applying hexadecylmethylammonium bromide, phenol, chloroform, and isoamylalcohol, and precipitated by addition of isopropyl alcohol.

Polymerase chain reaction. Oligonucleotide primers CYA359F and CYA781R were applied to selectively amplify 16S rRNA gene segments from cyanobacteria and plastids (Nübel *et al.*, 1997). Numbers in primer designations refer to 5'-ends of target signature sites in 16S rRNA genes [*Escherichia coli* nucleotide numbering (Brosius *et al.*, 1981)]. A 40-nucleotide GC-rich sequence was attached to the 5'-end of the primer CYA359F to improve the detection of sequence variation in amplified DNA fragments by subsequent denaturing gradient gel electrophoresis [DGGE (Nübel *et al.*, 1997)]. As templates for amplifications 10 ng of DNAs extracted from mat samples were added to each 100 µl reaction mixture.

Denaturing gradient gel electrophoresis and digital image analysis. Amplification products generated by duplicate polymerase chain reactions with the same template DNAs were pooled and subsequently purified and concentrated by using the QIAquick PCR purification kit (Diagen, Düsseldorf, Germany). DNA concentrations in resulting solutions were determined by comparisons to the Gibco low DNA mass standard

(Gibco, Eggenstein, Germany) after agarose gel electrophoresis. 500 ng were applied onto denaturing gradient gels. DGGE was performed as described previously (Nübele *et al.*, 1997). Briefly, polyacrylamide gels with a denaturant gradient from 20% to 60% were used, and electrophoreses were run for 3.5 h at 200 V. Subsequently, gels were incubated for 30 min in 1× TAE (40mM Tris-HCl [pH 8.3], 20 mM acetic acid, 1 mM EDTA) containing 20 mg/ml ethidiumbromide. Fluorescence of dye bound to DNA was excited by UV irradiation applying a UV transilluminator and photographed with a digital image gel documentation system (Cybertech, Berlin, Germany). Intensities of gel band fluorescences were measured on digital images by applying the gel plotting macro implemented in the NIH-Image software package version 1.62 (National Institutes of Health, Bethesda, Md.). To enable the transformation of fluorescence values into amounts of DNA in individual bands we designed and applied on each gel a DNA mass calibration standard for DGGE, which was a mixture of PCR products with known concentrations (Figure 4).

Carotenoid extraction and analysis. Frozen samples of mat layers (100 to 400 mg, representing approximately 60 mm² of mat surface) corresponding to photic zones were ground in a mortar while cooled by liquid nitrogen. Ground samples were extracted in 10–15 ml of degassed acetone for 24 h in the dark at 4 °C. Extracts were clarified by filtration on Whatman GF/F glass fiber filters and subsequently concentrated under a stream of N₂ gas. Concentrated pigment extracts were separated and analyzed by HPLC with online detection by diode-array based spectroscopy between 350 and 700 nm, allowing for the detection of typical carotenoid spectra. Detailed chromatographic conditions and equipment were essentially as previously described (Karsten & Garcia-Pichel, 1996).

Estimation of richness and diversity. The richness and diversity of morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophic microorganisms were estimated. Classes for grouping organisms and molecules were defined by unique cell and colony morphologies, nucleotide sequences, and pigment molecule structures. Cells and molecules from organisms other than oxygenic phototrophs were excluded from the analyses.

Morphotype analysis was performed by light microscopy. Diatoms and cyanobacteria could be distinguished from most other microorganisms due to their size and characteristic morphologies. Epifluorescence microscopy was used to identify putative members of the *Chloroflexaceae*. These green filamentous bacteria occur in marine microbial mats (Pierson *et al.*, 1994) and may be mistakenly identified as cyanobacteria, but were distinguished by their lack of visible fluorescence. Proportional abundances of morphotypes were calculated on the basis of cell numbers and, alternatively, cell volumes.

16S rRNA gene fragments were amplified by polymerase chain reaction (PCR) from cyanobacterial and plastid DNA after nucleic acid extraction from mat samples (Nübel *et al.*, 1997). Phylum-specific amplification enabled the exclusion from the analyses of DNA from organisms other than oxygenic phototrophs (Nübel *et al.*, 1997). Numbers and proportional abundances of unique rRNA gene segments amplified were estimated after DGGE analysis of PCR products. Carotenoids were extracted directly from mat samples and their analysis by HPLC enabled the determination of numbers and abundances of unique pigments. Quantification of the relative abundance of specific carotenoids was based on area integration of peaks resulting from absorption at 470 nm, and no attempts were made to introduce corrections for differences in extinction coefficient. Peak delimitation and area integration were carried out automatically by the instrument's software. Absorption peaks in these chromatograms corresponding to pigments other than carotenoids (Chlorophyll a, phaeophytins, diverse bacteriochlorophylls, scytonemin) were identified from the corresponding absorption spectra (350-700 nm) and deleted from the automated analysis *a posteriori*. An example of this procedure is shown in Figure 5. The assumption was made that the contribution to total carotenoids of pigments from heterotrophic and chemotrophic bacteria was negligible, as phototrophs make up the bulk of the biomass in the photic zone and they contain much higher specific amounts of carotenoids than non-phototrophic bacteria. In those samples displaying measurable amounts of bacteriochlorophylls, a correction for carotenoids stemming from anoxygenic phototrophic bacteria was carried out. These were identified by comparison of retention times and spectra to carotenoid standards from purple sulfur and green filamentous non-sulfur bacteria. However, these corrections were seldom necessary. The majority of carotenoids detected could be assigned to typical cyanobacterial and diatom standards from cultivated strains.

Using the approaches described above for triplicate samples from each oxygenic phototrophic mat community the numbers D and proportional abundances a_i of classes i were determined. Shannon-Weaver indices (H') (Ludwig & Reynolds, 1988; Shannon & Weaver, 1949) were calculated as:

$$H' = -\sum_{i=1}^D a_i \ln a_i$$

In the following, subscripts refer to the respective analyses of morphotypes (M), rRNA genes (R), and carotenoids (C). Corrections for crowding of bands and peaks in electrophoretic and chromatographic analyses transformed D_R and D_C into richness estimates S_R and S_C (see below). D_M was used directly as an estimate of the richness of the morphotypes S_M . Arithmetic means and standard errors were calculated from results of triplicate analyses. Statistical analysis was performed by using software SPSS 6.1 (SPSS, Erkrath, Germany).

Correction for crowding of the number of classes measured by electrophoresis and chromatography. With increasing numbers of bands or peaks detected in electrophoretic or chromatographic analyses, respectively, the probability increases that classes cannot be discerned because they run at identical positions in the gel or chromatogram. In the following we describe a way to approximate this probability distribution and, on that basis, to estimate how many classes are likely to have been missed in a given analysis due to crowding.

Let S be the number of specific classes present in a sample, D the number of classes actually detected in the analysis, and D_{max} the maximum number of classes that the analytical procedure can detect. Due to crowding within the chromatogram or electrophoretic gel, for any given S there exists a certain probability to detect only D classes, where $D \leq S$ and $D \leq D_{max}$. A distribution $P_S(D)$ describing this probability can be used to correct measured values for crowding. Assuming that there are no preferred sites of occurrence for the classes within the chromatogram or electrophoresis gel, i.e., crowding is homogeneous, and that all classes can be detected with equal resolution, then the probability, p , that two classes in a sample with $S = 2$ cannot be discerned is

$$p = P_2(1) = \left(D_{max}\right)^{-1}$$

The probability that we measure D classes in a sample with $S+1$ specific classes, is

$$P_{S+1}(D) = P_S(D)Dp + P_S(D-1)(1 - (D-1)p)$$

Because $P_1(1) = 1$, if p is known, one can inductively calculate any value of $P_S(D)$.

Alternatively, $P_S(D)$ can be computed directly as

$$P_S(D) = \left(\frac{1}{D_{max}}\right)^S \binom{D_{max}}{D} \sum_{i=0}^{D-1} \left\{ (-1)^i (D-i)^S \binom{D}{i} \right\}$$

Once the values of $P_S(D)$ are computed, a simple correction consists of calculating expectation values for D , D_{exp} , for any given S using $P_S(D)$, as

$$D_{exp} = \sum_{D=1}^D D P_S(D)$$

Values of S that yield D_{exp} corresponding to a measured value of D can be taken as corrected estimates of richness.

Values for D_{max} were estimated on basis of average peak width and total distance of separation for our experiments to be 200 for DGGE and 96 for HPLC. Accordingly,

needed to be raised by one to three classes (i. e., by up to 12%). It was not attempted to take into account the effect of varying class frequencies on crowding, or to correct the calculation of Shannon-Weaver indices, as such corrections can be extremely complicated. Thus, our values for H'_c tend to underestimate carotenoid diversity.

Results

Diversity of morphotypes. Morphotypes that were distinguished are illustrated in Figure 1. In total, 36 different morphotypes were observed, 25 of which were cyanobacteria, 10 diatoms, and one green alga. The number of morphotypes detected in each of the specimens is considered an estimate of the morphotype richness S_M of the respective oxygenic-phototrophic community. Proportional abundances based on cell counts and, alternatively, estimated cell volumes, were used to calculate Shannon-Weaver indices H'_M (Table 2). Figure 2 illustrates the proportional abundances of morphotypes in two of the eight mats on the basis of cell counts performed on triplicate randomly drawn subcores. Numbers labelling individual bars in the histograms refer to morphotype numbers in Figure 1.

A marked patchiness in mat community structure was observed at the scale of cyanobacterial colonies and filaments (10-100 μm). Therefore, a significant dependence of S_M and H'_M on the number of microscopic fields investigated had to be expected. The minimum number of cells needed to achieve representative subsamples was determined for each of the mats by randomly removing slides (microscopic fields) one by one from the respective analyses and subsequently determining richness and Shannon-Weaver indices for the resulting samples of reduced sizes. For a low number of cells included in an analysis both parameters increased (not necessarily monotonically) concomitantly with increasing sample size and then, provided that a large enough number of cells was investigated, leveled off. A subsample was assumed to be sufficiently representative, if its further enlargement caused no further increase of the diversity determined (Pielou, 1966; Tinnberg, 1979). Figure 3 shows plots of these analyses for two specimens. Sample sizes needed to detect all rare and localized morphotypes (Figure 3A) and to weight them according to their actual abundances (Figure 3B) were estimated from such plots to be 2000 to 3000 cells. Significant heterogeneity of the distribution of organisms at the scale of millimeters caused major differences among microscopic analyses of triplicate preparations from the same mat. For example the surface of the mat from pond 5 consisted of a loose film of diatoms (*Nitzschia* sp., morphotype no. 30) forming

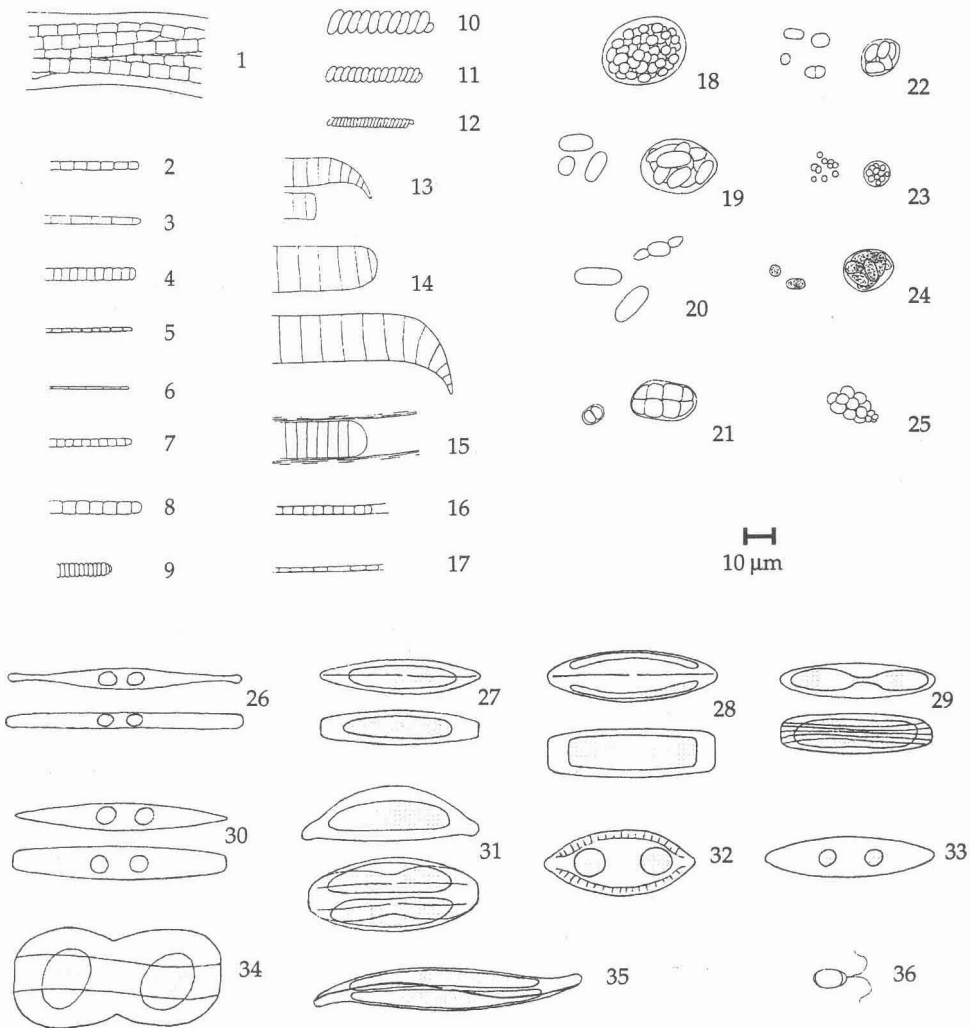


FIGURE 1. Morphotypes of oxygenic-photosynthetic microorganisms from eight mat communities as observed by phase-contrast light microscopy. Morphotypes 1 to 25 are cyanobacteria, 1 to 17 of which can be assigned to the *Oscillatoriales* and 18 to 25 to the *Chroococcales* (Castenholz and Waterbury, 1989). Morphotypes 26 to 35 are diatoms, to which the following generic assignments can be made (Round, et al., 1990): *Nitzschia*, 26, 30, 33; *Brachysira*, 27; *Navicula*, 28; *Amphora*, 31; *Mastogloia*, 32; *Entomoneis*, 34; *Gyrosigma*, 35. Morphotype 36 is a green alga (*Dunaliella* sp.).

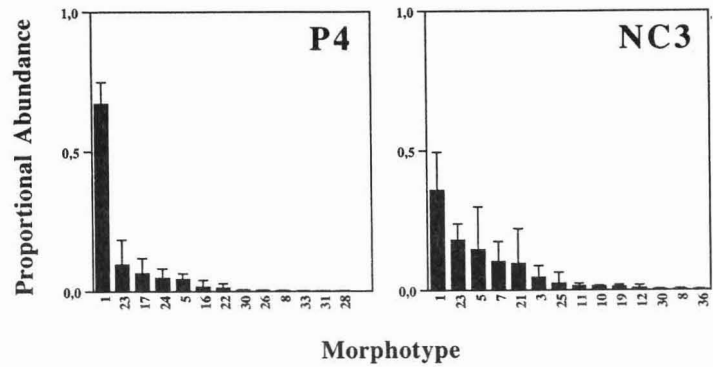


FIGURE 2. Proportional abundances of morphotypes based on cell counts. Morphotype numbers refer to Figure 1. Data are from analyses of communities in mats P4 and NC3. Because sets of morphotypes found in triplicate subscores from the same mat do not necessarily completely coincide, the cumulative number of morphotypes observed in a community may exceed the mean richness S_M (Table 2).

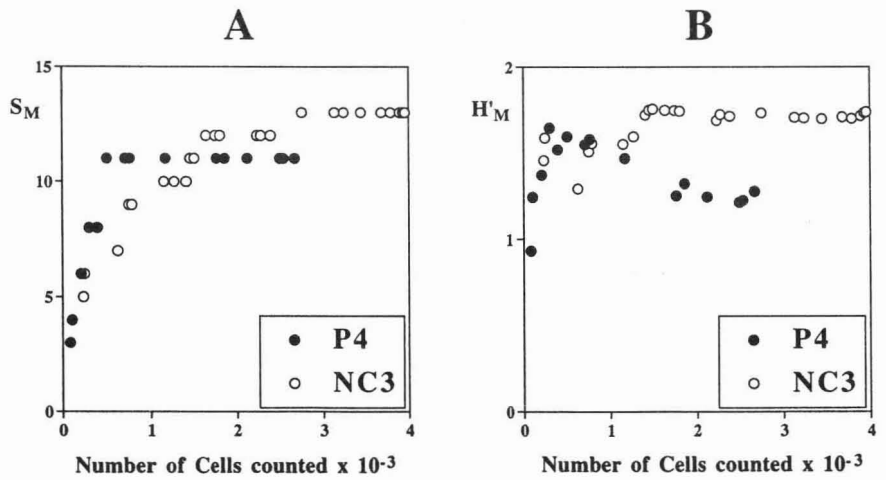


FIGURE 3. Relationship of S_M (A) and H'_M (B, based on cell counts) to the number of cells encountered. Data are from analyses of communities in mats P4 and NC3.

irregularly scattered tufts up to 1 mm in height and diameter. Concomitantly, depending on the exact site of sampling the relative proportion of diatoms in a randomly drawn subscore, representing only 0.5×0.5 mm mat surface, varied from 1.4 to 9.5 % of total cell numbers of oxygenic phototrophs. This level of patchiness resulted in large variances of estimates of both, abundances of individual morphotypes (Figure 2) and morphotype diversity as reflected in H'_M (Figure 7), whereas S_M was less affected. In all specimens the most rare morphotypes detected accounted for less than 1% of the total number of cells and biovolumes. Richness estimates and Shannon-Weaver indices varied almost two-fold among mats whereas coefficients of variation for each mat amounted to maximally 16%.

TABLE 2. Numbers of classes, richnesses, and Shannon-Weaver indices determined^a

Mat	Morphotypes			16S rRNA genes		Carotenoids		
	S_M	H'_M	H'_V	S_R	H'_R	D_C	S_C	H'_C
P2	10.67 ± 0.33	1.45 ± 0.09	1.82 ± 0.12	11.33 ± 0.66	1.38 ± 0.08	20.67 ± 0.66	22.67 ± 0.66	2.28 ± 0.03
P3/4	7.33 ± 0.33	0.98 ± 0.09	1.42 ± 0.17	4.00 ± 0.00	0.35 ± 0.02	9.33 ± 0.88	9.33 ± 0.88	1.62 ± 0.03
P4	10.33 ± 0.88	1.10 ± 0.14	1.07 ± 0.17	10.00 ± 0.00	1.75 ± 0.02	22.00 ± 1.15	24.00 ± 1.16	2.28 ± 0.02
P5	7.00 ± 0.58	1.02 ± 0.12	1.31 ± 0.03	6.67 ± 0.33	1.31 ± 0.09	17.00 ± 0.58	18.00 ± 0.58	1.97 ± 0.02
P6	9.67 ± 0.33	1.39 ± 0.05	1.72 ± 0.10	10.00 ± 0.00	1.90 ± 0.02	14.67 ± 0.33	15.67 ± 0.33	2.20 ± 0.03
NC2	12.00 ± 0.58	1.41 ± 0.21	1.68 ± 0.08	12.00 ± 0.00	2.09 ± 0.01	22.00 ± 1.00	24.00 ± 1.00	2.44 ± 0.02
NC52	12.00 ± 0.58	1.18 ± 0.13	1.65 ± 0.06	12.33 ± 0.33	1.74 ± 0.05	13.67 ± 0.66	14.00 ± 1.00	1.90 ± 0.03
NC3	12.67 ± 0.33	1.67 ± 0.04	1.29 ± 0.03	16.00 ± 1.00	2.08 ± 0.11	24.00 ± 1.00	26.67 ± 1.34	2.53 ± 0.02

^a D_C , number of classes detected; S , richness; H' , Shannon-Weaver index. The subscripts refer to the respective analyses of morphotypes (M), volumes of morphotypes (V), 16S rRNA genes (R), and carotenoids (C). The means and standard errors from triplicate analyses are shown.

Diversity of 16S rRNA genes. Total nucleic acids were extracted from triplicate mat samples and 16S rRNA gene segments were amplified from cyanobacterial and plastid DNA by applying a phylum-specific PCR that had recently been developed on the basis of published nucleotide sequences (Nübel *et al.*, 1997). The sequence-dependent separation of resulting amplification products by denaturing gradient gel electrophoresis (DGGE) (Muyzer *et al.*, 1993) generated band patterns that are characteristic for each of the mats (Figure 4). The number of bands visible in denaturant gradient polyacrylamide gels and corrected for crowding estimates the richness S_R . Fluorescence measurements and comparisons to a DNA mass calibration standard (Figure 4) allowed the calculation of amounts of DNA in individual gel bands, proportional abundances of sequence-defined populations, and Shannon-Weaver indices H'_R (Table 2). An exceptionally low diversity was detected in mat community P3/4. Among the other communities the variation of S_R was more than two-fold, and Shannon-Weaver indices varied from 1.31 to 2.09, with coefficients of variation derived from triplicate analyses smaller than 7%. The lower detection limit for ethidiumbromide-stained DNA in these gels was approximately 10 ng

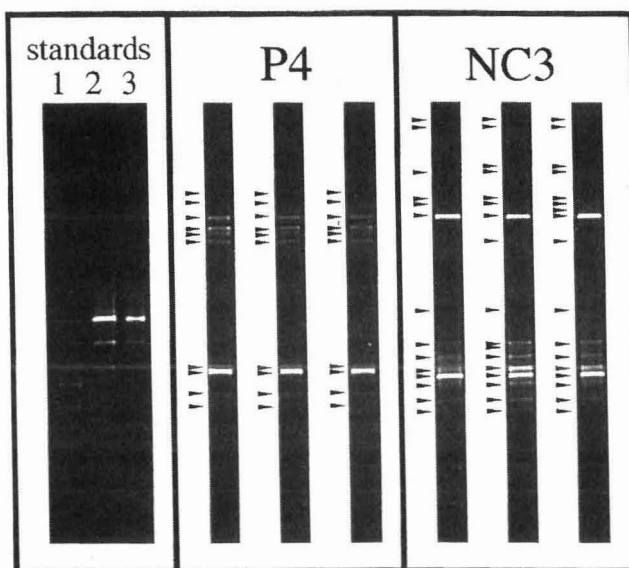


FIGURE 4. Composite figure of ethidium-bromide stained DGGE separation patterns of PCR-amplified segments of 16S rRNA genes. Mixtures of PCR products derived from five cyanobacterial strains were applied on each gel as standards (lanes 1 to 3, top to bottom: *Scytonema* sp. B-77-Scy.jav., *Synechococcus leopoliensis* SAG 1402-1, *Microcoleus chthonoplastes* MPI-NDN-1, *Geitlerinema* sp. PCC 9452 [*Microcoleus* sp. 10 mfx], *Cyanothece* sp. PCC 7418). The standard in lane 1 allows gel-to-gel comparisons. The DNA mass calibration standard in lanes 2 and 3 enables the transformation of band fluorescence values measured into amounts of DNA (lane 2, amounts of DNA in individual bands, top to bottom: 528 ng, 176 ng, 59 ng, 20 ng, 7 ng; lane 3, half of the amount of the standard applied as in lane 2). P4 and NC3: separation patterns of PCR products derived from triplicate sampling cores of microbial mats P4 and NC3. Arrowheads indicate bands included in the subsequent analyses.

per band, which is 2% of the total amount of PCR product applied onto each gel lane. To date DNA could be extracted, reamplified and sequenced from the majority of gel bands. This analysis should allow the determination of phylogenetic relationships among organisms forming the mats investigated and cultivated reference strains, which is outside the scope of the present paper. However, it may be significant to note that no "heteroduplex bands" (Ferris & Ward, 1997) could be detected and that all sequences determined were derived from cyanobacteria or plastids, so that, most probably, none of the bands represents any undesired amplification product.

Diversity of carotenoids. According to carotenoid pigment analysis, more than two-fold differences in richness could be observed among mats. Richness S_c was estimated to range from 9.33 ± 0.88 in mat P3/4 to 24.00 ± 1.00 in mat NC3 (Table 2). The corresponding estimates of the Shannon-Weaver diversity index, measured from the relative abundance of each carotenoid, varied between 1.62 and 2.53, almost two-fold. Crowding of peaks was likely to have affected the results of HPLC analyses (Fig. 5). While estimates of carotenoid richness could be corrected accordingly, this was not attempted for Shannon-Weaver indices. Thus, the latter may underestimate diversity. There may have been some misidentification events with carotenoids, particularly with the less abundant ones, as the spectra from small peaks were not always clear. These may have been either degradation products of original phototrophic carotenoids or may have stemmed from other bacteria.

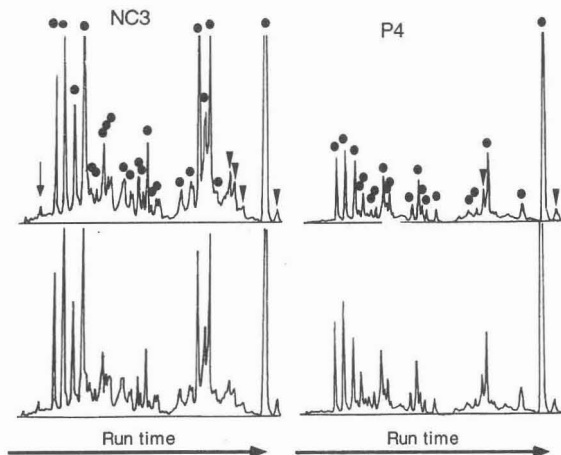


FIGURE 5. Carotenoid analysis by HPLC in two mats, P4 and NC3. Two independent analyses for each mat are shown (across). Each peak in the 470 nm-chromatogram was assigned to either a carotenoid (peaks crowned by solid dots), a tetrapyrrol (Chlorophylls, phaeophytin, crowned by arrowheads) or scytonemin (crowned by arrow) on the basis of absorption spectra obtained on-line.

Discussion

Units of biodiversity. Biologists studying the ecology of communities of plants or animals usually choose species as basic units for diversity estimates. For prokaryotes this is currently impossible because of the bacteriological practice to delineate species on the basis of cultivated strains (Wayne *et al.*, 1987). At present, probably far less than 5% of extant cyanobacterial species are successfully cultivated (Castenholz, 1992) and only a minority of these cultures is axenic (Castenholz & Waterbury, 1989). This disproportion reflects general problems of obtaining, within a scientist's lifetime, a collection of strains that represents microbial richness extant in an environmental sample. In addition, the current bacteriological species concept yields groupings, that are not equivalent to species of larger organisms (Staley, 1997), and that do not necessarily correspond to real ecological units (Palys *et al.*, 1997; Ward, 1998). In contrast, the traditional phycological taxonomy enables the identification of morphological species without the need of cultivation, which is advantageous for ecological studies (Anagnostidis & Komárek, 1985). But especially for organisms with less complex morphologies, such as unicellular or simple filamentous cyanobacteria, the power of this system of classification to identify evolutionarily and ecologically meaningful clusters may be rather limited (Castenholz, 1992; Komárek, 1996). However, the lack of any appropriate species concept does not hinder the estimation of microbial diversity. In fact, the predominant use in ecology of species as units of diversity is mainly due to practical reasons and may not always be the best solution possible (May, 1995; Tilman *et al.*, 1997).

We compared estimates of the diversity of oxygenic phototrophic microorganisms as reflected in morphotypes, carotenoids, and 16S rRNA gene segments. All three approaches have limitations and potential drawbacks. Morphological groupings of mat forming cyanobacteria may (Garcia Pichel *et al.* 1996) or may not (Ferriset *et al.*, 1996) represent phylogenetically coherent entities and sibling morphological species have also been described for eukaryotic microalgae (Medlin, 1995; Wood & Leatham, 1992). The potential variability of morphological traits with the conditions and state of growth may cause additional difficulties for identifications in the field (Garcia-Pichel *et al.*, 1998). Ribosomal RNA genes are strongly conserved in function and structure and their information content is, therefore, limited. Strains of bacteria with considerably different physiologies were reported to contain identical 16S rRNA genes (Palys *et al.*, 1997). On the other hand, slightly different rRNA gene sequences detected in an environmental sample do not prove the presence of several microbial populations, but may instead be derived from a single organism (Nübel *et al.*, 1996). Similarly, a single microalga or cyanobacterium usually produces a multiplicity of carotenoid types and, conversely, identical pigments may be extracted from several different algae.

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None of the approaches applied enables the indisputable determination of absolute numbers of ecologically distinct populations forming a community. Yet the congruity of the results obtained indicates that all characteristics analysed provide valuable information on the diversity of the organisms of interest. Therefore, their simultaneous investigation allows a meaningful comparison of the different communities in relative terms. Richness data obtained for the eight mat communities by applying three independent approaches are positively linearly correlated (Figure 6), meaning that, on average, an increase of the

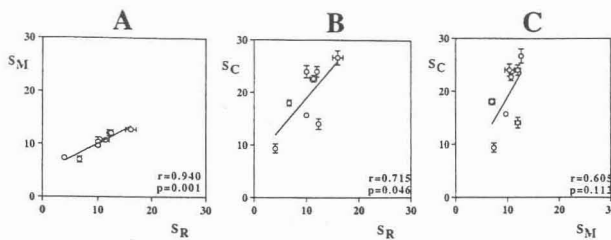


FIGURE 6. Relationships between estimates of the richness of oxygenic-phototrophic microorganisms in eight microbial mats based on triplicate analyses of morphologies (S_M), 16S rRNA genes (S_R), and carotenoids (S_C). Weighted means and 95% confidence intervals are shown for S_R and S_C , arithmetic means and standard errors for S_M . The Pearson correlation coefficient r and its statistical significance p have been calculated.

number of unique 16S rRNA genes is accompanied by an increase of the number of morphotypes and types of carotenoids. The correlation of morphotype and 16S rRNA richness is particularly strong and highly significant. Interestingly, for six of the eight mats the ratio of S_M to S_R is 1.0 (mean values of triplicate analyses). However, in the communities with the highest (NC3) and lowest (P3/4) richness according to carotenoid and genetic data, these ratios are 0.8 and 1.8, causing the slope of the respective regression curve to deviate from one. The richness determined on the basis of carotenoids for all mats is higher than the number of either morphotypes or rRNA gene sequence types, which is due to the fact that all phototrophs produce more than a single type of carotenoid (Goodwin, 1981). The ratio of S_C to S_R ranges from 1.1 to 2.7, causing a less significant positive correlation of these data, and possibly indicating, that the number of

pigments synthesized depends on the specific identity of the organism. Thus, for some communities richness estimates based on different approaches can be rather contradictory, which strongly confirms that the analysis of any single characteristic can be misleading. But, very importantly, the simultaneous application of three independent approaches unveils an underlying trend of richness among the various communities investigated, which may now be related to further mat characteristics or other environmental parameters.

Proportional abundances. The determination of abundances of microorganisms depends on meaningful units of counting, the choice of which is difficult and may to some extent depend on research objectives. Since microorganisms are clonal organisms some authors have argued that colonies or trichomes instead of cells should be considered individuals (Jordan & Staley, 1976; Tinberg, 1979). However, in the densely packed communities within microbial mats, boundaries of colonies often could not be recognized. Therefore, we simply counted cells and, as an alternative measure, weighted them by their respective individual volumes. When applying these alternative approaches, drastically variable cell sizes caused significantly divergent estimates of the proportional abundances of certain morphotypes, seriously affecting Shannon-Weaver indices. While abundances of cell components may reflect relative proportions of organisms, the number of marker molecules per cell depends on its specific identity and physiological state. The production of carotenoids depends on environmental conditions, with the quantity and spectral quality of incident irradiation being especially important (Paerl, 1984). The number of ribosomal RNA genes per cell varies with the number of copies per genome as well as the number of identical chromosomes (and replicated parts thereof) per cell. The latter may change considerably with growth conditions (Birky & Walsh, 1992; Wagner, 1994).

Because of practical problems with all of the methodologies applied, the relative abundance of any population detected may deviate from that actually present in a sample. Morphotype-based abundance determinations are biased in favour of large organisms since they are more likely to be encountered in focused microscopic planes. Procedures to extract nucleic acids and pigments from mat samples in principle may be selective. However, the application of alternative protocols employing mechanical disruption (bead beating) or enzymatic digestion (lysozyme treatment) of cells resulted in no detectable differences in DGGE band patterns (data not shown) and microscopic observations indicated complete lysis to be achieved by the protocol finally applied. The quantitative use of PCR may be compromised when the reactions reach a plateau phase of amplification. This typically occurs at a product concentration of 10^{-8} M (Sardelli, 1993; Suzuki & Giovannoni, 1996) which is approximately the final molarity yielded in our experiments. By performing amplification reactions using a series of template dilutions we

could confirm that above this limit the integrated amplification efficiency decreased, however, the proportional abundances of DNA molecules with different sequences remained unaffected (data not shown). Other potential sources of bias cannot be excluded, however. Primer degeneracies and greatly varying G+C contents of amplified DNA molecules have been suspected to cause differential amplification efficiencies (Von Wintzingerode *et al.*, 1997). Unknown target organisms may exist, the 16S rRNA genes of which do not contain the signature sites necessary for efficient amplification (Nübelet *et al.*, 1997). Hybridization techniques may allow to determine abundances of environmental nucleic acids more accurately than PCR. However, their application depends on the availability of suitable nucleic acid probes, which either require prior knowledge of the sequences to be detected (synthetic oligonucleotide probes (Amann *et al.*, 1995; Stahl *et al.*, 1988)) or need to be prepared from available reference DNA (polynucleotide probes (Holben *et al.*, 1988; Lanoil & Giovannoni, 1997)).

Despite the inherent difficulties of population abundance determinations, overall congruent estimates of diversity were obtained based on the analyses of 16S rRNA genes, morphotypes, and carotenoids. Shannon-Weaver indices calculated for the eight mat communities on the basis of the different approaches are positively linearly correlated (Figure 7). Mainly because of the relatively low 16S rRNA gene diversity detected in the

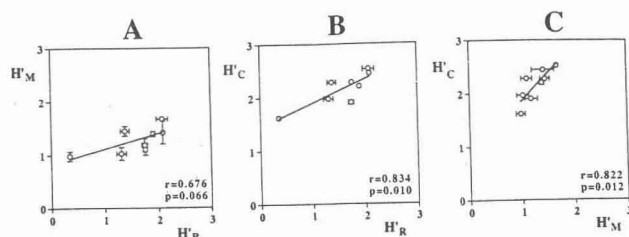


FIGURE 7. Relationships between Shannon-Weaver indices for communities of oxygenic-phototrophic microorganisms in eight microbial mats based on analyses of morphologies (H'_M), 16S rRNA genes (H'_R), and carotenoids (H'_C). Arithmetic means and standard errors from triplicate analyses are shown. The Pearson correlation coefficient r and its statistical significance p have been calculated.

community P3/4, slopes of the regression lines deviate from one. Similar to the richness results, carotenoid analyses for all mats yielded higher absolute values for Shannon-Weaver indices than other measurements. Shannon-Weaver indices based on proportional

volumes of morphotypes (not shown in Figure 7) are exceptional, since they are only weakly and insignificantly correlated to those based on cell number proportions ($r=0.35$, $p=0.39$) and not at all correlated to indices calculated on the basis of 16S rRNA genes ($r=0.09$) and carotenoids ($r=0.02$). Since individual cell volumes differed by up to three orders of magnitude the transformation of cell counts into biovolumes significantly changed the proportional abundances calculated for certain morphotypes. As was the case for estimates of richness, binary comparisons of Shannon-Weaver indices for particular communities are also contradictory when based on different methodologies. This again reflects the limitations inherent to all the identifying traits and their analyses.

Quantification of microbial diversity. Facing the numerous limitations inherent to the various methodologies currently available, many authors conclude that an ecological evaluation of microbial diversity has not yet been convincingly reported (O'Donnell *et al.*, 1995; Tiedje, 1995) or that it would be - at present - impossible (Steinberg & Geller, 1993). The identification of basic units of microbial diversity is difficult, and the determination of proportional abundances of microbial populations can be very ambiguous. However, the latter may be of interest because, generally, rare species in a community have little effect on the overall flux of energy and matter, but may instead become important under changing environmental conditions (Schulze & Mooney, 1993). Therefore, if any current activities of communities or ecosystems are investigated, diversity indices weighting populations by their proportional abundances may be more relevant than the number of distinct populations. Such indices also are less sensitive to detection limits of the respective methodologies applied. This feature may be especially useful for the study of habitats such as soil, with difficult to determine, tremendously high, microbial richness.

Many of the basic problems discussed in this paper are not specific for the exploration of the microbial world. For the majority of ecological collections the diversity can only be estimated and expressed in relative terms. A comprehensive census usually is not achievable and even random samples in many cases cannot be drawn (Pielou, 1966). Species concepts of larger organisms may be as controversial as their relevance for diversity estimates (Mayden, 1997). Depending on research objectives, it may be more fruitful to take into account the organisms' specific identities and their ecologically relevant properties (May, 1995; Tilman *et al.*, 1997). However, diversity is an inherent aspect of community structure and has been reported to be related to ecosystem functioning and predictability (McNaughton, 1993; Tilman & Downing, 1994), the study of which is considered to be a grand challenge of ecological research (Hanski, 1997). Our report demonstrates that the study of biomarker molecules enables the quantification of microbial richness and diversity in natural habitats. The analysis of pigments and

morphotypes cannot be generally applied or will be less informative for microorganisms other than phototrophs. However, other identifying features may be investigated such as cell wall components, fatty acids, enzyme activities, or other traits that can be related to a functional group of interest. Our approach can be considered analogous to the determination of species diversity for macroscopic organisms and makes achievable future synecological research on microbial diversity beyond purely descriptive studies.

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6

Diversity and functional stability in microbial mats.

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Manuscript in preparation

The proposition that the diversity of an ecosystem breeds stability has a long history in ecology. Early observational, theoretical and computational patterns of the behaviour of ecosystems in the face of environmental disturbance suggested the existence of such a relationship between diversity and stability (MacArthur, 1955; Margalef, 1963). Elton, (Elton, 1958) compiled some lines of evidence providing seed evidence for what has become one of the most central and controversial issues in ecology: simple Lotka-Volterra models failed to predict the stability of natural populations, simple laboratory "ecosystems" tended to be unstable and typically resulted in extinctions, natural habitats in islands (low diversity) tended to be more vulnerable to invasions, catastrophic pest events were more common in agricultural monocultures than in natural communities, insect outbreaks were a feature of temperate (low diversity) forests but not of tropical (high diversity) forests ... The possible relationship between diversity and stability in population dynamics and ecosystem functioning has been subject to a protracted debate ever since. Theoretical justifications have been sought (King & Pimm, 1983; May, 1973; Stone *et al.*, 1996; Tilman *et al.*, 1997), but few empirical studies have been attempted until recently (but see (McNaughton, 1977; McNaughton, 1978). While the issue remains controversial, strong empirical evidence favouring the diversity-stability hypothesis has been recently presented (McGrady-Steed *et al.*, 1997; Tilman & Downing, 1994).

In this respect, microorganisms have not been given much attention, probably due to the overwhelming difficulties associated with obtaining quantitative estimates of microbial (specially bacterial) diversity. But in laboratory experiments, where microbial (protistan) diversity could be artificially modified by partially controlled inoculation, it has been possible to obtain evidence for the relationship between diversity and predictability (a surrogate for stability) (McGrady-Steed *et al.*, 1997). The importance of microbial (fungal) diversity as a key factor determining the stability of complex ecosystems has recently also been demonstrated (van der Heijden *et al.*, 1998). The introduction of culture-independent molecular methods has opened new avenues for the study of the diversity of natural microbial communities (see Pace, 1997), albeit with few attempts to obtain quantitative estimates (Garcia-Pichel *et al.*, 1999). We have recently presented a method to obtain congruent estimates of diversity in natural populations of oxygenic phototrophic microorganisms (Nübel *et al.*, 1999). In contrast to the difficulties associated with quantifying microbial diversity, the assessment of organismal activities, from which potential estimates of functional stability can be gained, is generally much simpler for microbial than for macroorganismal communities. Here we present a comparative ecological study of entirely microbial ecosystems (microbial mats in hypersaline waters) in which correlations were sought, and found, between the natural diversity of microbial primary producers and the short-term functional stability against environmental change. Because microbial mats are the closest modern analogs of the earliest ecosystems on Earth, preserved as stromatolites in the fossil record (Schopf & Klein, 1992), our results provide evidence not only for role of diversity on

ecosystem stability in trophic-web poor ecosystems, but also for the continued importance of such relationship during the evolution and diversification of life on Earth.

As model ecosystems we used four different benthic microbial mats from hypersaline waters in saltern ponds or on tidal channels from Guerrero Negro, Baja California Sur, Mexico (see (Javor, 1989) for a description of the site). The primary producers were cyanobacteria and diatoms. Because our approach was comparative, and to avoid illicit comparisons, we restricted our analyses to mats that were similar in the following aspects (see also Table 1). They all grew under shallow water settings (0.2- 0.6 m deep) within the same microclimatic region (located within less than 25 km from each other), and were not subject to periodic drying events. Structurally they were all organic, vertically laminated, soft to leathery mat types with photic zones 1.5 -3 mm deep and contained similar areal biomass (Coefficient of variation in areal Chlorophyll *a* content of 9%). Functional measurements and experimentation were all carried out within less than two weeks, so that all mats had the same seasonal climate history. The communities differed in exposure to one single major physicochemical parameter: salinity. Two of the mats stemming from the salt works were exposed to relatively constant but different salinities, the other two, stemming from tidal channels, were exposed to tidal variations in salinity of different range. We characterised the richness and diversity of oxygenic phototrophs (primary producers) contained in these communities using three independent parameters: 16S rRNA genes, microscopically identifiable morphotypes and extractable carotenoids. While none of them is free of controversy or methodological problems, the combined use of three independent parameters has been shown to provide overall congruent estimates of richness and diversity in a larger survey of mats (Nübel *et al.*, 1999), where the methodology is also explained. Our approach has been to seek independent correlations of each one of the three types of diversity estimators with estimates of functional stability. In spite of the overall climatic and structural similarity of the communities, small but statistically significant differences in richness and diversity could be detected among mats (Table 1).

The functional performance of the mats with respect to the activities of oxygenic phototrophs was determined by measuring the O₂ export rate (net productivity in the light) into the overlying brine under standard conditions of illumination, temperature and flow (Garcia-Pichel *et al.*, 1999). The performance of each mat after an environmental disturbance was scored by measuring net productivity. Environmental disturbances consisted of two series of stepwise changes in brine salinity, one series for upshifts and one series for downshifts, imposed on two separate mat samples. The starting salinity for each series was that to which the mats were exposed in the field. In all, each mat was submitted to 7 salinities spanning the range 3-23 ‰ (w/v) total salts. From the collected performance scores an index of functional stability (Fig. 1)

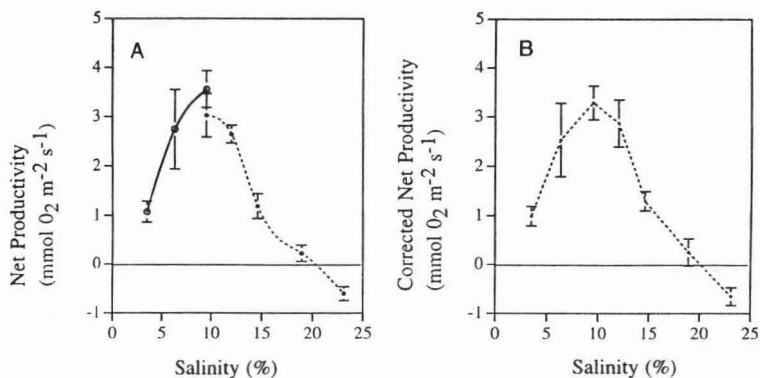


Figure 1. Calculation of the stability of net productivity against changes in salinity in mat communities (exemplary for mat P4). A: Uncorrected net productivity measured after step-wise decreases (solid line) or increases (dotted line) in salinity. Each series was measured on a different mat piece. B: Corrected data from A, in which both curves have been forced to share a corrected net productivity value at the initial salinity (9%) equal to the arithmetic mean of all measurements at that salinity, applying the corresponding multiplicative factor to all points within each curve. The result is a unified response curve to salinity for each mat. An index of stability is calculated as the coefficient of variation (standard deviation divided by the mean) of all means. This index is larger the more unstable the response, and it is independent of the absolute magnitude of productivity. Minimal values for the index are 0 indicating maximal stability (a flat response curve); maximal (theoretical) values are infinity. Both increases in net productivity above the initial and decreases below 0 (net oxygen uptake of the mat in the light) contribute to increase its value.

was calculated as the coefficient of variation in net productivity. We found large differences in stability among mats (Table 1). Crucial for the appropriate assignment of stability indices was the restriction of comparisons to mats that had very compact and thin photic zones (2-3 mm), which we measured in each case by using oxygen microelectrodes (not shown). While other mat communities were available in the field for comparison, these were of the so-called translucent or slimy type, presenting much thicker photic zones (5-8 mm). This small size differences resulted in very large deviations in the time needed for salinity change imposed in the overlying brine to reach the lower parts of the photosynthetically active mat. Modelling of diffusion based on Fick's law (not shown) shows that it takes around 3 hours for 75 % of a change in salinity of magnitude similar to those imposed in hour experiments to reach 2 mm depth, but ca. 20 hours for the same change to reach 5 mm depth. The latter is too long a period to avoid changes in community structure to play a role in the outcome.

Table 1. Characteristics of the mats compared. For the diversity indices, means and standard errors are given. The subscript denotes the experimental basis of the estimates (M: morphotypes; R: 16S rRNA gene sequences; C: carotenoids). Diversity data are from (Nübel *et al.* 1999). CV of net productivity have been computed from data in (Garcia-Pichel *et al.*, 1999).

Mat	Field Salinity (%)	Origin	Richness (S)			Shannon-Weaver Diversity (H')			CV of Net Productivity
			S _M	S _R	S _C	H' _M	H' _R	H' _C	
P2	6	Evaporation Pond	10.67±0.33	11.33±0.66	22.67±0.66	1.45±0.09	1.38±0.08	2.28±0.03	0.777
P4	9	Evaporation Pond	10.33±0.88	10.00±0.00	22.00±1.15	1.10±0.14	1.75±0.02	2.28±0.02	0.961
NC2	5-8	Tidal Channel	12.00±0.58	12.00±0.00	24.00±1.00	1.41±0.21	2.09±0.01	2.44±0.02	0.649
NC3	5-21	Tidal Channel	12.67±0.33	16.00±1.00	26.67± 1.34	1.67±0.04	2.08±0.11	2.53±0.02	0.202

We found that the coefficient of variation in net productivity correlated negatively in all cases with the mean values of the various indices of richness and Shannon-Weaver diversity, in spite of the small differences in diversity among mats. The correlations were statistically significant at the 90% level or better for 2 out of 3 estimators of richness and 2 out of 3 estimators of diversity (Table 2). Thus, this comparative approach provides evidence stemming from natural

Table 2. Statistics of regression analysis between the coefficient of variation (short term functional instability) of net productivity and oxygenic phototroph Richness or Diversity based on several estimators thereof.

	Slope	R ²	p
Richness based on			
morphotypes	-3.12	0.86	0.071
16S rRNA gene sequences	-7.96	0.99	0.004
carotenoids	-4.34	0.70	0.163
Shannon-Weaver Diversity based on			
morphotypes	-0.66	0.83	0.087
16S rRNA gene sequences	-0.62	0.35	0.403
carotenoids	-0.51	0.85	0.076

communities in favour of the diversity-stability hypothesis. It is interesting to note that according to the slopes of our regressions, and within the interval of stability covered, small increases in diversity result in large differences in stability (or vice versa, since causality is not granted from our data). Extrapolation of the regression lines obtained predict that a mat composed of a single strain of cyanobacteria or diatom (probably, but not certainly, represented by one morphotype, or one 16S rRNA gene sequence, or several 3 to 7- carotenoids and resulting in $H'_R = H'_M = 0$ or H'_C around 1) would present a CV of net productivity in the range of 2 -3, a prediction that lends itself to experimental verification. Should linearity hold beyond the bounds of our data range, H' indices slightly above those of mat NC3 should result in CV of 0, corresponding to maximal stability.

It is unlikely that the causative reason behind the stability -diversity correlation in our case relies on food web connectivity, as food webs in these ecosystems are very poorly developed due to the exclusion of higher level grazers. Rather a functional complementation of the primary producers with respect to salinity may well be behind this effect. Moderately halotolerant and extremely halotolerant cyanobacteria, as well as moderately halotolerant diatoms are known to inhabit this environments. While the salinity ranges for growth of strains isolated from

hypersaline mats differ substantially, there exist wide overlaps among these functional types (Garcia-Pichel *et al.*, 1998; Karsten, 1996). Recurrent changes in salinity conditions in nature may provide the nurturing grounds for the development of several populations of primary producers differing in salinity optima. This should result in a time-sharing of the contribution to productivity according to prevailing conditions, and in an overall functionally stable community before environmental fluctuations. The interpretation is consistent with the fact that the mats displaying overall highest stability and diversity were those stemming from tidal channels, where the ranges of tidal variations in salinity are continued and large. In the pond communities, where the bulk salinity of brines is constant, only processes of small scale stratification of the water column may be responsible for small range-variations in salinity. This mechanism must be applicable to any environmental variable and it might be the simplest, and oldest, mechanism by which environmental instability begets organismal diversity and results in ecosystem stability.

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**Spatial scale and the diversity
of benthic cyanobacteria and diatoms in a salina**

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Abstract

We characterized the richness of benthic cyanobacteria and diatoms in a salina system using traditional and molecular biological methods. After determining the different morphotypes and 16S rRNA genes present in various localities within this hypersaline system, an analysis of the increase of organismal richness as a function of numbers of samples considered was carried out. We found that the spatial scales of sampling yielding significant increases in cumulative richness are those at which significant variations in environmental parameters (salinity, vertical microgradients) are known to exist, indicating that the presence of environmental gradients contributes to increased biodiversity. Additionally, we could use this type of cumulative analysis for the estimation, through asymptotic extrapolation, of the total richness of oxygenic phototrophs present in the entire salina system, and for the estimation of the average degree of dissemination of community members within the system. We found interesting differences between analyses based on morphotypes or 16S rRNA genes. The cumulative number of rRNA gene sequences exceeded that of morphotypes more than two-fold. This indicates that many organisms possessing distinct 16S rRNA gene sequences could not be distinguished on the basis of morphology. Thus, some of the apparently widely distributed morphotypes may in fact conceal several ecologically independent genotypes.

Introduction

Reflecting on the notion that most microorganisms may have a cosmopolitan distribution, early microbiologists have coined the expression that with respect to bacteria, "everything is everywhere" (Baas-Becking, 1934). This hypothesis has pervaded microbiology until today. It is based on the assumption that microscopic organisms are efficiently dispersed over geographic distances, resulting in high local, but comparatively low global diversity (Fenchel, 1993). Indeed, small spatial areas may provide many ecological niches for microorganisms, and high local microbial richness has been described for many habitats (Pace, 1997; Tiedje, 1995). However, very limited data exist on the distribution of microorganisms in nature beyond the microscale. In fact, the impression of widespread occurrence of many microorganisms may to a considerable extent originate from poor taxonomic resolution.

We have investigated the richness and distribution of cyanobacteria and diatoms in a variety of microbial mats in a salina system in Baja California, Mexico. Detailed descriptions of this salina (Des Marais, 1995; Garcia-Pichel *et al.*, 1994; Javor, 1989) and of the morphology of the mats studied (Garcia-Pichel *et al.*, 1999; Nübel *et al.*, 1999) have been published elsewhere. Cyanobacteria and diatoms display considerable morphological variability, which is the basis for their current classification (Anagnostidis & Komárek, 1985; Castenholz & Waterbury, 1989; Round *et al.*, 1990). Accordingly, "floristic" studies have described the spatial distribution of morphology-defined taxa. For example, patterns of occurrence of morphotypes along environmental gradients in various hypersaline environments have been reported (Clavero *et al.*, 1994; Ehrlich & Dor, 1985; Jørgensen *et al.*, 1983; Montoya T. & Golubic, 1991). Moreover, for thermophilic cyanobacteria, the absence of particular morphotypes in some geothermal habitats suggests restrictions to dispersal at the global scale (Castenholz, 1996). However, limitations of morphology-based classification have also become apparent, particularly for morphologically more simple cyanobacteria (Castenholz, 1992; Komárek, 1996). Morphological groupings may (Garcia-Pichel *et al.*, 1996) or may not (Ferris *et al.*, 1996) represent phylogenetically coherent entities, and pleiomorphism may cause additional difficulties for microscopic identification (Garcia-Pichel *et al.*, 1998). Thus, depending on the organisms and on the habitat studied, the analysis of cell and colony morphology may be misleading to an unknown extent, if the study of organismic diversity and distribution is intended. We investigated the richness of oxygenic phototrophs as reflected by their morphology and 16S rRNA genes. We compared richness differences among and within localities, and, using cumulative analyses, evaluated the use of such techniques to determine the spatial scales at which diversity increases. Additionally, we used comparisons of data based on either morphotypes or rRNA gene sequences to evaluate the power of morphological classification to delineate ecologically coherent units in this habitat across environmental gradients.

Materials and methods

Sampling. Microbial mats were sampled during the second to fourth weeks of April of 1996 (mats P2, P4, P6, NC2, NC3) and 1997 (mats P3/4, P5, NC52). Sampling sites were located in evaporation ponds of the saltern in Guerrero Negro, Baja California Sur, Mexico, and in the salt marsh of the Ojo de Liebre Lagoon, as illustrated in Figure 1. Within the salina system, the samples analysed covered the entire spectrum of salinity at which microbial mats occurred, ranging from 6 to 16 ‰ (Figure 1). In addition, mats from upper tidal channels that had been exposed to natural diel variations of salinity were included (see Figure 1 for locations and ranges of salinity variations). Each of these sampling sites is referred to hereafter as a locality. Within each locality, triplicate cores 10 to 20 cm apart were subsampled. For light-microscopy, mat samples (core diameter, 4 mm) were fixed in 5% formaldehyde (wt/vol) and stored at 4°C. For extractions of nucleic acids, mat samples (core diameter, 25 mm) were frozen on site, transported to the laboratory in liquid nitrogen, and stored at -80°C until processed. Measurements of photosynthesis were performed in a field laboratory with microsensor techniques (Revsbech & Jørgensen, 1986). The maximum depth where gross photosynthesis was detectable varied from 1.5 to 4.8 mm (mean, 2.4 mm). These photic zones were sampled for the investigation of richness. Further results of these supplementary studies are reported elsewhere (Garcia-Pichel *et al.*, 1999).

Analysis of morphotypes. The layers corresponding to photic zones were cut from formaldehyde-fixed mat samples with scalpel blades, and sectioned vertically into subcores of approximately 0.5 × 0.5 mm mat surface area. These pieces were placed on glass slides in one drop of water and chopped and stirred to achieve even distributions. Of each subcore, 25 to 40 randomly chosen phase-contrast microscopic fields were photographed at 400-fold magnification and cell counts were performed on projections of the resulting slides. Depending on the respective mat, 2000 to 3000 cells needed to be investigated to detect all rare and localized morphotypes in a single subcore, and thus to achieve stable richness estimates (Nübel *et al.*, 1999).

Analysis of 16S rRNA genes. The layers corresponding to photic zones were aseptically cut from mat cores (100 to 400 mg, representing approximately 60 mm² of mat surface) and homogenized in Dounce tissue homogenizers (Novodirect, Kehl, Germany). Cell lysis and DNA extraction were performed as described previously (Nübel *et al.*, 1997). Briefly, the suspensions were repeatedly frozen and thawed, and subsequently incubated in the presence of sodium dodecyl sulfate and proteinase K. Cell lysis was controlled microscopically. DNA was extracted by applying hexadecyltrimethylammonium bromide, phenol, chloroform, and isoamylalcohol, and precipitated by addition of isopropyl alcohol. 10 ng of DNAs extracted

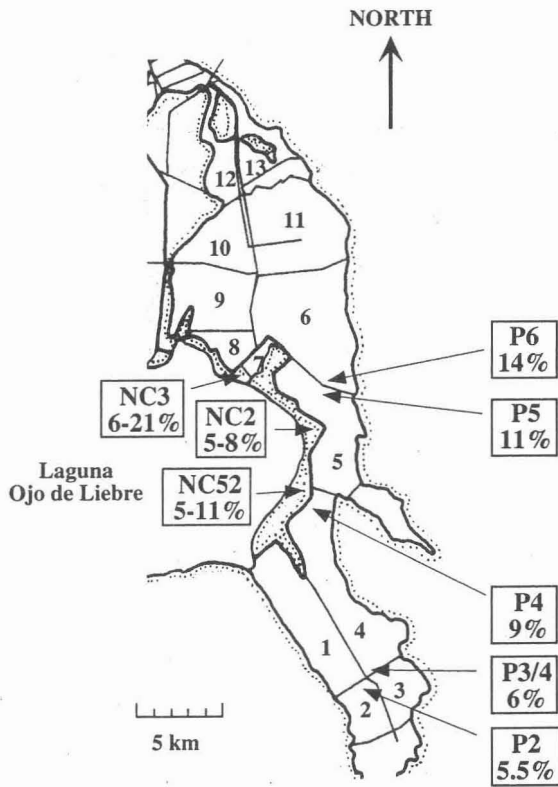


Figure 1. Map showing concentrator ponds of the saltern Exportadora de Sal, S. A. de C. V., Baja California Sur, Mexico. Sampling sites (localities) and salinities of brines, measured at time of collection, are indicated. In most localities salinity was virtually constant. In localities situated within tidal channels salinity varied with tidal cycles within the ranges indicated.

from mat samples were added as templates to each 100 µl polymerase chain reaction (PCR) mixture. Oligonucleotide primers CYA359F and CYA781R were applied to selectively amplify 16S rRNA gene segments from cyanobacteria and plastids (Nübel *et al.*, 1997). Numbers in primer designations refer to 5'-ends of target signature sites in 16S rRNA genes [*Escherichia coli* nucleotide numbering (Brosius *et al.*, 1981)]. A 40-nucleotide GC-rich sequence was attached to the 5'-end of the primer CYA359F to improve the detection of sequence variation in amplified DNA fragments by subsequent denaturing gradient gel electrophoresis [DGGE (Nübel *et al.*, 1997)]. Amplification products generated by duplicate polymerase chain reactions with the same template DNAs were pooled and subsequently purified and concentrated by using the QIAquick PCR purification kit (Diagen, Düsseldorf, Germany). DNA concentrations in resulting solutions were determined by comparisons to the Gibco low DNA mass standard (Gibco, Eggenstein, Germany) after agarose gel electrophoresis. 500 ng of DNA was applied onto denaturing gradient gels. DGGE separates DNA molecules with identical length but different sequences on the basis of their differential melting behaviour in a gradient of denaturants. It was performed as described previously (Nübel *et al.*, 1997). Briefly, polyacrylamide gels with a denaturant gradient from 20% to 60% were used, electrophoreses were run for 3.5 h at 60 °C and 200 V, and subsequently DNA was stained by using ethidium bromide.

Estimation of richness. The estimation of the richness and diversity of morphotypes and 16S rRNA genes of oxygenic phototrophic microorganisms is described and discussed in detail elsewhere (Nübel *et al.*, 1999). Briefly, cells and molecules were grouped into classes defined by unique cell and colony morphologies or nucleotide sequences, respectively. Ecological richness is considered the number of different classes. Cells and molecules from organisms other than oxygenic phototrophs were excluded from the analyses. Microscopically, diatoms and cyanobacteria could be distinguished from most other microorganisms due to their size, characteristic morphologies, and natural autofluorescence. 16S rRNA gene fragments were amplified by polymerase chain reaction (PCR) from cyanobacterial and plastid DNA after nucleic acid extraction from mat samples (Nübel *et al.*, 1997). Phylum-specific amplification enabled the exclusion from the analyses of DNA from organisms other than oxygenic phototrophs (Nübel *et al.*, 1997). Numbers of unique rRNA gene segments amplified were estimated after DGGE analysis of PCR products. With increasing numbers of bands detected in electrophoretic analyses the probability increases that classes cannot be discerned because they run at identical positions in the gel. However, based on the total distance of separation, average peak width, and the low numbers of peaks detected, this crowding effect was estimated to have only negligible effect on our DGGE results (Nübel *et al.*, 1999).

Cumulative richness and its extrapolation. With increasing number of samples investigated, novel classes (morphotypes or 16S rRNA gene sequences) detected that resulted in an increase of the cumulative richness. For example, the total number of different morphotypes detected in mats from localities P2 and P3/4 was 16 (five morphotypes occurred at both localities). Inclusion of locality P4 in the analysis added another five morphotypes that had not yet been observed in mats from P2 and P3/4, and so on. We pooled data obtained from individual localities and plotted the cumulative number of classes discovered as a function of the number of localities considered. The shape of the resulting accumulation curves depends on the sequence in which samples are added. In order to avoid this arbitrariness we computed and considered all possible accumulation sequences (Figure 2). The morphotype and gene sequence accumulation curves approach asymptotic maxima (S_{max}) which we estimated by extrapolation. For extrapolation two-parameter hyperbolas were fitted through means of cumulative richness calculated for an increasing number of localities as:

$$S(n) = \frac{S_{max}n}{K+n}$$

In this equation, S is the mean cumulative richness and n is the number of localities pooled. S_{max} and K are fitted constants, which were calculated by double inverse linear regression ($1/S(n)$ on $1/n$, Figure 2). $S(n)=S_{max}$ is the asymptote and S_{max} can be interpreted as the total richness to be expected in the system under study (Colwell & Coddington, 1994). We did not attempt to estimate confidence intervals for S_{max} and K because of questionable statistical validity of such a computation (Colwell and Coddington, 1994). The above equation is well known as the Michaelis-Menten equation of enzyme kinetics.

Results and discussion

Spatial heterogeneity. The analyses performed permitted investigation of the heterogeneity of the distribution and diversity of cyanobacteria and diatoms at various spatial scales. Considerable patchiness in the organismal distribution was observed at the scale of cyanobacterial colonies and filaments (10 to 100 μm). Detailed microscopic analysis indicated that at least 2000 to 3000 cells per subsample needed to be investigated to achieve stable and repeatable estimates of morphotype richness (Nübel *et al.*, 1999). In few cases, and to a lesser extent, this type of heterogeneity was still apparent at the scale of millimeters. For example, tufts of diatoms were macroscopically visible on the surfaces of some of the mats. However, for all eight localities investigated, richness estimates based on either morphotypes or 16S rRNA genes were consistent among triplicate subsamples that had been taken several centimeters apart. The coefficients of variation for triplicates within localities were typically 5%

TABLE 1. Numbers of morphotypes.

Locality	triplicate subsamples			cumulative total
P2	11	10	11	12
P3/4	7	7	8	9
P4	11	10	10	12
P5	7	6	8	8
P6	10	10	9	10
NC2	13	11	12	13
NC52	11	13	12	13
NC3	13	12	13	13

TABLE 2. Numbers of 16S rRNA genes.

Locality	triplicate subsamples			cumulative total
P2	10	12	12	12
P3/4	4	4	4	4
P4	10	10	10	10
P5	7	7	6	7
P6	10	10	10	10
NC2	12	12	12	13
NC52	13	12	12	13
NC3	14	17	17	18

and maximally 8%, indicating that heterogeneity was low within localities. As a result of this homogeneity, cumulative richness within localities was not much higher than that of the single subsamples (Tables 1 and 2). In contrast, we found marked differences among localities several kilometers apart. This is consistent with the assumption that the concentration of total salts in the brines overlying the microbial mats directly or indirectly determined the horizontal distribution of cyanobacteria and diatoms (Ehrlich & Dor, 1985; Golubic, 1980).

Estimation of regional richness. In the eight mat communities investigated, a total of 36 different morphotypes and 52 different 16S rRNA gene segments from oxygenic phototrophs were detected. Pooling of the data from an increasing number of localities resulted in increasing, but saturating, cumulative richness (Figure 2). Extrapolation of accumulation curves estimated the total numbers of different morphotypes and 16S rRNA genes from benthic oxygenic phototrophs probably present in the whole salina, S_{max} , to be 53 and 125, respectively. The mean richness detected at single localities, $meanS$, was 10.2 and 10.3 in the microscopic and molecular biological analyses, and the ratios $meanS/S_{max}$ accordingly were 0.19 and 0.08, respectively. These quotients are relative measures of the average dissemination of classes, theoretically ranging from zero (all classes are locally endemic) to one (all classes are ubiquitous). A dissemination quotient close to one may be reached in habitats such as soil, in which microbial community composition can be very homogeneous at a geographical scale of kilometers (Felske & Akkermans, 1998). The dissemination quotients determined in our study must be considered rather low, and are probably related to the marked physicochemical differences among sampling sites. However, it was observed that some morphotypes and rRNA genes were widespread among the various mat communities analysed, whereas others were found at few or single localities only (our unpublished results). While the former observation probably indicates that specific organisms are euryhaline, restriction to certain communities may be caused by various chemical, physical, and biological factors and interactions thereof. K , analogous to the half saturation constant K_m in enzyme kinetics, can be interpreted as the average number of samples needed to detect half of the richness in the system under study. These constants were found to be 3.6 and 11.5 for the analyses of morphotypes and 16S rRNA genes, respectively. In general, such estimates should have the potential for optimizing efforts in diversity surveys.

Morphotypes and 16S rRNA genes. We compared the richness of oxygenic phototrophs in microbial mats as reflected by their morphology and 16S rRNA gene sequences. Both approaches must be considered biased to an unknown extent, especially with respect to abundance determinations [discussed in detail in (Nübel *et al.*, 1999)]. However, when investigating samples within localities, both approaches repeatedly yielded similar results. Richness estimates based on 16S rRNA genes within localities correlated significantly with those obtained by morphotype analyses (Pearson correlation coefficient, $r=0.940$, $p=0.001$).

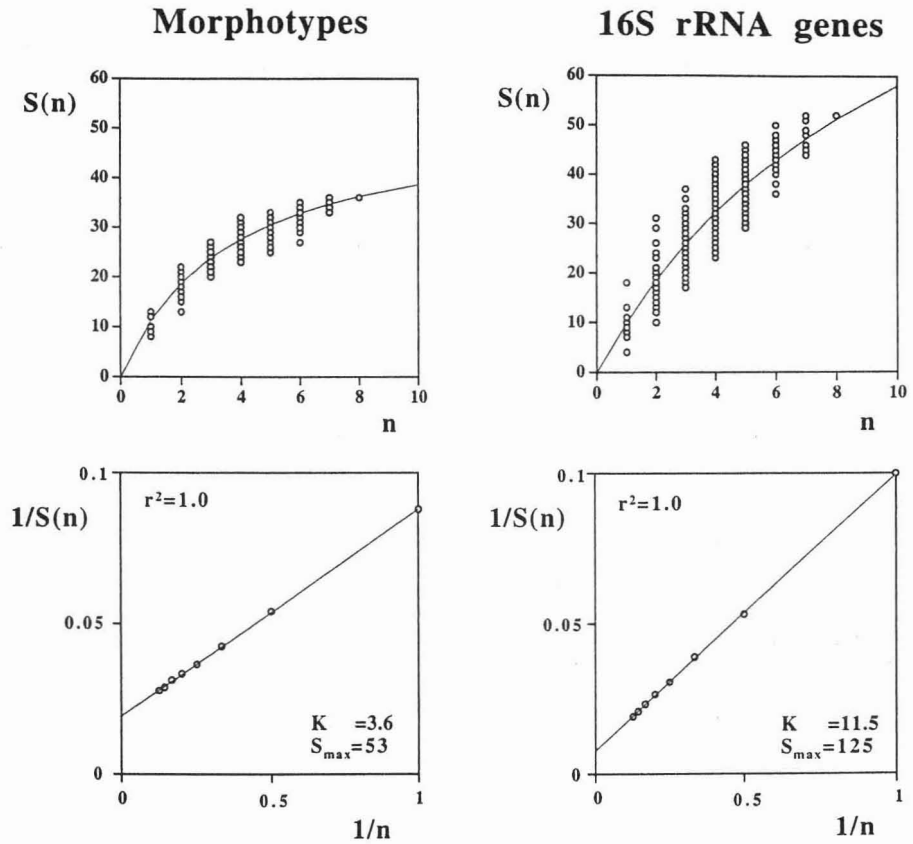


Figure 2. Cumulative number of classes, $S(n)$, plotted as functions of the number of localities, data from which were pooled, n . The left hand graphs show data from morphotype analyses, the right hand graphs show data from 16S rRNA gene analyses. In the upper graphs results from all possible combinations of localities were plotted to eliminate variation in curve shape due to accumulation order. Two-parameter hyperbolas were fitted through means of $S(n)$ based on double-reciprocal plots as shown in the lower graphs. Pearson correlation coefficients, r , estimates of asymptotic total regional richness, S_{max} , and half saturation constants, K , are given.

Interestingly, the ratio of both richness estimates (means of triplicate analyses) equaled 1 in six of eight localities (Nübel *et al.*, 1999). Thus, when analysing the diversity of cyanobacteria and diatoms at small spatial scale, morphotypes and 16S rRNA genes may seem equivalent. However, we found that morphology analysis detected significantly less richness than gene analysis when organism distribution was considered over larger spatial scales. The number of sequence-different 16S rRNA gene segments found in the eight microbial mats clearly exceeded the number of morphotypes identified. The lower degree of saturation (K , Figure 2) found for the latter analysis caused the estimates of S_{max} to differ more than two-fold.

The number of different 16S rRNA gene sequences found should be considered a minimum estimate of the underlying richness of 16S rRNA genes because oxygenic phototrophs may exist in the samples that do not contain the signature sequences for efficient PCR amplification (Nübel *et al.*, 1997), and because amplified DNA molecules potentially may occur at the same position in the denaturing gradient gel despite differential sequences (Nübel *et al.*, 1999). In turn, sequence analysis for the majority of bands indicated that most likely, none of the bands was derived from any undesired amplification product (data not shown). Slightly different 16S rRNA gene sequences may exist in some organisms (Nübel *et al.*, 1996), but this phenomenon seems to be rather rare among cultivated cyanobacteria and diatoms from hypersaline environments (our unpublished results).

With continued sampling sequence-different 16S rRNA genes accumulated at a faster rate and with a higher saturation value than morphotypes (Figure 2). Since the presence of strong limits to dispersal within this system of contiguous flow seem unlikely, the differences must indicate that many of the morphotypes concealed genotypes that are ecologically distinct and therefore occur at different sampling sites. Two reasons are plausible to explain the observation that these organisms have indistinguishable morphology, and probably both apply to some extent. First, morphology analysis may detect diversity at a phylogenetic level too high to correlate with ecophysiological diversity. In fact, once they are established, morphological features are rather conserved in phylogenetic lineages of bacteria in general (Siefert & Fox, 1998), and physiological differentiation may have occurred relatively later in the evolutionary history of the respective organisms. Alternatively, convergent evolution of morphology may have followed, or may have been part of, ecophysiological specializations. In any case, our results reflect the limitations of morphology-based classification, and indicate that the analysis of nucleic acid sequences may be more sensitive for the detection of the ecophysiological diversity of oxygenic phototrophic microorganisms. Clearly, there is a need for new cyanobacterial taxa, integrating information on the ecology and physiology of the respective organisms (Anagnostidis & Komárek, 1985; Castenholz, 1992; Golubic, 1980). For in-depth characterisations of ecophysiological capabilities of microorganisms, however, information retrievable from field observations will be insufficient in most cases. At best, differences in occurrence patterns may indicate some, but largely cryptic, divergence among distinguishable populations. In contrast,

the bacteriological system of cyanobacterial classification (Castenholz & Waterbury, 1989) allows much more detailed experimental investigations on individual strains, but severely suffers from the difficulties inherent in the need for isolation and cultivation and currently is far from being complete (Castenholz, 1992). Molecular biology, however, offers powerful tools for the comparison of cultivated strains and their counterparts in the wild, as well as for guiding future isolation attempts towards the discovery of as yet unknown microorganisms.

Conclusions and outlook. It seems unlikely that limits to dispersal may isolate microbial populations only a few kilometers apart. The differential distribution of organisms must have been determined by their specific ecological demands. Accordingly, large increases in cumulative richness, detected by continued sampling, occurred at a spatial scale which included measurable variations in environmental parameters. We cannot assert at this point if this apparent relationship between microbial richness and environmental heterogeneity will hold in general for microbial communities, but it seems plausible in the light of niche specialization theory. If it does, then it should indicate that the spatial scales important for the accumulation of diversity may depend on the spatial structure of environmental gradients or boundaries. In habitats which show considerable physical heterogeneity at smaller scales (millimeter, centimeter), but not necessarily at larger scales (meter, kilometer), microbial richness may increase at different scales than those that were important in our system. It should prove interesting to extend this type of study to the global scale, as this may offer important information on extant global microbial diversity, for which data are simply non-existent. If cumulative analysis of similar habitats in widely separated geographical localities (for example, salina systems worldwide) should reveal small cumulative richness increases, this would speak for efficient dispersal at planetary scales. The dissemination quotients should give some information on the potential existence of limits to dispersal for a portion of the community members. Additionally, this type of analysis may facilitate the distinction of the presence of climatic borders for microbial communities or the determination of the space/time scales at which microbial dispersal occurs. It must be noted, however, that the primary structure of ribosomal RNA genes is highly conserved, and to identify ecologically coherent populations, it may even be necessary to analyse more variable nucleic acid sequences, such as those encoding proteins (Palys *et al.*, 1997). Generally, ecologically and evolutionarily more meaningful species concepts for microorganisms may be developed in the future (Ward, 1998). Only then, when based on more equivalent units, the diversity and distribution in space of metazoa and unicellular organisms can be meaningfully compared.

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