Phylogenetic characterization of the blue filamentous bacterial community from an Icelandic geothermal spring

Cristina Danielle Takacs a, Marissa Ehringer b, Renée Favre c, Michaele Cermola c, Gudmundur Eggertsson d, Astridur Palsdottir e, Anna-Louise Reysenbach a,*

* Department of Biology, Portland State University, Portland, OR, USA
b Human Medical Genetics Program, University of Colorado Health Sciences Center, Denver, CO, USA
c International Institute of Genetics and Biophysics, Consiglio Nazionale delle Ricerche, Naples, Italy
d Institute of Biology, University of Iceland, Grensitvegur 12, Reykjavik, Iceland
e Institute for Experimental Pathology, University of Iceland, Keldur, Reykjavik, Iceland

Received 11 September 2000; received in revised form 30 November 2000; accepted 30 November 2000

Abstract

Molecular phylogenetic analysis of a blue filamentous community from an alkaline thermal spring (79–83°C) in Iceland revealed that the blue filaments were affiliated with the Aquificales. The dominant sequence type, pIce1, was most closely related to a sequence (SRI-48) found in a white filamentous community from a separate Icelandic thermal spring and the pink filaments (EM17) from Yellowstone National Park. Fluorescent in situ hybridization with clone-specific oligonucleotide probes showed that the sample analyzed was essentially a monoculture of a single phylotype.

© 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Thermophile; Phylogeny; S-layer; Aquificales

In the past decade, knowledge of microbial diversity of high temperature ecosystems has expanded substantially [1–8]. This increased knowledge is largely due to the application of molecular phylogenetic techniques to microbial ecology, in particular the analysis of phylogenetically informative macromolecules such as small subunit rDNA (16S rDNA) genes. For example, analysis of the archaeal and bacterial diversity of Obsidian Pool, Yellowstone National Park (YNP), yielded a plethora of novel sequence types (‘phylotypes’), which substantially expanded the crenarchaeal and bacterial 16S rDNA sequence database and led to the proposal of a third kingdom within the archaean domain [3]. Similarly, the phylogenetic diversity of the conspicuous pink filamentous community associated with the outflow of Octopus Pool, YNP was determined. This organism had intrigued researchers for many years, and attempts by independent researchers to enrich for this organism were unsuccessful. Phylogenetic analysis of the pink filamentous organism (EM17) revealed it to be a close relative of the organisms in culture, Aquifex pyrophilus and Hydrogenobacter thermophilus, and it was identified in situ using fluorescent oligonucleotide probes (FISH, [2]). Thermocrinis ruber, a close relative of EM17 (98.7%), subsequently was isolated from Octopus Spring [9]. Additionally, studies of white and yellow filamentous sulfur mats in Icelandic and Japanese thermal springs detected several phylotypes within the Aquificales that were closely related to organisms detected in YNP geothermal springs [7,10].

Another filamentous community, which has a blue coloration when submerged, is associated with many of the neutral-alkaline geothermal springs in Iceland. These organisms have also been targets for cultivation attempts that have yet to be successful. In this study we identified the phylogeny of the blue filamentous community and characterized the prevalent S-layer associated with these organisms. Samples were collected from the fast flowing outflow of Haegindi and Fluidir Springs in western Iceland for electron microscopy and molecular phylogenetic analysis. The filaments were present in the stream between 79–83°C. The pH of the water was 8.8 and the conductance was 0.46 mS cm⁻². Samples were fixed in 2%
glutaraldehyde for electron microscopy or fixed in ethanol:PBS (80:20; PBS contains per l 18 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, and 0.24 g KH2PO4, pH 7.4) and stored at −20°C for fluorescence in situ microscopy (FISH). DNA extraction samples were frozen at −80°C.

For the electron microscopy, approximately 30 µl of the glutaraldehyde-fixed filaments were placed on a formvar-carbon-coated grid for 10 s. The grids were stained either with 1% phosphotungstic acid (PTA) for 1 min or coated with carbon at an angle of 45°. Additional samples were prepared for freezing and freeze substitution. The pelleted, fixed filaments were touched onto a 2.3 mm copper grid (40 mesh), which was laid between two specimen carriers for freeze-fracture preparations (Balzers-Furstenstum, Lichtenstein). The carriers were placed into liquid propane and then quickly transferred to liquid nitrogen. The

![Fig. 1. The blue filaments: (a) carbon-coated rod showing the S-layer, 26 000×, (b) carbon-coated rod partially missing its outer envelope, which revealed the underlying paracrystalline layer, 35 000×, (c) section of a blue filament, 80 000×, (d) section of a blue filament, 250 000×, (e) dominant form, 60 000×, (f) short large rod, 60 000×, (g) thin filament, 60 000×, (h) PTA negative stain of the S-layer, 90 000×. Arrow depicts S-layer, arrowhead = outer envelope.](https://academic.oup.com/femsec/article-abstract/35/2/123/534441)

---

Fig. 2. Phylogenetic analysis of the major phylotype (pIce1) from the blue filament community. The phylogenetic tree was determined by maximum likelihood analysis of conserved regions of the 16S rRNA gene. The scale bar represents 0.1 fixed mutations per nucleotide position. Numbers at nodes represent bootstrap values for that node (based on 100 bootstrap resamplings). Sequence accession numbers are given in parentheses.
carriers were separated and stored for 48 h at −80°C in dry acetone containing 2% OsO₄. The samples were then slowly warmed up to room temperature, washed out of the holders and off the grids, and embedded in Polybed 812 (Polyscience Inc. Warrington). Ultrathin sections were prepared on a Reichert OMU2 microtome and stained with uranyl citrate and lead citrate. The sections were viewed with a Siemens E102 electron microscope, which revealed the filaments to be long and variable in length (often more than 10 μm), with a diameter of approximately 0.38 μm. Some very long and thin filaments as well as a few shorter broader organisms were present in all samples viewed. The presence of a paracrystalline layer (S-layer) was observed on many cells. The S-layer was visible only if the more external bacterial envelope was lost as shown in Fig. 1b, c, d. Even if the external amorphous envelope was only partially destroyed, the underlying S-layer was apparent (Fig. 1b). The outer envelope was easily detached from the S-layer (Fig. 1c, d). The ultrastructure and lattice periodicity (27 nm as determined at high magnification) of the S-layer was identical on all morphotypes and samples examined. The hexagonal lattice of the S-layer is illustrated in Fig. 1h.

Community DNA was extracted from samples stored at −80°C and two sets of primers were used to specifically amplify bacterial and archaeal 16S rDNA by PCR [2]. No archaeal 16S rRNA genes were amplified using the 4F and 1492RPL primers [8]. Bacterial products were cloned into pBluescript KS− (Stratagene, La Jolla, CA, USA) and clones containing inserts of the appropriate size (1–1.5 kb) were identified by agarose gel electrophoresis of small-scale plasmid preparations. Forty insert-containing clones were analyzed by single nucleotide (ddT-terminated) sequencing patterns [11] and unique clone types (phytopotypes) were identified. Four unique bacterial clone types were detected by single nucleotide (ddT) sequencing patterns of the plasmid preparations: 36 of pIce1 (90%), one of pIce2 (2.5%), one of pIce3 (2.5%), and two of pIce4 (5%). To ascertain the diversity of the bacterial clones, approximately 700–800 nucleotides of each type were sequenced by the Sanger dideoxy chain termination method using a suite of forward and reverse primers to sequence both strands [8]. Sequences were later confirmed by automated sequencing using a SequiTherm long-read cycle sequencing kit-LC (Epitrend Technologies) and a Licor sequencer. The secondary structure of each of the clones was analyzed to verify the integrity of the sequence and to identify evidence of chimeras or PCR-induced artifacts.

The 16S rRNA sequences were aligned by hand with a subset of bacterial and archaeal 16S rRNA sequences obtained from the Ribosomal Database Project (RDP, [12]). The alignments were based on the established secondary structure and conserved sequence regions of the 16S rRNA, thus ensuring only homologous regions were compared. Comparisons of the sequences revealed less than 1% sequence difference between the phylotypes. The secondary structures of each phylotype did not reveal any inconsistencies that may have resulted from PCR-induced artifacts. Because pIce1 represented the dominant phylotype, both strands of the entire gene were sequenced (GenBank accession number AF301907). Of the approximately 1500 nucleotides, 1212 bases were used in the phylogenetic analysis. The phylogenetic position of the blue filaments was explored using parsimony, nearest neighbor, and maximum likelihood analyses using PAUP (4.0). Tree topologies did not differ among the analyses tested and maximum likelihood was used to construct our phylogenetic tree. Corresponding bootstrap proportions were estimated using fastDNAm [13].

The phylogenetic analysis of the pIce1 sequence placed the phylotype within the deeply-branching Aquifilales group (Fig. 2). Two lineages within the Aquifilales are shown in the maximum likelihood tree: the ‘black bacteria-EX-H1’ and Aquifex lineages. These two groups and the general arrangement of the tree are firmly supported by bootstrap values from 100 maximum likelihood trees. Lower, but still significant bootstrap values resulted within the two Aquifilales lineages, and placed pIce1 as a close relative of SRI-48 (98.9%), a phylotype from a geothermal sulfur spring in Grensdalur, Iceland [10]. The closest relative to these two Iceland phylotypes was EM17 (97.2%), a phylotype detected in the pink filaments from Octopus Spring, Yellowstone National Park [2]. Additionally, pIce1 was closely related to Hydrogenobacter thermophilum (96.5%), Hydrogenobacter subterranea (96.4%), Caldorobacterium hydrogenophilum (96%), and SS5H1 (95.3%). SS5H1 is a Calderobacterium recently isolated from Calcite Springs, Yellowstone National Park, capable of growth at 70°C by aerobic hydrogen oxidation (Takacs and Reysenbach, unpublished results).

In order to establish that the PCR products were representative of the blue filamentous community a fluorescently labeled oligonucleotide probe was designed that was specific for the pIce phylotypes and was complementary to 1474–1447 (Escherichia coli numbering) region of the 16S rRNA (5′-GTCCCCTGCCTCCCTTGC-3′). The samples were hybridized with the pIce probe, as well as bacterial, universal, and control probes, and viewed as previously described [2]. All organisms hybridized with the pIce-Fig. 3-and bacterial-specific probes. No hybridization occurred with the archaeal-specific probes or the negative RNA-like probe (data not shown). The observed sequence variation appears to be an emerging trend in microbial community analyses where a particular phylotype is represented by a cluster of variants rather than a single rRNA [14,15]. Differences among the phylotypes may depict variation among closely related populations within the community or may represent multiple 16S rRNA gene copies with different sequences within the same organism. The two Iceland clones (pIce1 and SRI-48) are closely related to EM17, and were obtained from very similar, yet geographically distant environments. This is reminiscent of
the often quoted ‘Everything is everywhere, the milieu selects’ [16], and further illustrates how similar environments select similar physiological types. Near-neutral terrestrial geothermal springs are emerging as a niche that is dominated by members of the *Aquificales* that are closely related based on their 16S rRNA sequences [2,6–10]. However, it is possible that analysis of other genes will indicate that these populations are distinct [17].

Based on the diversity detected by electron microscopy, the analysis of the clones, and the in situ hybridization study, the blue filamentous community appeared to be essentially a monoculture at the time of collection, comprised of one dominant phylotype that may be represented by several variants or ‘ecotypes’ [14]. Filamentous bacterial communities from other near-neutral geothermal springs (e.g. the black filaments [8] and the pink filaments [2]) are also dominated by a single phylotype. However, clone libraries of the pink, black, and white/yellow [7,10] filaments revealed a greater bacterial diversity than detected in this study. Relative diversity differences among the communities may be attributed to the stream flow or temporal variation of the community structure.

The blue filamentous bacteria represent another example of an *Aquificales* community from a near-neutral-alkaline sulfur hot spring. All known isolates within this order are capable of hydrogen oxidation with oxygen as the electron acceptor. The majority of the *Aquificales* are chemolithotrophs. Presumably, the various filamentous communities differ with respect to color owing to ambient geochemical differences and consequently, physiological activity. These organisms are conspicuous members of near-neutral geothermal springs, and as a group probably...
contribute significantly to the productivity of high temperature terrestrial ecosystems. Because of their phylogenetic position and importance in the ecology of thermal springs, determining the diversity, distribution, and physiology of members of the *Aquificales* may be instrumental in understanding the early evolution of life on Earth.

Acknowledgements

The authors thank Norman Pace for his support. This research was partially funded by an NSF grant to A.-L.R. (OCE 99-96160).

References


