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Cytomorphological and Genetic Characterization of Troglobitic *Leptolyngbya* Strains Isolated from Roman Hypogea[▽]

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Six *Leptolyngbya* strains, isolated from the archaeological surfaces of hypogean sites, were phenotypically and genetically characterized by light and electron microscopy and 16S rRNA gene and 16S-23S internally transcribed spacer (ITS) sequencing. Three phycoerythrin-rich (red) and three phycocyanin-rich (green) isolates were assigned to different operational taxonomic units (OTUs). Among the green isolates, one strain showed an OTU intraspecific variation due to differences in the ITS sequences and genomic polymorphism. Within the ITS sequence, variable regions, conserved domains and $tRNA^{Ile}$ and $tRNA^{Ala}$ genes showed high sequence identity among the phylotypes. Together, these data indicated a relatedness of the six strains to other *Leptolyngbya* from subaerophytic and geothermal environments and allowed the definition of novel *Leptolyngbya* OTUs.

Archaeological hypogea, such as Roman catacombs and the necropolis, are man-made underground sites illuminated from natural openings to the outside and/or from artificial lights that permit visits by tourists. The low-light conditions (photosynthetic photon flux density of <2 μmol m^{-2} s^{-1}), however, still sustain the development of phototrophic biofilms on calcareous substrata (2, 3, 16). These microbial communities are composed of chroococcal and filamentous cyanobacteria, a few green algae, diatoms, mosses, and bacterial populations, which are all embedded in common extracellular polymeric secretions (9, 10, 17, 26, 47, 55). The development and metabolic activity of these biofilms play a significant role in the biodeterioration of the art works of great historical and artistic value that are present in hypogea. Aesthetic damage to stuccoes, frescoes, and marble is the result of the colored patinas of microbial origin. Structural damage is due to the ability of microbial biofilms to mobilize constituent elements from mineral substrata (21, 48).

The phototrophic communities of Roman hypogea are dominated by abundant populations of subaerophytic epilithic cyanobacteria belonging to the genera *Eucapsis*, *Leptolyngbya*, *Scytonema*, and *Fischerella* that have been identified on the basis of their cytological and ultrastructural features (8, 10). Some of these taxa were never recorded outside of these habitats and for this reason were defined as "troglobitic," i.e., obligate cavernicole taxa unable to survive outside of caves or other low-light environments (27). Subaerophytic (35) troglobitic cyanobacteria belonging to the genus *Leptolyngbya* are particularly abundant in phototrophic biofilms present in Roman hypogea. To date, different *Leptolyngbya* species have been detected in all the hypogea investigated, but their phenotypic simplicity made their correct identification difficult (6, 34), and molecular data are scarce. The 16S rRNA gene sequences of only two troglobitic *Leptolyngbya* isolates, strains VRUC135 and VRUC184, have been reported (15, 39). The genus *Leptolyngbya* was first described by Anagnostidis and Komárek (11). This genus includes groups of filamentous cyanobacteria in LPP group B that exhibit very thin trichomes (<3 μm), exemplified by *Lyngbya*, *Plectonema*, and *Phormidium* (45). However, the heterogeneity of this genus has since been questioned (7, 35). Molecular data are needed to clarify its phylogenetic position. In the last few years, the 16S rRNA gene sequences of several *Leptolyngbya* strains isolated from desert, marine, and freshwater environments have become available (57). *Leptolyngbya* species are very common and yet difficult to identify because of the controversial position of this genus among *Cyanobacteria*. It has been recently proposed to divide the genus *Leptolyngbya* into at least two genera, each with six ecological groups, for easier identification, since the majority of the 91 species so far described have distinct and delimited ecological requirements (35). A polyphasic approach combining morphological, ultrastructural, molecular, and ecophysiological studies is the most progressive method in the modern taxonomic study of cyanobacteria (19, 20, 29, 35, 38, 56). This approach is especially suitable for the taxonomy of species belonging to genera such as *Leptolyngbya*, which are morphologically very simple.

In the present study, six strains of *Leptolyngbya* isolated from five Roman hypogea, namely, the catacombs of St. Callistus, St. Sebastian, Domitilla, and Priscilla and the Domus Aurea (2), all located in Rome (Italy), were investigated by means of a polyphasic approach. The cytomorphological features were analyzed by light microscopy and transmission electron microscopy (TEM), while the genetic diversity was investigated by 16S rRNA gene and 16S-23S internally transcribed spacer (ITS) sequencing. Molecular approaches to the study of cyanobacterial systematics have focused primarily on the analysis of 16S rRNA gene sequences (57); however, this gene is highly conserved, and its use in estimating relationships at the subgeneric level is limited (24). On the other hand, the ITS region, which is more variable than the 16S rRNA gene and less subject to selection pressure, is considered a useful tool in cyanobacterial taxonomy (12, 14, 18, 23, 37, 43). All these data allowed the successful identification of two phylotypes among six members of the genus *Leptolyngbya* and highlighted their intraspecific genetic variation.

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TABLE 1. *Leptolyngbya* strains used in this study

Strain designation	Isolation date	Isolation site
VRUC184 CSC7-1/Albertano et Bruno	1994	Catacombs of St. Callistus, on tufa
VRUC201 CD2/Kovacik et Albertano	1992	Catacombs of Domitilla, on plaster
VRUC206 CSC8/Kovacik et Albertano	1992	Catacombs of St. Callistus, on tufa
VRUC192 CP9-1/Albertano	1992	Catacombs of Priscilla, on tufa
VRUC198 CSS6-3/Albertano	1992	Catacombs of St. Sebastian, on tufa
VRUC135/Albertano	1985	Domus Aurea, on frescoes

MATERIALS AND METHODS

Cyanobacterial strains. Six nonaxenic strains of *Leptolyngbya* belonging to the Vergata Rome University Culture Collection (VRUC) and isolated from biofilms present in five Roman hypogea were studied (Table 1). All strains were cultured in liquid BG11 medium (45) at 20 ± 1°C, 60% relative humidity, and a photosynthetic photon flux density of 5 μmol photons m^{-2} s^{-1} (measured with a Licor LI-185B quantum/radiometer/photometer equipped with an LI-190SB quantum sensor) provided by fluorescent cool-white lamps (Philips HPL-N150W) with a dark-light cycle of 12:12 h.

Microscopy. Microscopic investigations of fresh samples were performed using a Zeiss Axioskop light microscope equipped with differential interference contrast. Images were taken using a Nikon Coolpix 5000 digital camera. Drawings were made after light microscopy observations in order to illustrate the key characteristics of each strain. For the morphological characterization of cyanobacterial strains, the diacritic traits used for description of botanical species were considered (35). In order to characterize cell size, at least 50 measurements were made for each biometrical characteristic. The six *Leptolyngbya* strains were also observed by TEM after fixation in 0.2 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde and subsequent postfixation in 1% osmium tetroxide, dehydration in ethanol series, and embedding with an 812 resin kit (Multilab, England). Ultrathin sections were stained with uranyl acetate and lead citrate (44) and observed using a Zeiss CEM 902 electron microscope at 80 kV.

Amplification of the 16S rRNA gene and ITS. Genomic DNAs were extracted from *Leptolyngbya* cells as described previously (15). The amplification of the 16S-23S rRNA operons from the six *Leptolyngbya* strains was performed using primer 1 (5′-AGAGTTTGATCCTGGCTCAG-3′ [59]), corresponding to nucleotides 8 to 27 of the 16S rRNA gene in *Synechocystis* sp. strain PCC 6301, and primer 18m (5′-TCTGTGTGCCTAGGTATCC-3′ [49]), corresponding to nucleotides 26 to 45 of the 23S rRNA gene in *Synechocystis* sp. strain PCC 6301. PCRs were carried out in 25-μl aliquots containing approximately 100 ng DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/10 volume of the supplied 10× buffer) supplemented to give a final concentration of 3 mM $MgCl_2$, 1 U of *Taq* polymerase (Amersham, Pharmacia), and 0.5 pmol of each primer. Reactions were cycled with a Mastercycler gradient (Eppendorf) as follows: 1 cycle at 94°C for 3 min; 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 3 min; and a final step at 72°C for 7 min. PCRs resulted in products of ~2,000 bp, which were purified on an agarose gel with a Qiaquick gel extraction kit (Qiagen) and used as templates to amplify the 16S rRNA gene and the ITS region.

The 16S rRNA genes were amplified using as the forward primer the cyanobacterial specific primer CYA359 (41) and the universal primer C (5′-ACG GGCGGTGTGTAC-3′) corresponding to *Escherichia coli* positions 1406 to 1392. Final PCR concentrations were as described above. Amplifications were run in a GeneAmp PCR system 2700 (Applied Biosystem) as follows: 1 cycle of 2 min at 94°C; 30 cycles of 1 min at 94°C, 40 s at 45°C, and 1 min at 72°C; and a final elongation step of 7 min at 72°C. After purification from the agarose gel using a Qiaquick gel extraction kit (Qiagen), the PCR products (~1,100 bp) were cloned into pGEM-T Easy vector (Promega) and sequenced using primers CYA359, C, AC552F (5′-CAGCCGCGGTAATAC-3′), and AC552R (5′-GTA TTACCGCGGCTG-3′).

PCR amplifications of the ITS regions were performed using the forward primer 16S3′F (49) and reverse primer 18m (58). Amplifications were run in a GeneAmp PCR system 2700 (Applied Biosystem) as follows: 1 cycle of 3 min at 94°C; 30 cycles of 1 min at 94°C, 40 s at 45°C, and 1 min at 72°C; and a final elongation step of 7 min at 72°C. PCR products of about 600 bp were purified with a Qiaquick gel extraction kit (MinElute gel extraction kit; Qiagen) and commercially sequenced independently on both strands using primers 16S3′F, ALAF (5′-GGTTTAGCTCAGTTGGT-3′), and 18m. Conserved domains and tRNAs were searched along the ITS sequences for the six strains using the cyanobacterial ITS alignment proposed by Iteman et al. (32) as a reference.

Phylogenetic analysis. 16S rRNA gene and ITS sequences obtained in this study were first analyzed by a similarity search using the BLAST software (www.ncbi.nlm.nih.gov/blast), and then the two most similar sequences were selected. Next the sequences were aligned with a representative data set of sequences of other *Leptolyngbya* strains available from GenBank, both manually and with the ClustalW program (www.ebi.ac.uk/clustalw). Maximum-parsimony trees were generated using a heuristic search constrained by random sequence addition, steepest descent, and tree-bisection-reconnection branch swapping using the PAUP* 4.0 b10 software package (51). Bootstrap values were obtained from 500 replicates with one random sequence addition to jumble the data using the PAUP* software. A maximum-likelihood tree was constructed using MODELTEST v 3.7 (42) with corrected invariable sites (I) and gamma shaper parameters (G). Distance analysis using the HKY85 distance method was also performed.

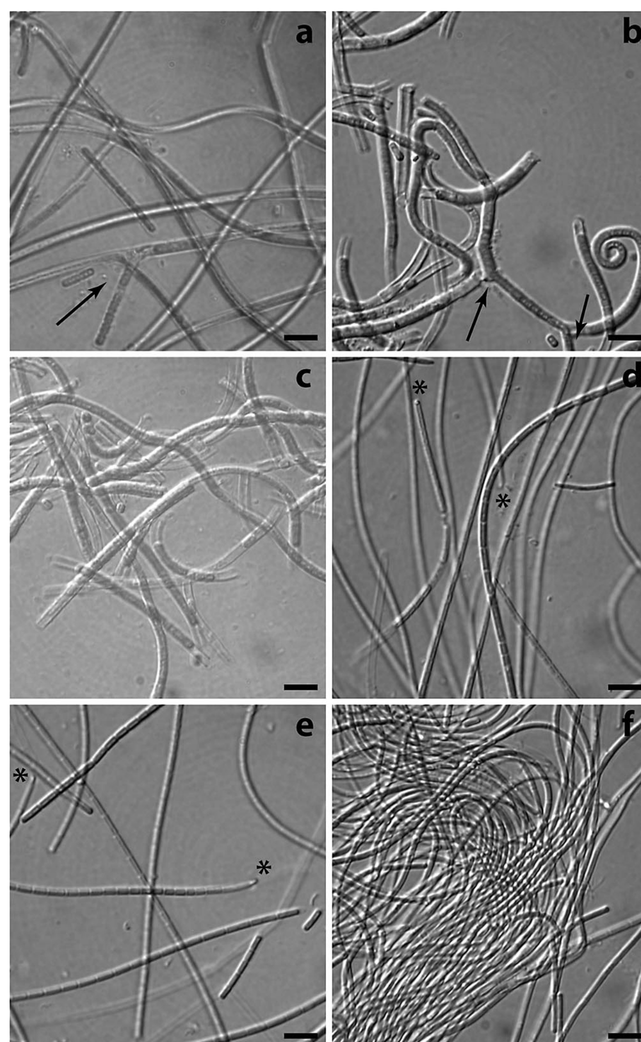

FIG. 1. Photomicrographs illustrating the morphological features of the six *Leptolyngbya* strains. On the basis of their pigmentation they could be divided into phycocyanin-rich strains (green) VRUC184 (a), VRUC201 (b), and VRUC206 (c) and phycoerythrin-rich strains (red) VRUC192 (d), VRUC198 (e), and VRUC135 (f). Arrows indicate false branching (a and b), and asterisks indicate the eyespot-like structure at the tip of the apical cell (d to f). Bars, 5 μm.

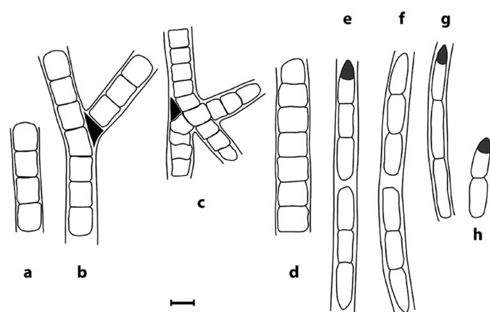

FIG. 2. Line images of the six *Leptolyngbya* strains, showing the most relevant morphological features. All strains exhibited trichome fragmentation or hormogonia (a and h). Strains VRUC184 (a and b) and VRUC201 (c) formed false branching in correspondence with the position of necridial cells, while VRUC206 (d) lacked them. VRUC192 (e), VRUC198 (f), and VRUC135 (g and h) had cells longer than wide and an orange spot at the tip of the apical cell. Bar, 2 μm.

The 16S rRNA gene sequence of *E. coli* K-12 was used as an outgroup for the construction of trees with the 16S rRNA gene sequences obtained in this study and the corresponding sequences of several *Leptolyngbya* strains available in GenBank. The ITS sequences were aligned with the closest related strains available from GenBank for which the alignment with our ITS sequences seemed meaningful. *Oscillatoria* sp. strain PCC 9240 was arbitrarily defined as an outgroup. The trees were edited using TREEVIEW version 1.6.6 (R. D. M. Page, distributed by the author at http://Taxonomy.zoology.gla.ac.uk/rod/rod.html.).

PositionsThe 405 to 780 of the *E. coli* 16S rRNA gene were used to find operational taxonomic units (OTUs) using a threshold of 97.5% identity among our strains (53). Furthermore, the identified OTUs were divided in two categories (52): "new" OTUs, composed of only our sequences exhibiting less than 97.5% identity with GenBank sequences, and "cosmopolitan" OTUs, including our sequences and others from different environments. The OTUs defined were compared with near-complete 16S rRNA gene and ITS region sequences to show phylogenetic relationships within the trees.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences were deposited in the GenBank database under accession numbers AY769961 (VRUC184) (an update of a shorter sequence), DQ295207 (VRUC198), DQ295208 (VRUC192), DQ295209 (VRUC201), and DQ295210 (VRUC206). ITS sequences were deposited with access codes EF560651 (VRUC135), EF560652 (VRUC192), EF560653 (VRUC198), EF560654 (VRUC184), EF560655 (VRUC206), and EF560656 (VRUC201).

RESULTS

Morphology and ultrastructure. The six *Leptolyngbya* strains were observed by light and electron microscopy. On the basis of their pigmentation (Fig. 1) the strains could be divided into two phenotypes: strains VRUC184, VRUC201, and VRUC206 were green or blue-green in color, while VRUC135, VRUC192, and VRUC198 were red, due to a high phycoerythrin content. All the strains appeared as long filaments, surrounded by uncolored sheaths open at each end, and were able to form hormogonia as reproductive cells (Fig. 2). The three green strains had isodiametric cells and trichome fragmentation by randomly occurring death of individual cells within a filament (Table 2), while two of them, VRUC184 and VRUC201, showed false branching (Fig. 1a to c). The trichomes of the red strains were thinner than those of the green strains (Fig. 1d and e; Table 2), with evident constrictions at the cross walls, cells which were longer than wide, and a conical apical cell characterized by an orange spot at the tip (4). Cells of the green strains varied between 2.0 and 2.3 μm in width and 1.9 and 2.7 μm in length, and those of the red strains varied between 1.3 and 1.5 μm in width and 3.5 and 5.8 μm in length.

The six *Leptolyngbya* isolates showed similar ultrastructural features typical of the genus (Fig. 3). The number of thylakoids varied from 5 to 6 in all the red strains, from 6 to 8 in the green strains VRUC201 and VRUC206, and from 6 to 10 in the green strain VRUC184. The latter had inner thylakoids which were more densely packed than the peripheral ones. The thylakoidal arrangement was parietal in all six strains, although in the green strain VRUC201 the thylakoids had anchorage points attaching them to the cell membrane. The nucleoplasm was characterized by the presence of carboxysomes, cyanophycin, and lipid globules, and interthylakoidal glycogen granules were also observed. Ultrastructural differences between the strains included the structure and the thickness of the envelopes. In fact, the green-pigmented strains VRUC201, VRUC206, and VRUC184 (Fig. 3a, b, and c) all possessed a multistratified compact sheath with fibrils parallel to the long axis of the filament. In contrast, the red strains VRUC192, VRUC198, and VRUC135 (Fig. 3d, e, and f) had a thin, bilayered sheath; the internal layer was made up of fibrils running parallel to the long axis of the filament, while the external layer was formed by disordered fibrils that were sometimes grouped into bundles. Several cells were observed to be actively dividing by centripetal invagination of the cell wall. More than one necridic cell was observed in the trichomes of strains VRUC184, VRUC201, and VRUC206. Coccoid or rod-shaped bacteria were sometimes visible in the cultures, often associated with the outermost sheath layers.

Phylogenetic analysis of the partial sequences of the 16S rRNAs and ITSs. The 16S rRNA gene sequence (~1,025 bases) was obtained for four *Leptolyngbya* strains, namely, VRUC192, VRUC198, VRUC201, and VRUC206. The 16S rRNA gene sequence of strain VRUC135 was previously re-

TABLE 2. Morphological features of the six *Leptolyngbya* strains observed with a light microscope[a]

Strain	Trichome fragmentation/ hormongonia	Necridic cells	False branching	Constrictions	Sheath	Filament diam (μm), mean ± SD	Cell width (μm), mean ± SD	Cell length (μm), mean ± SD	Apical cell shape	Pigmentation
VRUC184	+	+	+	+	+	2.4 ± 0.4	2.2 ± 0.4	2.7 ± 0.6	Rounded	Green
VRUC201	+	+	+	−	+	2.2 ± 0.3	2.0 ± 0.4	1.9 ± 0.3	Rounded	Blue-green
VRUC206	+	+	−	+	+	2.4 ± 0.3	2.3 ± 0.4	2.2 ± 0.5	Rounded	Blue-green
VRUC192	+	−	−	++	+	2.1 ± 0.5	1.4 ± 0.2	3.5 ± 0.7	Conical	Red
VRUC198	+	−	−	++	+	1.7 ± 0.4	1.5 ± 0.3	4.0 ± 0.7	Conical	Red
VRUC135	+	−	−	++	+	1.8 ± 0.2	1.3 ± 0.2	5.8 ± 0.9	Conical	Red

[a] −, absent; +, present; ++, abundant.

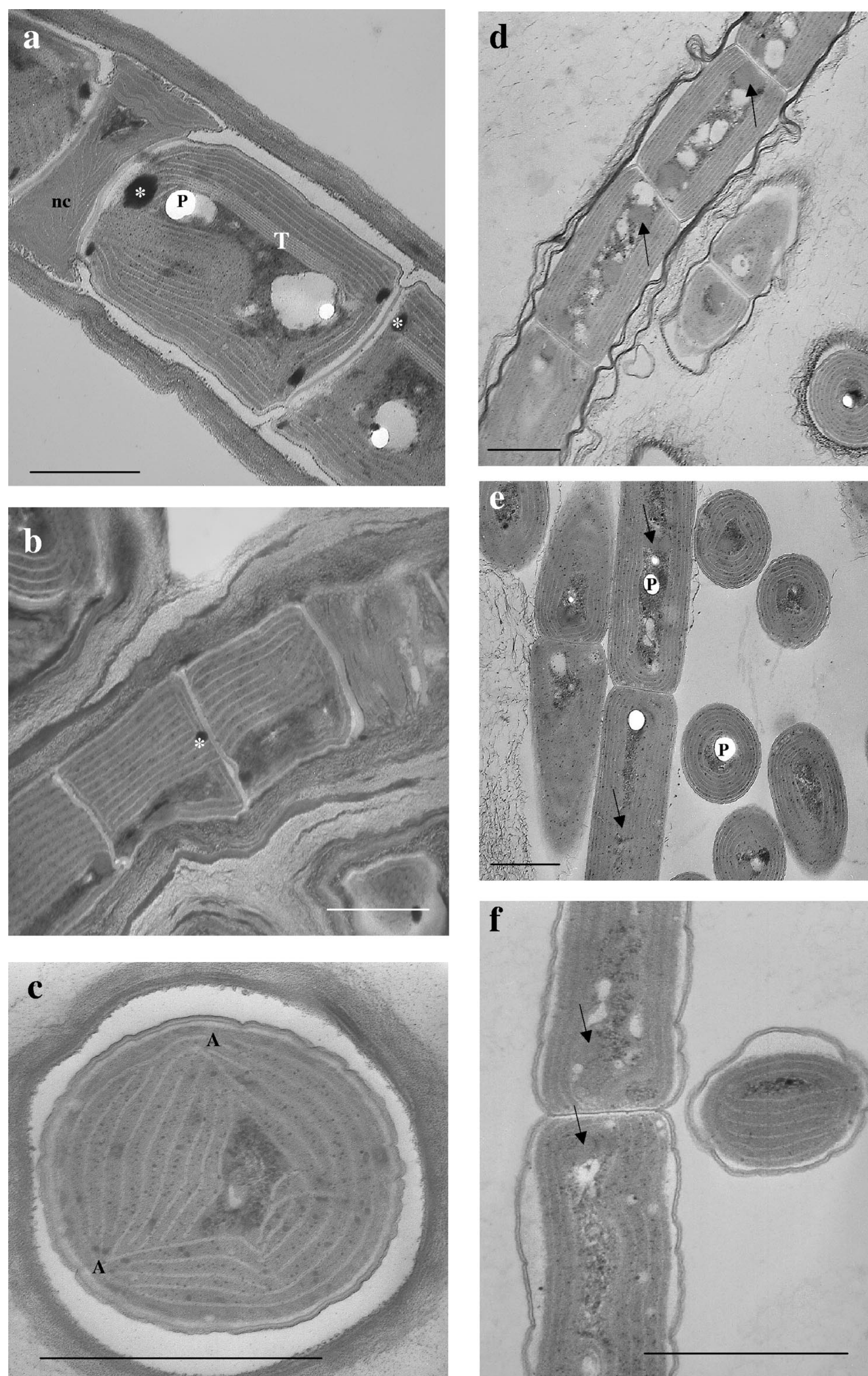

FIG. 3. TEM micrographs of strains VRUC184 (a), VRUC201 (b), VRUC206 (c), VRUC192 (d), VRUC198 (e), and VRUC135 (f). Note the presence of parietal thylakoids, polyphosphates (P), cyanophycin granules (asterisks), and carboxysomes (arrows) in the cytoplasm. In strain VRUC206 (c) the thylakoids were connected to the cell membrane (A), while in VRUC184 (a) the central thylakoids (T) were more densely packed than the peripheral ones. In the latter, a necridic cell (nc) is also visible. Bars, 1 μm.

ported (39), while a shorter 16S rRNA sequence of strain VRUC184 (15) has now been extended up to 1,027 bp. The red *Leptolyngbya* strains, VRUC135, VRUC192, and VRUC198, showed high DNA sequence identity (99.3% to 99.6%), as did the green strains VRUC184, VRUC201, and VRUC206 (98.4% to 99.5%). The sequence identity between the red and the green strains was about 92%. The neighbor-joining trees (Fig. 4) based on a data set of 45 16S rRNA gene sequences

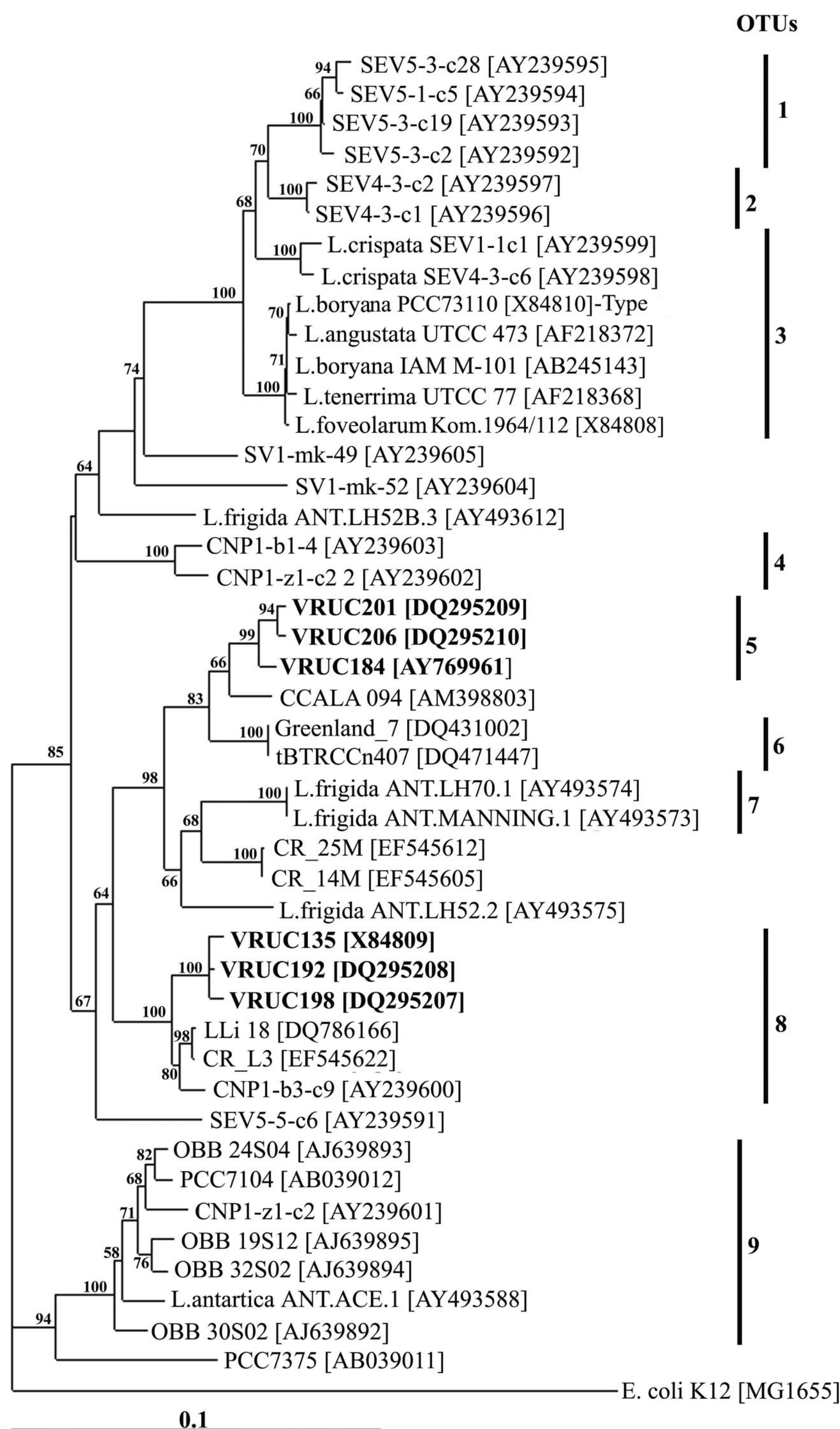

FIG. 4. Neighbor-joining tree inferred from 45 16S rRNA gene sequences (~ 916 bp). The numbers at the nodes indicate bootstrap values as percentages greater than 50% obtained using distance as an optimality criterion with 500 replicates. Numbers 1 to 9 indicate the OTUs inferred from partial 16S rRNA gene sequences (*E. coli* positions 405 to 780; threshold value, 97.5% identity). All the strains are *Leptolyngbya* sp. except where the species name is given. The sequences determined in the present study are indicated in bold. The *E. coli* K-12 sequence was designated as an outgroup. GenBank accession numbers are in brackets. The scale marker represents 0.1 nucleotide substitution per sequence position.

had the same topology of the trees inferred by the maximum-likelihood and the maximum-parsimony methods (not shown). As previously described (19), the well-supported clade of aquatic *Leptolyngbya* taxa, including the type species *L. boryana* (Gom.) Anagn. and Kom. PCC 73110 was sister to a clade containing *Leptolyngbya* strains from desert soils. In another paraphyletic branch of the phylogenetic tree, the red and green isolates of subaerophytic *Leptolyngbya* formed two different

clusters whose near-terminal branches were supported by high bootstrap values. Notably, the green strains clustered into a group that was sister to a clade formed by two strains of *Leptolyngbya*, one epilithic on granite in Nepal (CCALA 094, GenBank accession no. AM398803 [38]) and one benthic in Artic hot springs (Greenland_7, GenBank accession no. DQ431002 [46]), plus an unidentified filamentous strain from thermal springs in Jordan (tBTRCCn407, GenBank accession no. DQ471447 [30]). The first of these strains showed 97.4 to 97.6% pairwise identity with the green strains, while the second and the third had less than 95% identity. The red strains were sister to a clade formed by three strains of *Leptolyngbya* sp.: two from geothermal waters of Costa Rica (LLi 18, GenBank accession no. DQ786166 [K. Finsinger and W. R. Hess, unpublished]; CR_L3, GenBank accession no. EF545622 [S. Morales et al., unpublished]) and one from Canyonland National Park (Utah) (CNP1-b3-c9, GenBank accession number AY239600 [19]) whose ecology was not disclosed. The pairwise identity of these three strains with the red strains ranged from 97.5% to 98.0%.

Based on the alignment of shorter 16S rRNA sequences, corresponding to positions 405 to 780 of the *E. coli* 16S rRNA gene, nine OTUs were found (Fig. 4) using a threshold of 97.5% identity. The red strains grouped in OTU8 with strains LLi 18, CR_L3, and CNP1-b3-c9 (pairwise identity of 97.8 to 98.3%); thus, we define this as a "cosmopolitan OTU" because it included our sequences and others from different environments. The green strains grouped in OTU5; strain CCALA 094 was near the threshold value (pairwise identity of 97 to 97.5%) for assignment to this OTU. The sequence identity within each OTU varied from 97 to 100% for the longer sequence of the 16 rRNA gene sequences (~916 nucleotides) and from 97.5 to 100% for the OTUs (data not shown). OTU7 corresponded to OTU9 determined in a previous study (52).

The PCR amplification of the ITS regions yielded one dominant band of the expected size (Fig. 5): 600 nucleotides for the three red strains, 564 nucleotides for strain VRUC184, 630 nucleotide for strain VRUC206, and 631 nucleotides for strain VRUC201. This corresponded to the length of the ITS plus 22 bp of the 16S rRNA gene and 44 bp of the 23S rRNA gene, since the first primer recognition site is located ~50 bp before the 3′ end of the 16S rRNA gene and the second is ~50 bp after the 5′ end of the 23S rRNA gene. Sometimes minor bands were obtained, similar to those of cyanobacterial strains in which the presence of heteroduplex formation and multiple operons had been shown (32, 33). The sequences obtained for the 600-bp PCR product showed that the ITS regions of each of the six *Leptolyngbya* strains contained both $tRNA^{Ala}$ and $tRNA^{Ile}$ genes and the conserved domains D1, D1′, D2, D3, box A, D4, and D5, as described by Iteman et al. (32) (Fig. 6). In all six strains we found 100% sequence identity within these conserved regions, except for box A and D5, where only a few gaps and nucleotide substitutions were present. This distinguished the strains belonging to OTU5 from the strains belonging to OTU8. The polymorphism was found in the more variable regions (V2, box B, and V3) with some different nucleotide substitutions. The red strains VRUC192, VRUC198, and VRUC135 showed high pairwise identity (99.6 to 100%) and were readily separated from the green strains VRUC184, VRUC201, and VRUC206, with lower than 74% identity. Two

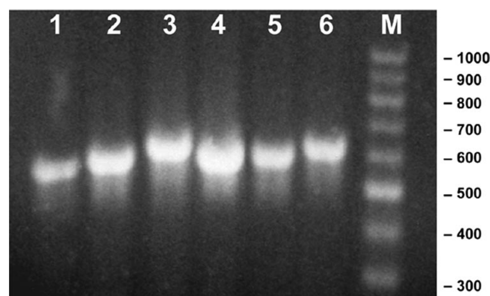

FIG. 5. Agarose gel showing the PCR products obtained by amplification of the ITS regions with the six *Leptolyngbya* strains. One principal band was obtained for all the strains. Lanes: 1, VRUC184; 2, VRUC192; 3, VRUC201; 4, VRUC135; 5, VRUC198; 6, VRUC206; M, 1-kb molecular size ladder.

green strains, VRUC201 and VRUC206 (with 98.2% pairwise identity), were distinct from strain VRUC184 with 80% sequence identity.

When the ITS sequences of these six strains were compared to a selection of *Leptolyngbya* ITS sequences available from GenBank, the results (Fig. 7) were comparable with the grouping inferred from the 16S rRNA gene sequences. The epilithic *Leptolyngbya* strain CCALA 094 (GenBank accession no. AM398976) clustered with the green strains; they had 73.7 to 77.1% sequence identity for the full-length ITS, but 100% sequence identity was observed within the conserved domains (Fig. 6). The thermal *Leptolyngbya* strain LLi 18 (GenBank accession no. DQ786166), belonging to OTU8, clustered with the red strains, sharing 80% sequence identity for the full-length ITS, with 100% sequence identity in the conserved domains. In both cases the degree of dissimilarity was higher in the polymorphic regions and in particular in region V2. Unfortunately, no ITS sequences are available for the other two strains, CNP1-b3-c9 and CR_L3, belonging to the same OTU. The ITS sequences of the two strains of *Leptolyngbya frigida* belonging to OTU7 clustered together.

DISCUSSION

In this study, we report on the cytomorphological and genetic diversity of six subaerophytic troglobitic *Leptolyngbya* strains isolated from five Roman hypogea, as revealed by combining morphological and ultrastructural observations with 16S rRNA gene and ITS sequencing.

Six strains of *Leptolyngbya* could be separated into two morphotypes based on pigmentation and cell diameter. However, within the same morphotype, the differences observed, i.e., the presence or absence of false branching, cell sizes, and constrictions at the cross wall, did not allow a net distinction among the strains. Furthermore, the six *Leptolyngbya* strains also shared common ultrastructural features. In all cases, the parietal arrangement of thylakoids was typical of this genus, as it is in *Pseudoanabaenaceae* (11). Most of the morphological and ultrastructural features of subaerophytic *Leptolyngbya* strains have been previously studied, because of their ecological relevance in Roman hypogea (1, 4, 5, 6, 8). In fact, these are the most common cyanobacteria in extreme low-light environments and are the major agents of biodeterioration of under-

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                  D1
VRUC135    AGGGAGACCTACCCGCTCAAATT----------------------------------------C 24
VRUC198    AGGGAGACCTACCCGCTCAAATT----------------------------------------C 24
VRUC192    AGGGAGACCTACCCGCTCAAATT----------------------------------------C 24
Lli 18     AGGGAGACCTACCCATTCAGATT----------------------------------------C 24
CCALA 094  AGGGAGACCTACCCACTCTATGA----------------------------------------C 24
VRUC206    AGGGAGACCTACCCGCTTGAGAAGGATGAAGGGTAAAGGATGAAGGATGAAATAAGCCCGAAGCC 65
VRUC201    AGGGAGACCTACCCGCTTGAGAAGGATGAAGGGTAAAGGATGAAGGATGAAACAAGCCCGAAGCC 65
VRUC184    AGGGAGACCTACCCGCTCTATAA----------------------------------------T 24

                                                     D1'
VRUC135    TGAAAGCACAGTG-CAAATAGGAAAT-------GAGTTGGTCATCCCAAGGTCGTTCGAGGTATG 81
VRUC198    TGAAAGCACAGTG-CAAATAGGAAAT-------GAGTTGGTCATCCCAAGGTCGTTCGAGGTATG 81
VRUC192    TGAAAGCACAGTG-CAAATAGGAAAT-------GAGTTGGTCATCCCAAGGTCGTTCGAGGTATG 81
Lli 18     TGAAAGCACAGAG-CAAATAGGGACT-------GAATTGGTCATCCCAAGGTCGTTCGAGGTATG 81
CCALA 094  TGAACGCAATCAG-CAAATAAGTTAA-------GAGTTGGTCATCCCAAGGTCGTTCGAGGTTTA 81
VRUC206    TGAAACTCTTCAGATATACACATTACTTCTTCCAAGTTGGTCATCCCAAGGTCGTTCG-AGTATA 129
VRUC201    TGAAACTCTTCAAACATACACATTACTCCTTCCAAGTTGGTCATCCCAAGGTCGTTCG-AGTATA 129
VRUC184    TGAAAGCACTCAG-CAAATAAATCAA-------GAGTTGGTCATCCCAAGGTCGTTCGGAGTATG 81

                  D2           D3                      tRNA^Ile
VRUC135    AGTGTGAG-GCTTTCAAACTATTAAAGGTTCGGAT--ATGGGCTATTAGCTCAGGTGGTTAGAGC 143
VRUC198    AGTGTGAG-GCTTTCAAACTATTAAAGGTTCGGAT--ATGGGCTATTAGCTCAGGTGGTTAGAGC 143
VRUC192    AGTGTGAG-GCTTTCAAACTATTAAAGGTTCGGAT--ATGGGCTATTAGCTCAGGTGGTTAGAGC 143
Lli 18     AGTGTAAG-GCTTTCAAACTATTAAAGGTTCGGAT--ATGGGCTATTAGCTCAGGTGGTTAGAGC 143
CCALA 094  AGTATCAG-GCTTTCAAACTATTT--GGTTCGGTTTCATGGGCTATTAGCTCAGGTGGTTAGAGC 143
VRUC206    AGTGTCTGAGCTTTCAAACTATTC--GGTTCGGTTTCATGGGCTATTAGCTCAGGTGGTTAGAGC 192
VRUC201    AGTGTCTGAGCTTTCAAACTATTC--GGTTCGGTTTCATGGGCTATTAGCTCAGGTGGTTAGAGC 192
VRUC184    AGTGTCTGAGCTTTCAAACTATTTT-GGTTCGGTTTCATGGGCTATTAGCTCAGGTGGTTAGAGC 145

                              tRNA^Ile
VRUC135    GCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCAGGATGGCCCACCTTAAG-AAGGACGAA 207
VRUC198    GCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCAGGATGGCCCACCTTAAG-AAGGACGAA 207
VRUC192    GCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCAGGATGGCCCACCTTAAG-AAGGACGAA 207
Lli 18     GCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCAGGATGGCCCACCTTAGC-GA------- 200
CCALA 094  GCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCAGGATGGCCCACCTTCAC-A-------- 199
VRUC206    GCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCAGGATGGCCCACCTTTACCGAGGACGAA 257
VRUC201    GCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCAGGATGGCCCACCTTTACCGAGGACGAA 257
VRUC184    GCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCAGGATGGCCCACCTTA------------ 198

                                 V2
VRUC135    AGAGAGAAGAGAGAAGAGAGAGGTTTAACCTCTGTCCTCCAGCTTCAACTTTCATTCTTTAGAGC 272
VRUC198    AGAGAGAAGAGAGAAGAGAGAGGTTTAACCTCTGTCCTCCAGCTTCAACTTTCATTCTTTAGAGC 272
VRUC192    AGAGAGAAGAGAGAAGAGAGAGGTTAAACCTCTGTCCTCCAGCTTCAACTTTCATTCTTTAGAGC 272
Lli 18     ------------------------------------------TTAATATT---------GCAC 212
CCALA 094  -------------------------------------------------------------CCT 202
VRUC206    GTGCTGAGGACTGAGGACTGAGTAGGAATATTCATCTTTCATCCTTTATTCTTCATCCTTTACAT 322
VRUC201    GTGCTGAGGACTGAGGATTGAGTAGGAATATTCATTTTTCATCCTTCATTCTTCATCCTTTACAT 322
VRUC184    --GCAAGGGATTTTGGATTGAGTC----CATCCATCTAAGATCCAGAATT-----------GCAT 244

                              tRNA^Ala
VRUC135    GGGGGTTTAGCTCAGTTGGTAGAGCGCCTGCTTTGCAAGCAGGATGTCAGCGGTTCGAGTCCGCT 337
VRUC198    GGGGGTTTAGCTCAGTTGGTAGAGCGCCTGCTTTGCAAGCAGGATGTCAGCGGTTCGAGTCCGCT 337
VRUC192    GGGGGTTTAGCTCAGTTGGTAGAGCGCCTGCTTTGCAAGCAGGATGTCAGCGGTTCGAGTCCGCT 337
Lli 18     GGGGGTTTAGCTCAGTTGGTAGAGCGCCTGCTTTGCAAGCAGGATGTCAGCGGTTCGAGTCCGCT 277
CCALA 094  GGGGGTTTAGCTCAGTTGGTAGAGCGCCTGCTTTGCAAGCAGGATGTCAGCGGTTCGAGTCCGCT 267
VRUC206    GGGGGTTTAGCTCAGTTGGTAGAGCGCCTGCTTTGCAAGCAGGATGTCAGCGGTTCGAGTCCGCT 387
VRUC201    GGGGGTTTAGCTCAGTTGGTAGAGCGCCTGCTTTGCAAGCAGGATGTCAGCGGTTCGAGTCCGCT 387
VRUC184    GGGGGTTTAGCTCAGTTGGTAGAGCGCCTGCTTTGCAAGCAGGATGTCAGCGGTTCGAGTCCGCT 309

                                                                 BoxB
VRUC135    AACCTCCACTGGG---AGTTAATAGGGCAAA--AATTAG--------AGGAACAATTCAGCAACT 389
VRUC198    AACCTCCACTGGG---AGTTAATAGGGCAAA--AATTAG--------AGGAACAATTCAGCAACT 389
VRUC192    AACCTCCACTGGG---AGTTAATAGGGCAAG--AATTAG--------AGGAACAATTCAGCAACT 389
Lli 18     AACCTCCACTGG----AGTTGATAGAGCAAGCGAGATTG--------AGGAACAGTTCAGCAACT 330
CCALA 094  AACCTCCACTGG-----GTTTTATGCCCAAATAGTATTT--------GGAGTTAGCTCAGCAACT 319
VRUC206    AACCTCCACTGGGCAAATTGCCCAATATGAAGCAATTTT-------AGAGA-G--TTCAGCACT 442
VRUC201    AACCTCCACTGGGCAAATTGCCCAATATGAAGCAATTTT-------AGAGA-GAGTTCAGCAACT 444
VRUC184    AACCTCCACTGGGCGGATTGCCCAACCCGAAGCAAGATTCAAATTGAGAGATAAATTCAGCAACT 374

                       BoxB                                  BoxA
VRUC135    TATCTAG------------------GTTTGGCTAGGAAGTCTGCTGGATTA-GTCCAGCCAGAAC 435
VRUC198    TATCTAG------------------GTTTGGCTAGGAAGTCTGCTGGATTA-GTCCAGCCAGAAC 435
VRUC192    TATCTAG------------------GTTTGGCTAGGAAGTCTGCTGGATTA-GTCCAGCCAGAAC 435
Lli 18     TATCTAG------------------GGAATGCTAGAGAGCCTGCTGGGATT-GTCCAGCCAGAAC 376
CCALA 094  TATCTAGTTAGTATCGATTGAATACATTAAGCTAGAGAGCCTGCTGGATTA-ATCCAGCTAGAAC 383
VRUC206    TATCTGGATAGTCTGGTC----AAAGACTGTTGAGAGAGCCTGCTGGATTTTA-CCAGCCAGAAC 502
VRUC201    TATCTGGATAGTCTGGTC----AAAGACTGTTGAGAGAGCCTGCTGGATTTTA-CCAGCCAGAAC 504
VRUC184    GGTCTGGAGAGTTCAGT-----AAGAACTCTTGAGGAAGCCTGCTGGGTTTTATCCAGCCAGAAC 434

            BoxA   D4               V3
VRUC135    CATGAAAACTGCATAGCGAAAGAAGATTTCTGTCAGGTAGTAGAGATTCATTTGACCCAGGTTGA 500
VRUC198    CATGAAAACTGCATAGCGAAAGAAGATTTCTGTCAGGTAGTAGAGATTCATTTGACCCAGGTTGA 500
VRUC192    CATGAAAACTGCATAGCGAAAGAAGATTTCTGTCAGGTAGTAGAGATTCATTTGACCCAGGTTGA 500
Lli 18     CATGAAAACTGCATAGCGAAAGAAGATTTCTGTCAGGTAGTAGAGATTCATTTGATTATTGTTAA 441
CCALA 094  CTAGAAAACTGCATAGCGATCAA------TTGTCAGGTAGTAAATATCA--------------- 427
VRUC206    CTAGAAAACTGCATAGCGAGAAA------TTGTCAGGTAGTAAAATATCA--------------- 546
VRUC201    CTAGAAAACTGCATAGCGAGAAA------TTGTCAGGTAGTAAAATATCA--------------- 548
VRUC184    CTAGAAAACTGCATAGCGAAAAAA-----TTGTCAGGTAGTAAAATATCA--------------- 479

                                D5
VRUC135    ATGAATCGTCAACACAGACACCAATGAATTTTGT 534
VRUC198    ATGAATCGTCAACACAGACACCAATGAATTTTGT 534
VRUC192    ATGAATCGTCAACACAGACACCAATGAATTTTGT 534
Lli 18     ATGAATC---AACACAGACACCAATGAATTTTGT 472
CCALA 094  ----------------GACACCAATGT-TGTAGT 444
VRUC206    ----------------GACACCAATGT-TGTAGT 563
VRUC201    ----------------GACACCAATGT-TGTAGT 565
VRUC184    ----------------GACACCAATGT-TGTAGT 498
```

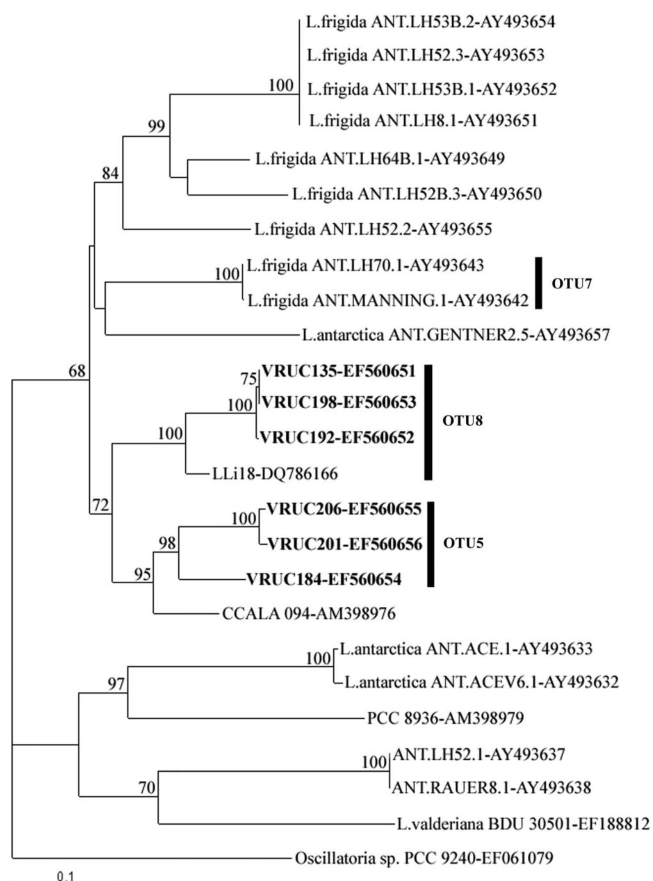

FIG. 7. Distance tree (HKY85) constructed from an alignment of the ITS region sequences obtained for the six *Leptolyngbya* strains (in bold) along with other ITS sequences (*Leptolyngbya* sp. except where the species name is given) available from GenBank. *Oscillatoria* sp. strain PCC 9240 was arbitrarily used as an outgroup. Numbers above branches indicate parsimony bootstrap values (percentages) of greater than 50% based on 500 replicates. Three clusters represent the same strains as in the tree obtained with the 16S rRNA gene sequences and the definition of OTU5, -7, and -8. The GenBank accession number is indicated after the strain name. The scale marker represents 0.1 nucleotide substitution per sequence position.

ground archaeological sites attributable to the presence of acidic and sulfated groups in the heteropolysaccharides that form their sheaths, which have the ability to remove cations from stone substrata (13).

The current deficient state of cyanobacterial taxonomy makes a reevaluation of diagnostic traits based on a combination of thylakoidal patterns and molecular phylogenetic analyses timely (28, 36). In this respect, the thylakoid arrangement in our *Leptolyngbya* strains is in agreement with the position of this taxon in the phylogenetic tree of cyanobacteria. The 16S rRNA gene sequence identity among the green and the red strains was 92%. This would support a tentative assignment of the three green strains to the genus "*Leptolyngbya*" Komárek and Anagnostidis sensu stricto (35) and of the red strains to a different taxon.

Phylogenetic analysis based on longer (~916 nucleotides) and shorter (~400 nucleotides) sequences of the 16S rRNA gene confirmed the well-known polyphyletic nature of the genus *Leptolyngbya* (19, 31, 40, 54). In fact, different clusters and nine OTUs were obtained when 44 *Leptolyngbya* sequences were aligned. The type species, *L. boryana* (Gom.) Anagn. et Kom. PCC 73110 and *L. foveolarum* Komárek 1964/112, assigned to the genus "*Leptolyngbya*" (35), were present in a paraphyletic branch and in a different OTU than the green strains although they were assigned to the same genus. This highlights the heterogeneity of this genus and the likely presence of more generic entities still to be defined.

The phylogenetic analyses also indicated that the three troglobitic red strains clustered with three strains of *Leptolyngbya* sp. from subaerophytic and geothermal environments and belonged to the same OTU8, sharing more than 97.5% sequence identity (in the region corresponding to *E. coli* positions 405 to 780). Based on these data and on the species definition of Stackebrandt and Goebel (50), in which sequence identity among strains of less than 97.5% indicates that they represent different species, all these six strains may belong to the same species.

The phylogenetic relationships deduced from the 16S rRNA gene sequencing were in agreement with the ITS grouping as well with the grouping of the different OTUs. The analysis of the ITS sequences of the six strains identified three clusters: the first containing the red strains VRUC135, VRUC192, and VRUC198; the second the green strains VRUC201 and VRUC206; and the third the green strain VRUC184. Indeed, the ITS sequencing not only supported the high morphological and genetic identity shown for the red strains but discriminated among the green strains.

Complete sequence identity was found in the conserved domains in all six strains along with the *Leptolyngbya* strains CCALA 094 from Nepal and LLi 18 from Costa Rica. These domains are important sites for folding of the rRNA transcripts or for the transcriptional antitermination and the encoding of $tRNA^{Ile}$ and $tRNA^{Ala}$ (32). High identity in the conserved domains and polymorphisms in the variable regions of the ITS were also found in benthic and pelagic *Microcystis* colonies from a French storage reservoir (29). Nonetheless, the more variable regions (V2, box B, and V3) facilitated the discrimination among the six strains, defining three clusters, one with the red strains, one with the two green isolates VRUC201 and VRUC206, and another one with the green strain VRUC184. It appears that the secondary structure of these variable regions is more important than the primary sequence (32), and according to secondary structure predictions, variations are mostly confined to regions corresponding to loops or hairpin structures (22, 32). Furthermore, these

FIG. 6. Nucleotide sequence alignments of full-length ITSs of the eight *Leptolyngbya* strains (six catacomb strains and strains LLi 18 and CCALA 094). The conserved (D1, D1′, D2, D3, D4, and D5) and variable (V2 and V3) domains, the antiterminator (box B and box A), and the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes are indicated. One hundred percent sequence identity is indicated in grey within the conserved domains and is shaded within the variable regions.

variable regions showed the close relationship of the red strains with strain LLi 18 (92% sequence identity). This finding together with the 16S rRNA gene sequence data indicated that the two strains belong to the same species, although more knowledge of the morphological characters of the LLi 18 strain are needed to confirm this. The ITS relatedness of strains CNP1-b3-c9 and CR_L3 cannot be determined because of a lack of the corresponding sequences. However, because of the high sequence identity of the 16S rRNA gene sequences of these two strains with the LLi 18 strain (>98%), we could infer that these strains may also belong to the same species of the red *Leptolyngbya* strains.

OTU5 represents a new phylotype, because our sequences did not exhibit more than 97.5% identity with GenBank sequences. The genetic comparison of the 16S rRNA and ITS sequences of CCALA 094 with those of the green strains showed sequence identities of less than 97.5% and 77%, respectively. This strain also has a different cytomorphology, with cells shorter than wide and peripheral ondulating thylakoids (38). Based on these data, CCALA 094 was excluded from OTU5, and thus this should be considered a "new" OTU.

All these results demonstrate the utility of the polyphasic approach in cyanobacterial taxonomic studies. The ITS pattern configuration identified in the epilithic troglobitic *Leptolyngbya* strains, containing both $tRNA^{Ile}$ and $tRNA^{Ala}$, has been reported as the most common for cyanobacteria. It was found also in almost all plastids investigated to date and is probably the same which the rRNA operon of the cyanobacterial ancestor may have possessed (14). This is in accordance with the hypothesis of an early origin of the *Oscillatoriales* and *Chroococcales* with respect to the heterocystous cyanobacteria (57) and confirms that the 16S-23S region represents a potentially powerful tool for studies of phylogeny and molecular evolution of cyanobacteria (14, 25, 43).

The comparison with other sequences available in the GenBank database showed that some genotypes are conserved in troglobitic *Leptolyngbya* strains as well as in subaerophytic and geothermal isolates such as CCALA 094 from Nepal, CNP1-b3-c9 from Utah, and LLi 18 and CR_L3 from Costa Rica, probably because of the extreme environmental conditions of their habitats.

In this study, we found a good resolution of the genetic variability among these strains using ITS domain sequencing. Since ITS differences reflect the geographic distribution of cyanobacteria, as has been reported for aquatic strains (18, 52), more ITS sequences of subaerophytic *Leptolyngbya* strains are needed to better understand their evolution and biogeography. To our knowledge, this is the first study in which a polyphasic approach, combining morphological and ultrastructural observations with 16S rRNA gene and ITS sequencing, was employed to resolve the diversity of *Leptolyngbya* strains and to assess intraspecific variation.

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