Cyanobacterial sequences retrieved directly from the Great Sippewissett Salt Marsh, MA, USA

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The microbial mats of Great Sippewissett Salt Marsh (Cape Cod, MA, USA) have been studied previously by Nicholson *et al.* (1987) and Pierson *et al.* (1987, 1990). These investigations included the use of light and electron microscopy, pigment analysis, and fiber optic analysis of spectral irradiance within the mats. These techniques have provided very valuable information. However, communities are difficult to fully describe by microscopy and pigment analysis alone. Thus, they remain poorly understood and only broadly characterized (Turner *et al.*, 1989; Zehr *et al.*, 1995).

During the "Microbial Diversity" course of the Marine Biological Laboratories (Woods Hole, MA) in July 1996, a preliminary molecular study of the diversity of the multi-layered, laminated microbial mats from the Great Sippewissett Salt Marsh was carried out. Samples from the upper green-colored cyanobacteria-dominated layer and the pink-colored, purple sulfur bacteria layer below it were sampled by coring and scraping individual layers with a razor blade. Cell material was suspended in sterile seawater. After washing in TE buffer to remove the sand grains from the cell material, the cells were resuspended in 50 µl of TE buffer. 15 µl of Gene Releaser™ (5-3 Prime Inc.) was added to extract DNA following the thermocycler method described in the manufacturer's instructions (65°C 30 sec, 8°C 30 sec, 65°C 90 sec, 97°C, 180 sec, 8°C 60 sec, 65°C 180 sec, 97°C, 60 sec, 65°C 60 sec, 80°C hold) in an Ericomp Powerblock™. 5 µl of this lysis product was used for PCR in a total volume of 100 µl, containing PCR buffer 1X (Fisher Products), 2.5 mM

$MgCl_2$, 200 µM dNTPs, and 0.6 µM of primers. One Ampliwax bead (Perkin-Elmer) was added to each tube. Two primer pairs were used, the universal pair: AGAGTTTGATYMTGGC and GYTACCTTGTTACGACTT (B. Paster, pers. com.) and the cyanobacterial-specific pair: GAGAGTTTGATYCTGGCTCAG and TCTGTGTGCCTAGGTATCC (B. Paster pers. com.; Wilmotte *et al.*, 1993). In the case of the universal pair, the 16S rRNA gene was amplified between the positions 24 and 1491 of *E. coli*, whereas for the cyanobacterial-specific pair, the 16S rRNA, ITS and the start of the 23S rRNA genes were amplified between positions 28 of the 16S rRNA and 25 of the 23S rRNA of *E. coli* (Brosius *et al.*, 1981). A hot-start procedure was followed, with an incubation at 94°C for 10 min followed by 5 min at 35 °C to solidify the wax, during which 2.5 U Taq Polymerase (Fisher Products) was added. The PCR cycles were 35 repetitions of 94°C 1 min, 50°C 1 min, 72°C 2 min followed by 5 min at 72°C. The PCR products were purified using the Promega Wizard™ PCR Prep kit and then cloned into the pCNTR vector using the blunt-end ligation protocol of the General Contractor™ DNA Cloning System (5-3 Prime, Inc.). In short, 14.5 µl of PCR products were blunted and phosphorylated before ligation. The plasmids were checked for proper insertions by restriction with BamH1. With this cloning kit, 1 ng of vector pUC18 yielded about 1000 blue colonies. Unfortunately, cloning efficiency was quite low in this study. For example, 14.5 µl of PCR product from the pink layer amplified with the universal primers yielded about 300 colonies, of which 20 were blue. However, only 2 out of 8 clones from white colonies had an insert with the expected size after BamH1 digest.

Two cyanobacterial clones, WH7B (green layer, cyanobacterial-specific primers) and WH12 (green layer, universal primers), were further analyzed. Plasmid DNA purification was performed on 1.5 ml of an overnight culture using the Quantum Prep Plasmid DNA Purification protocol (BioRad, USA) and the sequence was determined bidirectionally (Genome Express, France). The EMBL accession numbers are AJ007374 (WH7B) and AJ007375 (WH12). The sequences were aligned manually with their closest relatives in an alignment of 31 complete cyanobacterial sequences. It appeared that the circa 200 bp at the 5' end of the 16S rRNA sequence of WH12 have a different origin than the rest of the sequence and shows 174 identities out of 185 positions with a phototrophic bacterium (DSM 2111). This sequence thus seemed to be a chimera, as confirmed by the "Chimera Check" analysis of the RDP server (http://rdpwww.life.uiuc.edu/). For submission to EMBL and construction of a distance tree, the first 223 bases were therefore excluded. Pairwise evolutionary distances were calculated using the formula of Jukes and Cantor (1969), correcting for multiple mutations. Positions corresponding to *E. coli* 237 to 1392 were used (Brosius *et al.*, 1981). This distance matrix was used to construct a tree topology by the Neighbor-joining method (Saitou & Nei, 1987) implemented in the software package TREECON (Van De Peer & De Wachter, 1993). *Escherichia coli* was used as an outgroup. Indels were not taken into account and a bootstrap analysis involving 500 resamplings was performed.

The neighbor-joining tree in Fig. 1 shows that both cyanobacterial sequences, WH7B and WH12, appear to belong to one lineage containing narrow

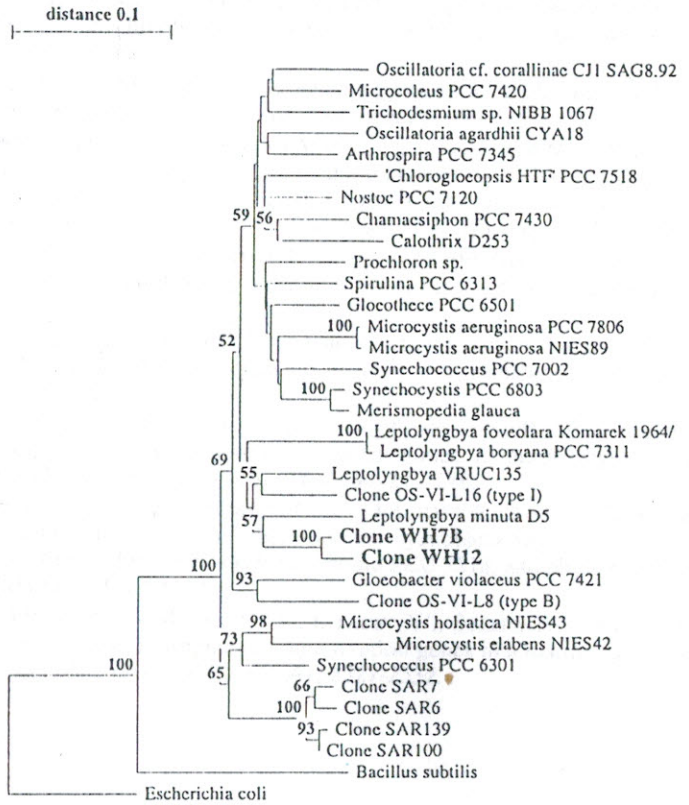

Figure 1. Neighbor-joining tree based on 16S rRNA sequence positions 237 to 1392 (*E. coli* numbering) from 33 cyanobacteria, *Bacillus subtilis*, and *Escherichia coli* which is used for rooting the tree. The two clones, WH12 and WH7B obtained during this study are indicated in bold. Bootstrap percentages obtained after 500 resampling are given besides the corresponding nodes when they are higher than 50%. Branches supported by less than 50% of bootstrap support are drawn as unresolved. The distances between two strains are obtained by summing the lengths of the connecting horizontal branches, using the scale on the top.

filaments of diameters around 2-3 µm (*Phormidium* following Geitler, *Leptolyngbya* following Anagnostidis and Komárek), though with a bootstrap support of only 57 %. The uncorrected dissimilarity values used for the tree construction are 2.4 % between WH7B and WH12, 8.4 % between WH7B and *Leptolyngbya minuta* D5, 7.6 % between WH7B and *Leptolyngbya* VRUC, and 8.2 % between WH7B and clone 0S-VI-L16. The cyanobacterial clones are thus most similar, but not closely related, to *Leptolyngbya minuta* D5, which is a marine, epiphytic cyanobacterium isolated from the Baleare Islands in Spain (Wilmotte, 1991) and *Leptolyngbya* VRUC135 which was

isolated from Roman frescoes (Albertano, 1991). The morphology of the clone 0S-VI-L16 retrieved from Octopus Spring, Yellowstone (Ward *et al.*, 1992) is unknown. During microscopic observations of the mats, we frequently have observed narrow filamentous cyanobacteria with the same type of morphology as *L. minuta* D5 and VRUC 135.

This preliminary study represents one of the first attempts to use molecular tools based on the rRNA sequence to describe salt marshes microbial mats. Several difficulties were encountered in isolating and amplifying DNA from these environmental samples. One problem was in sampling individual layers of the mat as the coring and scraping method was not as precise as could be desired. The layers in a single mat were quite variable in thickness and numbers at distances of a few centimetres. Another difficulty involved the cell lysis and DNA extraction method which did not pull out DNA from the heavily sheathed cyanobacterial types like *Microcoleus chthonoplastes* which was common in the mats. In future work, mechanical disruption or another more efficient DNA extraction method should be used instead. The rather low cloning efficiency should also be addressed. An additional purification of the PCR products to get rid of shorter non-specific fragments produced during amplification would probably improve efficiency. While these difficulties should be noted, cyanobacterial sequence data were obtained from these samples and it is quite clear that molecular methods are providing another valuable set of tools in characterizing this still poorly understood salt marsh habitat.

ACKNOWLEDGEMENTS

We are grateful to the Bernard Davis Fund, the Office of Naval Research (grant 3N000014-95-1-0463), and S.O. Mast Memorial Fund for financial support to attend the Microbial Diversity Course at MBL. We thank the course directors, A. Salyers and E. Leadbetter, the faculty members and teaching assistents for help, ideas, and support.

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