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Phenotypic and Phylogenetic Analyses Show *Microcoleus chthonoplastes* To Be a Cosmopolitan Cyanobacterium

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We used micromanipulation to isolate from their environment representative samples of seven geographically distant field populations fitting the description of *Microcoleus chthonoplastes* (a cyanobacterium) and obtained seven corresponding cultured strains. Samples of both field populations and cultures were phenotypically characterized by microscale techniques, and their partial 16S rRNA gene sequences were compared by denaturing gradient gel electrophoresis and in some cases by sequencing. All field populations and strains were phenotypically extremely coherent, and their 16S rRNA sequences were indistinguishable by DGGE. The sequences determined were identical or virtually identical. Thus, *M. chthonoplastes* represents a single, well-delimited taxon with a truly cosmopolitan distribution. Comparison with three culture collection strains originally assigned to *M. chthonoplastes* revealed that strain PCC 7420 belongs to the same tightly delimited group, both phenotypically and in 16S rRNA gene sequence, but that strains SAG 3192 and 10mfx do not.

Understanding the role of a particular cyanobacterium in nature requires that it be correctly identified, but identification of field populations within the cyanobacteria is problematic. The bacteriological approach to cyanobacterial classification, based on the study of axenic strains in culture, is at present insufficient for use in ecological research, because the number of strains studied is too restricted. On the other hand, the traditional botanical approach makes identification to the species level possible in most cases; however, because it is based on morphological criteria alone, those morphotypes may or may not correspond to phylogenetically coherent taxa.

Considerable ecological insights may be gained by the extrapolation to natural conditions of the results of physiological experiments carried out on cultures. This powerful tool assumes that the isolates used are representative of the natural field populations. A potential problem arises here with the misidentification of cultured strains. For more than 50% of the strains in the culture collections, the taxonomic names do not correspond to the morphological description of the taxon (19). In addition, comparative analysis of 16S rRNA sequences derived from environmental cyanobacterial DNA has demonstrated that the cyanobacterial strains kept in culture are in general not representative of those found in their natural habitat (see, e.g., references 14 and 40).

A potential step forward in clarifying this situation is the systematic study of a multiplicity of field populations and their correspondence to cultured strains by comparing phenotypic and phylogenetic characteristics, or by using the polyphasic approach of Colwell (6). In the present study, we have used such an approach to characterize geographically distant field populations, isolates, and culture collection strains of cyanobacteria either corresponding to the botanical description of *Microcoleus chthonoplastes* or variously assigned to this taxon. This filamentous oscillatorian cyanobacterium is a dominant constituent of microbial mats in shallow marine intertidal and hypersaline environments the world over and has been the subject of much attention (reference 27 and references therein). Its ability to form large bundles of tightly interwoven trichomes, which are enclosed in a common sheath, makes it a conspicuous, easily identifiable cyanobacterial morphotype. However, some of the traits which define the taxon are not shared by strains assigned to *M. chthonoplastes* in several culture collections, and significant phylogenetic divergence in the 16S rRNA gene sequence has even been found between two isolates (25), casting doubt on either the phylogenetic coherence of the taxon or the validity of the strain identification. This species has been tentatively included in the last edition of *Bergey's Manual of Systematic Bacteriology* because of its ecological importance (5), but, interestingly, it is not treated in the botanical revision of the *Oscillatoriales* (2). Our goal was to characterize the biological diversity contained within the taxon, which should help us interpret the previously available information but carried out on a variety of geographically separated field populations or on several isolated strains of uncertain affiliation.

(Preliminary results of this study have been published previously in the proceedings of a NATO workshop [27].)

MATERIALS AND METHODS

Field sampling and isolation of clean bundles. Natural samples of marine benthic microbial mats from intertidal or hypersaline environments containing populations of *M. chthonoplastes* were collected from the following sites (a three-letter code and the environment type are given in parentheses): Alte Mellum Island, German Wadden Sea (MEL, intertidal); Norderney Island, German Wadden Sea (NDN, intertidal); Ebro River Delta, Catalonia, Spain (EBD, natural hypersaline pool); Rachel Carson Estuarine Reserve, Barrier Islands, N.C. (NCR, intertidal); Solar Lake, Sinai Peninsula, Egypt (SOL, hypersaline pond); Laguna Ojo de Liebre, Guerrero Negro, Baja California Sur, Mexico (GNL, intertidal); and salt-concentrating pond, Exportadora de Sal, Guerrero Negro, Baja California Sur, Mexico (GN5, hypersaline). The mat samples were transported to the laboratory, where bundles of *M. chthonoplastes* were pulled out with watchmaker's forceps and cleaned by being dragged through an agarose gel. Details of this procedure have been published elsewhere (27). Microscopic examination revealed that these cleaned bundles were free of other cyanobacteria, diatoms, and, to a large extent, heterotrophic bacteria. These cleaned bundles represent our "field population" samples.

Strains in culture. Culture was performed without an enrichment step, since the cleaned bundles from the field populations were used directly as the inoculum. Cultivation was successful in F/2 medium (15) with filter-sterilized North Sea water as a base; attempts to use defined media such as ASN-III (31) yielded no or very poor growth. The strains thus isolated retained the morphological

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characteristics of the field populations, including the ability to form bundles of filaments enclosed in a common sheath. None of the strains could be isolated in axenic form during the work. Seven strains, corresponding to each of the seven field populations, were studied, and this group of strains will be referred to below as the core strains.

Additional cultured strains, assigned in the literature to *M. chthonoplastes*, were also studied for comparison. Axenic strain PCC 7420 (Collection Nationale de Microorganismes, Institut Pasteur, Paris, France), was isolated from a microbial mat in Massachusetts. Strain SAG 3192 (Sammlung von Algenkulturen, University of Göttingen, Göttingen, Germany), most commonly referred to as strain 11 (35), was isolated from an intertidal mat in Alte Mellum Island, Germany, and has been widely used in physiological studies. Finally, *Microcoleus* strain 10mfx, also commonly used in laboratory studies as a representative of *M. chthonoplastes*, originally isolated from a microbial mat in Australia, was obtained through Elaine Muñoz, (NASA Ames Research Center, Moffett Field, Calif.). These three strains are referred to below as reference strains. An *Oscillatoria* sp. (strain MPI 940S01) and a *Lyngbya* sp. (strain MPI 94LA02), isolated by us from GN5 and EBD microbial mats, respectively, were also used in some experiments as outgroups.

Microscopy and microscale analyses. Cleaned bundles from both natural populations and cultures were characterized. Special attention was given to documenting and quantifying morphological traits that are defining in the botanical species description (trichome width and shape of both intercalary and end cells). Visual observation of motility was aimed at determining the presence of filament rotation on gliding. This was done by observing eccentrically placed intracellular granules, which appear to move from side to side as they come in and out of focus if rotation occurs. Visible and UV absorption spectra were recorded by a filter technique based on that of Amesz et al. (1), miniaturized as follows to allow in vivo spectral analysis of one to several intact live bundles. A fiberglass filter soaked in growth medium was sandwiched between a quartz microscope slide and a piece of aluminum foil. A small rectangular hole (ca. 4 by 1 mm) was bored in the foil to act as a slit. Cleaned bundles were carefully placed on the filter through the slit, so that an optimal coverage of the orifice was attained. The slide was fixed in the spectrophotometer as close as possible to the sensor, with the slit (and the cells) centered on the impinging measuring beam, and a spectrum was measured. A blank had been obtained by using the same procedure but excluding the cells. Measurements were made on a Shimadzu UV160a recording spectrophotometer. The presence of a particular compound, as indicated by in vivo spectroscopy, was confirmed by extraction of batch cultures. Phycobilins were partially purified by the method of Tandeau de Marsac and Houmard (38), and mycosporine-like compounds were partially purified by the method of Garcia-Pichel et al. (11). Photoalignment of the bundles was checked by incubation on petri dishes containing 1.5% (wt/vol) agarose F/2 medium under a highly directional light field of approximately 45 μmol photon of white light m^{-2} s^{-1} over 1 to 2 weeks. Catalase activity was measured as the rate of O_2 production in response to the addition of 0.03% (vol/vol) hydrogen peroxide to agar-immobilized cleaned bundles via custom-built Clark-type oxygen microelectrodes (29) inserted within the bundle.

Extraction of DNA and PCR amplification. Cleaned bundles were lysed by a modification of the method of Atlas and Bej (3). Briefly, 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 9], 15 mM $MgCl_2$, 500 mM KCl, 0.1% [wt/vol] gelatin, 1% [vol/vol] Triton X-100), 1 μl of 200 mM dithiothreitol, 1 μl of 0.01% (wt/vol) sodium dodecyl sulfate, and 42 μl of sterile water (Sigma, Deisenhofen, Germany) were added to the cleaned bundle (or cell pellet) in a 500-μl tube. The tubes were subjected to three freeze-thaw cycles. Thereafter, 1 μl of proteinase K (10 μg/ml) was added, and the tubes were incubated at 55°C for 1 h. After incubation, they were briefly centrifuged and the supernatant was transferred to a clean tube and stored at −80°C for further use. The cell lysate was used directly in the PCR (32) to amplify a 550-bp rDNA fragment, which was analyzed by denaturing gradient gel electrophoresis (DGGE). PCR amplifications were carried out as described by Muyzer et al. (23).

DGGE analysis of PCR-amplified 16S rDNA fragments. In DGGE, DNA fragments of the same length are separated on the basis of their nucleotide sequence. The fragments migrate through the denaturant gradient until they reach a concentration at which the double strand opens and migration halts. The concentration at which the fragment stops migrating depends on its melting behavior and thus on its sequence. For short DNA fragments (up to 250 bp), DGGE can resolve fragments with single base pair differences, but the actual resolution of the technique may be variable, depending on other factors such as the length of the fragments. DGGE was performed as described by Muyzer et al. (22). PCR products were applied directly either onto polyacrylamide gels containing a gradient from 20 to 60% urea-formamide or onto polyacrylamide gels with a narrower gradient, i.e., from 40 to 50% urea-formamide. Electrophoresis was performed at a constant voltage of 200 V and a temperature of 60°C. After electrophoresis, the gels were incubated for 15 min in Milli-Q water containing ethidium bromide, rinsed for 10 min in Milli-Q water, and photographed with a CS1 digital camera system (Cybertech, Berlin, Germany) under UV transillumination.

DNA sequencing of PCR fragments and sequence comparison. Amplified DNA fragments were separated from free PCR primers by electrophoresis in 2% (wt/vol) agarose gels with the E91 electrophoresis unit (Biometra, Göttingen, Germany) (22). Direct sequencing of the purified PCR products was performed as previously described (22). The 16S rRNA sequences thus obtained were manually aligned with the 16S rRNA sequence of *M. chthonoplastes* PCC 7420 (24) by using the sequence editor SeqApp (13). A distance matrix from the aligned sequences (corrected for missing bases) was obtained by using the appropriate option of the PAUP 3.1 software program (37).

RESULTS

The results of the morphological, biochemical, and physiological investigations that could be performed for both the field populations and the core strains are presented in Table 1. The corresponding results for the three reference strains are also included.

Morphology. All field populations and core strains showed elongated intercalary cells, with shallow (less than one-quarter of the cell diameter) but clear constrictions at the cross-walls between cells and with conical (bullet-shaped) apical cells (Fig. 1). Both conical and rounded apical cells were observed in all field population samples and cores strains. The trait is defined here as the presence or absence of conical end cells and implies that many, but not all, end cells have this shape. Similarly, recently divided cells could be quadratic (roughly as long as wide), but fully developed cells were always elongated. Small but in some cases significant differences were found among field populations in the absolute cell (trichome) width (Table 1). The core strains tended to have somewhat wider cells than did their respective parent field strains, indicating that environmental conditions may have an effect on this parameter. The cell widths were constricted to the range of 3 to 6 μm. All field populations and core strains formed bundles of several to many trichomes, which were held together in a common sheath, a characteristic that was retained for more than 2 years of continued culturing in nonaxenic form. Strain NCR-1 has apparently lost this ability upon cultivation in axenic form (41). Strain NDN-1, which we could only recently render axenic, also concomitantly lost the bundle formation ability.

The morphological characteristics studied here would suffice to assign all of the field populations and core strains to the botanical species *M. chthonoplastes*. This species is defined (12) as a benthic marine oscillatorian cyanobacterium, with trichomes between 2.5 and 6 μm in diameter, having elongated intercalary cells with constrictions at the cross-walls and conical end cells and forming bundles of many closely intertwined trichomes enclosed in a common sheath. By contrast, none of the reference strains studied would conform rigorously to the species description, since none forms bundles of trichomes enclosed in a common sheath. Strain PCC 7420, however, conforms to the description in every other characteristic. Strain SAG 3192 conforms in two or perhaps three of the five criteria, and strain 10mfx conforms in only one or perhaps two criteria. The formation of bundles, however, seems to be an unstable characteristic, since two of our strains recently rendered axenic no longer form bundles. It is possible that bundle formation is triggered by the presence of certain bacterial types and thus is not expressed in pure cultures. Further investigation is needed to answer this question.

Ultrastructure. Whenever the ultrastructure of natural populations of *M. chthonoplastes* has been studied, a radial arrangement of the thylakoids has been observed (7–9, 36). Because the field sites (and hence the field populations) in some of these studies were the same as ours, we have included these results in Table 1. We included unpublished data (21) demonstrating by electron microscopy the presence of a radial thylakoid arrangement in our strains MEL-1 and GNL-1 and the presence of a concentric thylakoid arrangement in strains 10mfx and SAG 3192.

Spectroscopy. All field population samples had virtually

TABLE 1. Properties of the field populations samples and cyanobacterial strains analyzed[a]

Characteristic[b]	Result for[c]:																
	Field population sample and core strain pair														Reference strain		
	MEL	MEL-1	NDN	NDN-1	EBD	EBD-1	SOL	SOL-1	GNL	GNL-1	GN5	GN5-1	NCR	NCR-1	PCC 7420	SAG 3192	10mfx
Morphology																	
Cell width (μm) (SD)	3.7 (0.35)	4.1 (0.51)	3.8 (0.48)	5.0 (0.27)	3.95 (0.48)	5.6 (0.34)	4.5 (0.39)	4.0 (0.4)	3.6 (0.21)	4.2 (0.43)	4.5 (0.43)	4.0 (0.26)	3.1 (0.32)	3.4 (0.21)	3.7 (0.23)	3.3 (0.21)	2.5 (0.11)
Cell shape	E	E	E	E	E	E	E	E	E	E	E	E	E	E	E	Q	E
Conical end cells	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Contrictions at cross-wall	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	−
Ensheathed bundle formation	+	+	+	+[d]	+	+	+	+	+	+	+	+	+	+[d]	−	−	−
Ultrastructure																	
Radial thylakoids	ND	+[e]	ND	ND	+[f]	ND	+[g]	ND	ND	+[e]	+[g]	ND	ND	ND	ND	−[e]	−[e]
Pigments																	
PEC shoulder	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
MAA (332 nm)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±	−	−
Catalase activity	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	ND	ND
Motility																	
Gliding motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	±
Speed (μm s^{-1})	0.6	0.4	0.4	0.5	ND	0.3	0.6	0.3	ND	0.4	0.3	0.4	ND	0.3	0.3	2.7	<0.001
Rotation	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	NA
Bundle photoalignment	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	NA	NA	NA

[a] The table includes traits that could be analyzed both in field populations and in cultures. See Materials and Methods for field population sample and strain designations. See Results for particular explanations regarding each trait.
[b] SD, standard deviation.
[c] E, elongated; Q, quadratic; ND, not determined; NA, not applicable.
[d] Recently obtained axenic cultures of NDN-1 (our data) and NCR-1 (41) do not form bundles.
[e] Reference 21.
[f] Reference 9.
[g] Reference 7.

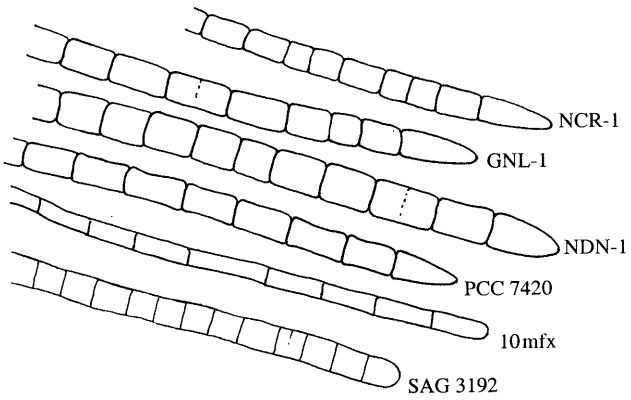

FIG. 1. Major morphotypes of the strains studied. The drawings were obtained by tracing projections of micrographs. Note the shape of the end cells and the constrictions at cross-walls typical of core strains and strain PCC 7420. Acronyms correspond to strain denomination. Scale, 5 μm.

identical in vivo absorption spectra (Fig. 2). Besides the peaks around 680 nm, corresponding to the red band of chlorophyll *a* and at 620 nm, corresponding to phycocyanin (a light-harvesting phycobiliprotein), a more or less marked shoulder at around 585 nm, consistent with the presence of phycoerythrocyanin, a phycobiliprotein of rather restricted occurrence (4),

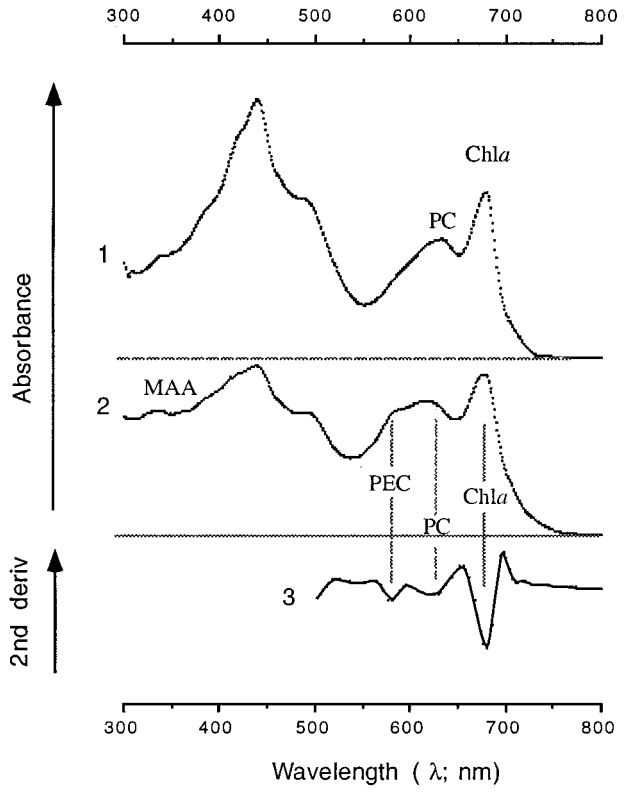

FIG. 2. In vivo absorption spectra. 1, Absorption spectrum of a batch culture of strain 10mfx. 2, Absorption spectrum of clean bundles of strain NDN-1. 3, Second-derivative (2nd deriv) spectrum of spectrum 2 with major absorbing components. The minima in the second-derivative spectrum correspond to major absorbing components in the original spectrum. Assignment of major features is as follows: Chl*a*, chlorophyll *a*; PC, phycocyanin; PEC, phycoerythrocyanin.

was visible. Second-derivative analysis of the spectra indicates the presence of a component with a maximum absorbance at around 580 nm. This shoulder, absent in strains SAG 3192 and 10mfx, was also found in strain PCC 7420. Absorption and fluorescence spectroscopy of the phycobiliprotein fraction extracted from batch cultures confirmed that the shoulder around 580 nm corresponds to a phycobiliprotein resembling phycoerythrocyanin. Characterization of this phycoerythrocyanin-like phycobiliprotein is now in progress.

A characteristic trait of field populations and core strains was the relatively high UV absorption (Fig. 2). Typically, cyanobacteria without specific UV-absorbing compounds present a minimum absorbance in the near UV (10). A peak in absorbance was found at 330 nm in all cases; absorption maxima in these region usually correspond to mycosporine-like amino acid derivatives (MAA), which are common in some cyanobacteria (11). Extraction of the MAA fraction in the core strains revealed the presence of MAAs with a maximum absorbance at 332 nm. Of the reference strains, only strain PCC 7420 contained small amounts of these compounds; strains SAG 3192 and 10mfx did not contain the compounds. Further separation and purification of MAA from these and other isolates have shown that the MAA peak is composed of one to several different molecules, which are not identical in all isolates (18).

Catalase activity. Cyanobacteria have two known hydrogen peroxide-removing enzymes, i.e., catalase and ascorbate peroxidase (39). Whereas ascorbate peroxidase may not be universal in cyanobacteria (20), catalase is usually considered a universal enzyme in all aerobic organisms (17). However, we did not measure any significant catalase activity in cleaned bundles from any of the field populations or from core strains; the catalase activities were at least 3 orders of magnitude lower than those measured in other filamentous cyanobacteria (used as positive controls and belonging to the genera *Lyngbya* and *Scytonema*) by the same procedure. This test could not be performed with the reference strains, because they did not form bundles and could not be mechanically isolated from other bacteria. Axenic strain PCC 7420 did not generate measurable amounts of oxygen upon addition of hydrogen peroxide to a centrifuged cell pellet, which indicates the absence of significant catalase activity.

Motility and photoresponses. Gliding motility was observed in all field population samples and core strains, with speeds ranging between 0.3 and 0.6 μm s^{-1}. A more detailed examination of the motility of strain NCR-1 showed no evidence of photokinesis (28), indicating that the numbers given here are good estimates. In addition, gliding motility occurred in the absence of rotation along the trichome axis in all field population samples and in the core strains. Gliding in the oscillatorian cyanobacteria is thought to be accompanied by rotation of the trichome and gliding without rotation typical of the heterocystous, filamentous forms (16), but some *Pseudanabaena* strains are also able to glide without rotating (30). Among the reference strains, only PCC 7420 showed gliding without rotation and with speeds comparable to those of our field population samples and cultures. Strain SAG 3192 also glided, but with speeds almost an order of magnitude greater than those of our field population samples and core strains and with left-handed rotation of the trichome along its long axis. Strain 10mfx was immotile by microscopic observation but showed very slow movements after long-term incubation on agar plates, from which a plausible speed has been calculated (Table 1).

When cultures of field population samples were incubated under a directional light field, the bundles would align themselves parallel to the direction of the incoming light during

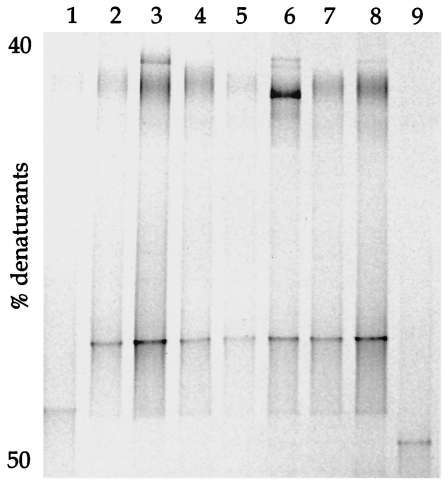

FIG. 3. DGGE comparison of core strains. A negative image of an ethidium bromide-stained DGGE pattern of PCR-amplified 16S rDNA fragments from the core strains and outgroups is shown. Lanes: 1 *Lyngbya* sp. strain MPI 94LA02; 2, MEL-1; 3, NDN-1; 4, GN5-1; 6, NCR-1; 7, EBD-1; 8, SOL-1; 9, *Oscillatoria* sp. strain MPI 94OS01.

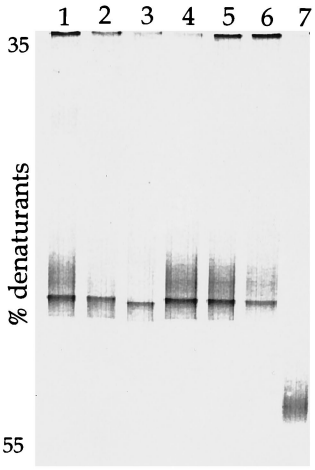

FIG. 4. DGGE comparison of field population samples and corresponding core strains. A negative image of an ethidium bromide-stained DGGE pattern of PCR-amplified 16S rDNA fragments from the core strains and their corresponding field populations is shown. Lanes: 1, NDN-1; 2, NDN-field; 3, EBD-field; 4, EBD-1; 5, GNL-field; 6, GNL-1. *Desulfovibrio gigas* (lane 7) was used as an outgroup.

growth. Whether they were growing toward the light or away from it depended on the intensity of the incident light. Strain MEL-1, for example, aligned its bundles away from the light if exposed to visible light irradiances higher than 17 μmol photon m^{-2} s^{-1}. The mechanism behind this response is unclear, but it seems that both direct tactic responses and the way in which the extracellular sheath is deposited must be involved. This phenomenon could not be properly compared with that of the reference strains, because their trichomes form no bundles.

Additional observations. Strains NDN-1 and MEL-1 produced considerable amounts of the odoriferous volatile metabolite geosmin (detectable by smell). Strains MEL-1, NCR-1, and NDN-1 displayed a sudden and massive production of single, short trichomes, which remained in suspension in the batch culture for several days, but these phenomenon was not observed in the other strains. Differences in the sugar moiety attached to the myxoxanthophylls, but not in their overall carotenoid complement, occur in these strains (18).

DGGE analysis of 16S rDNA fragments. We succeeded in using DNA extracted from one clean bundle of each field population and core strain to amplify 16S rDNA fragments by PCR (32). DGGE was used to analyze the PCR products and to determine the purity of the amplified fragments. DGGE analysis of some samples yielded a single band (Fig. 3, lanes 2, 4, 5, and 7), indicating that the cleaning procedure was sufficient to avoid amplification of DNA from contaminating bacteria. However, in other samples, multiple bands, probably due to PCR products amplified from contaminant organisms, were observed (lanes 3, 6, and 8). Coelectrophoresis of the PCR-amplified 16S rDNA fragments from all of our core strains showed indistinguishable migration properties; i.e., they halted migration at a denaturant concentration of about 48% (Fig. 3). This result demonstrates that the fragments have identical melting properties and points to virtual identity of their sequences. By contrast, the DNA fragments from our core strains could be easily separated from those obtained from other oscillatorians, i.e., *Lyngbya* sp. strain MPI 94LA02 and *Oscillatoria* sp. strain MPI 94OS01, which halted at 48.5 and 49.0% denaturant, respectively (lanes 1 and 9). Thus, these two sequences were different from each other and from those of the core strains.

DGGE comparisons of field populations and cultures (not all data shown) showed identical melting behavior of all DNA fragments, indicating that only one sequence or nearly identical sequences were present in all core strains and field population samples. Figure 4 shows a DGGE analysis of PCR products of some core strains and their corresponding field populations.

Figure 5 compares the melting behavior of the rDNA fragments of one of our core strains (MEL-1 [lane 1]) with those from the reference strains (PCC 7420, 10mfx, and SAG3192 [lanes 2, 3, and 4, respectively]). A discrete band at the same position in the gel was found for each MEL-1 (lane 1) and PCC 7420 (lane 2), again indicating the same sequence. However, bands at different positions in the gel were found for the two other reference strains, 10mfx (lane 3), and SAG 3192 (lane 4), demonstrating divergent sequences.

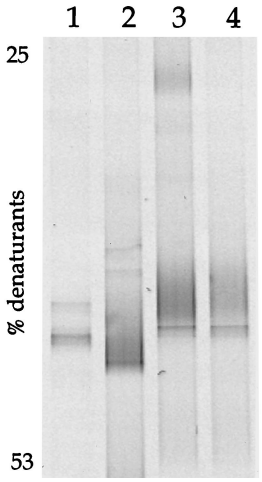

FIG. 5. DGGE comparison of reference strains. A negative image of an ethidium bromide-stained DGGE pattern of PCR-amplified 16S rDNA fragments from core strains and reference strains. Lanes: 1, 10mfx; 2, SAG 3192; 3, MEL-1; 4, PCC 7420.

TABLE 2. Percent similarity matrix in partial 16S rDNA sequence (540 bp compared) for *M. chthonoplastes* and related cyanobacterial strains[a]

Strain	% Similarity with strain:								
	MEL-1/EBD-1	GN5-1/PCC 7420	PCC 7515	PCC 6304	10mfx	PCC 7419	PCC 7105	*O. limnetica*	*E. coli*
M. chthonoplastes MEL-1* *M. chthonoplastes* EBD-1*	100	99.8	90.8	89.9	89.9	89.6	88.8	88.4	75.1
M. chthonoplastes GN5-1* *M. chthonoplastes* PCC 7420		100	91.1	90.1	89.7	89.6	88.6	88.6	74.9
Oscillatoria strain PCC 7515			100	89.0	87.6	89.3	89.0	89.0	71.7
Oscillatoria strain PCC 6304				100	88.4	89.5	89.9	86.4	73.7
"*M. chthonoplastes*" 10mfx					100	89.5	98.0	88.4	74.5
Lyngya strain PCC 7419						100	93.1	88.2	71.6
Oscillatoria strain PCC 7105							100	88.9	73.7
Oscillatoria limnetica								100	74.2
Escherichia coli									100

[a] Sequences marked with an asterisk were determined in this work. All other sequences can be retrieved from the EMBL nucleotide sequence library. The sequences of strains MEL-1 and EBD-1 and those of GN5-1 and PCC 7420 are identical and so have been assigned a common entry.

16S rRNA sequence comparison. We sequenced the DGGE bands of three strains (GN5-1, EBD-1, and MEL-1) to demonstrate rigorously the sequence similarities. The sequences (550 bp from *Escherichia coli* positions 341 to 927) of EBD-1 and MEL-1 were identical. The partial sequence found for GN5-1 was identical to that of the corresponding fragments of strain PCC 7420 (24). A single difference was found between the EBD-1/MEL-1 (adenine at position 629) and the GN5-1/PCC 7420 (thymidine at position 629) sequences. The complete sequence for PCC 7420 has been deposited in the EMBL nucleotide sequence library with accession number X70770 (24), and thus our partial sequences can be reconstructed from it. A distance matrix between the sequences obtained for some of our strains and the corresponding sequences of several closely related cyanobacteria is presented in Table 2. A phylogenetic tree based on 16S rRNA sequences constructed from cyanobacterial sequences (data not shown) places this cluster in a branch that contains only simple filamentous cyanobacterial forms, which corresponds to branch F in the general tree of Wilmotte (42).

DISCUSSION

***M. chthonoplastes* as a cosmopolitan cyanobacterium.** In all the phenotypic traits studied here, the field population samples, the core strains, and strain PCC 7420 showed a remarkable degree of similarity. The molecular studies indicate that their relatedness must be at least at the genus level, since this is probably the limit of resolution of 16S rRNA sequence comparisons (34). In the light of present estimates of the 16S rRNA molecular clock (26), suggesting bacterial substitution rates of 1% every 50 million to 60 million years, this group of strains of *M. chthonoplastes* may have diverged as recently as 9 million years ago. The coincidence in some traits that are highly variable, even among closely related cyanobacteria, such as the phycobiliprotein complement, the presence of mycosporine-like compounds, and the motility and photoresponse traits, also points to a high degree of phylogenetic coherence of the geographically separated populations. A study of the possible biogeographical or ecotypic diversification by monitoring chemotaxonomic markers in these and other strains of *M. chthonoplastes* is in progress (18). Even though this group of strains can be separated chemotaxonomically into several groups, no clear evidence is yet available to contend that they can be separated into several species. At this stage, it seems that the most conservative and logical conclusion is to regard the isolates as a single species. Thus, field populations of *M. chthonoplastes*, even of single trichomes, can be identified with the conviction that one is dealing with a monophyletic, coherent group of organisms. The closest partial sequence to the *M. chthonoplastes* cluster of strains remains *Oscillatoria* strain PCC 7515 (only 90.8 to 91.1% similarity [Table 2]), a strain that is morphologically quite different from *M. chthonoplastes* (42). It should prove interesting to determine if other morphotypes in the botanical genus *Microcoleus* or allied morphotypes (i.e., *Schizothrix*) might span this apparent gap.

Identity of collection strains of *M. chthonoplastes*. One of the main conclusions of our work is that the strain PCC 7420 undoubtedly belongs to the *M. chthonoplastes* group and that both SAG 3192 and strain 10mfx do not. The use of the last two strains as representatives of *M. chthonoplastes* in laboratory studies should be strongly discouraged. Strains PCC 7420, NDN-1, or NCR-1, which are presently available in axenic form, should be used instead. Our results demonstrate that when working with cyanobacterial strains obtained from culture collections, the specific or even the generic epithet by which the strains have been labeled is not always reliable. The use of the strain number designation remains necessary for proper replication of the work and to avoid confusion.

Significance for the concept of diversity in cyanobacteria. Ward et al. have demonstrated that the apparently monospecific cyanobacterial populations of "*Synechococcus lividus*" in a single hot-spring microbial mat, are actually composed of at least seven divergent 16S rRNA cyanobacterial "phylotypes" (40), of which only one was phylogenetically close (99.3% sequence similarity) to the collection strains in culture. A sim-

ilar case is that of the marine picoplanktonic *Synechococcus* sp. from open-ocean waters. Giovanonni et al. (14) and Schmidt et al. (33) retrieved several 16S rRNA cyanobacterial sequences from field populations in the Sargasso Sea and in the North Pacific. None of the sequences retrieved were identical to the sequences of cultured strains or to each other. All of the marine *Synechococcus* sequences, however, clustered in a relatively wide but monophyletic group which included cultivated strains. These studies indicated that "morphological species" contain a high degree of biodiversity that is expressed even at the 16S rRNA level. The results obtained here with *M. chthonoplastes* are in marked contrast to those obtained with both *Synechococcus* types, since we could detect virtually no sequence diversity, even from geographically separated sources. The results are congruent with those obtained by Wilmotte et al. (43) in a study of cultured strains of marine oscillatorian cyanobacteria with narrow trichomes. In that study, good agreement was found between morphology and the 16S rRNA sequence, with some sequences being virtually identical, even though strains of different geographical origins were used. Thus, the genetic diversity contained within cyanobacterial morphotypes must be variable, depending on the specific case analyzed. It is possible that simple morphotypes, such as small rods (*Synechococcus* spp.), offer too little morphological basis for the recognition of "phylotypes" other than with molecular analyses but that the correlation between morphotypes and phylotypes becomes gradually better as morphologically more complex forms are considered.

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