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Investigation of the Microbial Ecology of Intertidal Hot Springs by Using Diversity Analysis of 16S rRNA and Chitinase Genes†

Cédric F. V. Hobel,[1] Viggó T. Marteinsson,[1]* Gudmundur Ó. Hreggvidsson,[1,2] and Jakob K. Kristjánsson[1]

Prokaria Ltd., Gylfaflöt 5, IS-112 Reykjavik,[1] and Institute of Biology, University of Iceland, Sturlugata 7, IS-101 Reykjavik,[2] Iceland

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The microbial diversity of intertidal hot springs on the seashore of northwest Iceland was examined by combining directed in situ enrichments, artificial support colonization, and mat sampling. Analysis of 16S rRNA genes revealed the presence of clones related to both marine and terrestrial, thermophilic, mesophilic, and psychrophilic microorganisms scattered among 11 bacterial divisions. No archaea were found. The species composition of the enrichments was affected by the length of the hot periods experienced at low tide and was very different from those found in the biomass. A total of 36 chitinase genes were detected by molecular screening of the samples with degenerate primers for glycoside hydrolase family 18. The chitinase gene diversity was at least twofold higher in the enrichment samples than in the controls, indicating that a much higher diversity of hydrolytic genes can be accessed with this approach.

The coastal hot springs located on the Reykjanes peninsula in northwest Iceland can be regarded as one of the most extreme microbial environments on Earth. A series of freshwater hot springs are located on the seashore, from the tidal zone to about 100 m off the coast. The geothermal system is essentially supplied with deep fresh and slightly alkaline groundwater. It is also low in sulfide and slightly mixed with seawater in the hot spring outlets (chloride, ≤600 μg/ml). The temperature ranges from 45 to 95°C in different springs, and many are covered by the sea at high tides (9). The tides are as high as 4 m in this area, and therefore the organisms living in those hot springs can be subject to almost 100°C fluctuations twice per day, in addition to severe changes in salinity, light, and oxygen concentration. This type of habitat is quite rare, and no studies have been conducted on the unculturable microflora in these environments until now. Furthermore, the effects of drastic temperature and salinity fluctuations remain so far unknown.

Chitin is an abundant polysaccharide in the marine environment. Information on microbial chitinases has been prominently restricted to a few culturable microorganisms belonging to β- and γ-proteobacteria, gram-positive bacteria, and the domain *Archaea* (3, 5, 6, 21). Yet, the use of chitinase genes as molecular markers has demonstrated the presence of a large pool of uncultured chitinolytic microorganisms in marine and soil environments (3, 13, 14, 24).

In this report, we used molecular diversity methods, combined with in situ enrichments and artificial support colonization, to investigate the apparent microbial diversity of 16S rRNA genes and chitinase genes. 16S rRNA gene analysis of natural samples has shown that the number of microbial species present can differ drastically in occurrence and that organisms that are below 0.1% can hardly be detected (2). However, combining cultivation methods and direct molecular techniques enables access to more diversity than does either method alone (7, 16). By applying different sampling methods we analyzed both the diversity of 16S rRNA genes and the diversity of chitinase genes promoted by selective growth of chitinolytic microorganisms via in situ enrichments. This strategy allowed access to a remarkable diversity of microbes and a high diversity of valuable novel chitinase genes from a unique extreme environment.

Sample collection, artificial support colonization, and in situ enrichments. Three in situ enrichments (IE102, IE103, and IE105) and two artificial support colonization experiments (AS106 and AS107) were conducted over 82 h in four hot spring outlets, all gathered in an area of 10 m in diameter, within the intertidal section on the shore. In situ enrichments were performed using sterile 1-liter polyethylene flasks which were inoculated with untreated hot spring water and supplemented with 2% (wt/vol) coarse chitin flakes (Sigma). The flasks were closed to prevent medium and biomass loss and maintained in the hot spring with a 500-g weight. Sample IE102 was obtained from an 80 to 82°C hot spring, 30 cm deep and located at 2 m in depth below the high tide line. Water flow was high, and a very thin microbial mat was visible. Samples IE103 and IE105 were obtained from two springs, ≤70°C and ≤65°C, respectively, both located at a depth of 4 m below the high tide level. Hot fluid was unevenly coming through a heterogeneous mixture of gravel, sand, and broken seashells on the shore. Holes were dug to bury the polyethylene flasks, and the flasks were re-covered to maintain them in the sand. Woolen fibers (50 to 100 cm^2) were used to create two artificial supports for biomass colonization while samples were inserted in hot spring outlets. Artificial support sample AS106 was deployed in the same hot spring as IE102 and served subsequently as a reference sample to evaluate the efficiency of the in situ enrichment. Sample AS107 was obtained from a rock fissure located in the intertidal zone at about 1 m in depth from

* Corresponding author. Mailing address: Prokaria Ltd., Gylfaflöt 5, IS-112 Reykjavik, Iceland. Phone: (354) 570 7900. Fax: (354) 570 7901. E-mail: viggo@prokaria.com.

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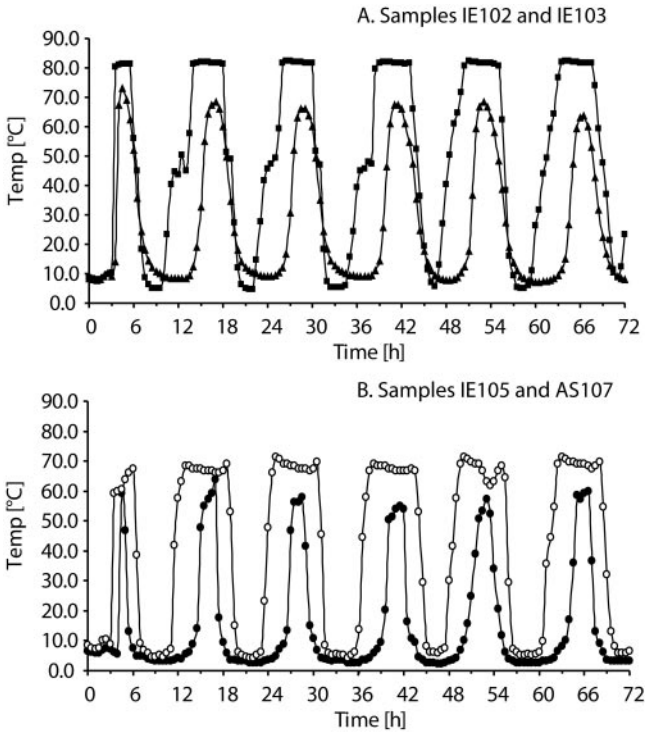

FIG. 1. Temperature measurement of hot spring fluid in in situ enrichments IE102, IE103, and IE105 and biomass sample AS107. Temperature was recorded every 10 min over 82 h, but only 72 h are displayed here. A. ■, IE102; ▲, IE103. B. ●, IE105; ○, AS107.

high tide. The fissure was surrounded with a thick algal mat except in the zone of 70°C hot water discharge.

The temperature of the hot spring water was checked every 10 min using temperature logging probes (Dickson HT100; Dickson, Addison, Ill., and HOBO Optic Stowaway Temp; Onset Computer, Bourne, Mass.) placed inside all in situ enrichment flasks or attached to the artificial support AS107. The recorders reported that the experiment was conducted over more than five complete tidal cycles (Fig. 1). The average duration of the high-temperature period (≥55°C) was much longer for samples AS107 and IE102 (6 h 30 min and 6 h, respectively) than for samples IE103 and IE105 (2 h 35 min and 1 h 50 min, respectively). Conversely, the low-temperature period (≤10°C) was longer for samples IE103 and IE105 (4 h and 7 h 50 min, respectively).

Samples of an orange/green microbial mat (B110) were also collected from a 45 to 60°C hot spring diffusing through gravel. The mat was located on a rock formation, a few meters away from sampling site AS107, on the opposite side of the shore at about 2 m in depth from the high tide line. All collected samples were stored below 10°C until reaching the laboratory. Cells from the enrichment fluids were collected by centrifugation. Woolen colonization supports were washed thoroughly with sterile saline water (0.87% [wt/vol] NaCl), and cells were concentrated by centrifugation. The bacterial mat sampled on site was used as such. DNA extraction was performed as previously reported (12).

Phylogenetic analysis of the microbial diversity. Analysis of the 16S rRNA genes was conducted according to the method of Hobel et al. (7) using the ARB package (10). The CHECK-CHIMERA (11) and Bellerophon (8) programs were used to search for chimeric artifacts.

All samples resulted in the amplification of bacterial 16S rRNA genes, but no archaeal 16S rRNA genes were found despite the use of various PCR conditions (decreased annealing temperature and larger number of cycles). Six libraries were constructed with a total of 560 clones that were partially sequenced with primer R805, and the sequences were identified with BLASTN searches (see Table S1 in the supplemental material). Sequences were manually edited, and 41 operational taxonomic units (OTUs or phylotypes) were created by pairwise similarity calculations using a 98% DNA-DNA similarity cutoff. Three chimeric sequences were identified and omitted from the subsequent microbial diversity analysis. One representative of each OTU was selected, and its sequence was aligned with its 10 closest relatives. The analysis of the constructed phylogenetic tree (Fig. 2) revealed the presence of clones branching among 11 bacterial divisions (shown by asterisks), including proteobacteria (α, β, γ, and δ subdivisions), cyanobacteria, bacteroidetes, acidobacteria, planctomycetes, *Deinococcus-Thermus*, *Thermomicrobium*, chloroflexi, *Firmicutes*, thermotogae, and aquificae. Fifteen clones showed high to very high database matches (≥99% identity) and clustered well with their closest relative in the tree. Seven clones displayed very low identity scores (≤92 to 93%), and their respective identification and branching remained only putative (19).

The overall microbial diversity in all samples was predominantly thermophilic and moderately thermophilic, and the species composition in all samples reflected the duration of the high- and low-temperature periods. Samples IE102, AS106, and AS107, which were subject to the longest exposure to hot fluid (Fig. 1), contained a majority of clones (OTUs) belonging to the typical terrestrial thermophilic genera *Thermus*, *Meiothermus*, *Chloroflexus*, and *Thermonema*, but very few clones belonged to psychrophilic and mesophilic species. The low-temperature-period samples IE103 and IE105 were in contrast dominated by moderate thermophiles such as *Anoxybacillus*, *Ureibacillus*, *Fervidobacterium*, and *Hydrogenophilus*, plus a few clones related to mesophilic marine or terrestrial proteobacteria. It is clear that the hot-temperature periods apparently favored the growth of a majority of thermophiles whereas the overall lower temperature found in samples IE103 and IE105 allowed only moderate thermophiles to grow. The presence of psychrophilic marine-related microorganisms in samples AS106 and AS107 was very likely due to the colonization of the supports by sea microbes during the high tide period. This suggests, however, that those psychrophilic microorganisms have gained a resistance to heat since the supports were continuously subject to overflow with 70 to 83°C hot water for several hours at low tide. The mat sample B110 was composed only of terrestrial species typically found in green to orange microbial mats from alkaline hot springs (cyanobacteria, *Chloroflexus*, planctomycetes, and *Thermus*) (17), and no marine-related species were detected in this sample although the hot spring was subject to long exposures to cold seawater. This indicates that the fast-growing terrestrial photosynthetic thermophiles have overgrown the psychrophilic marine species. Moreover, this community may have developed a tolerance to high salt concentrations since the mat had an appearance and

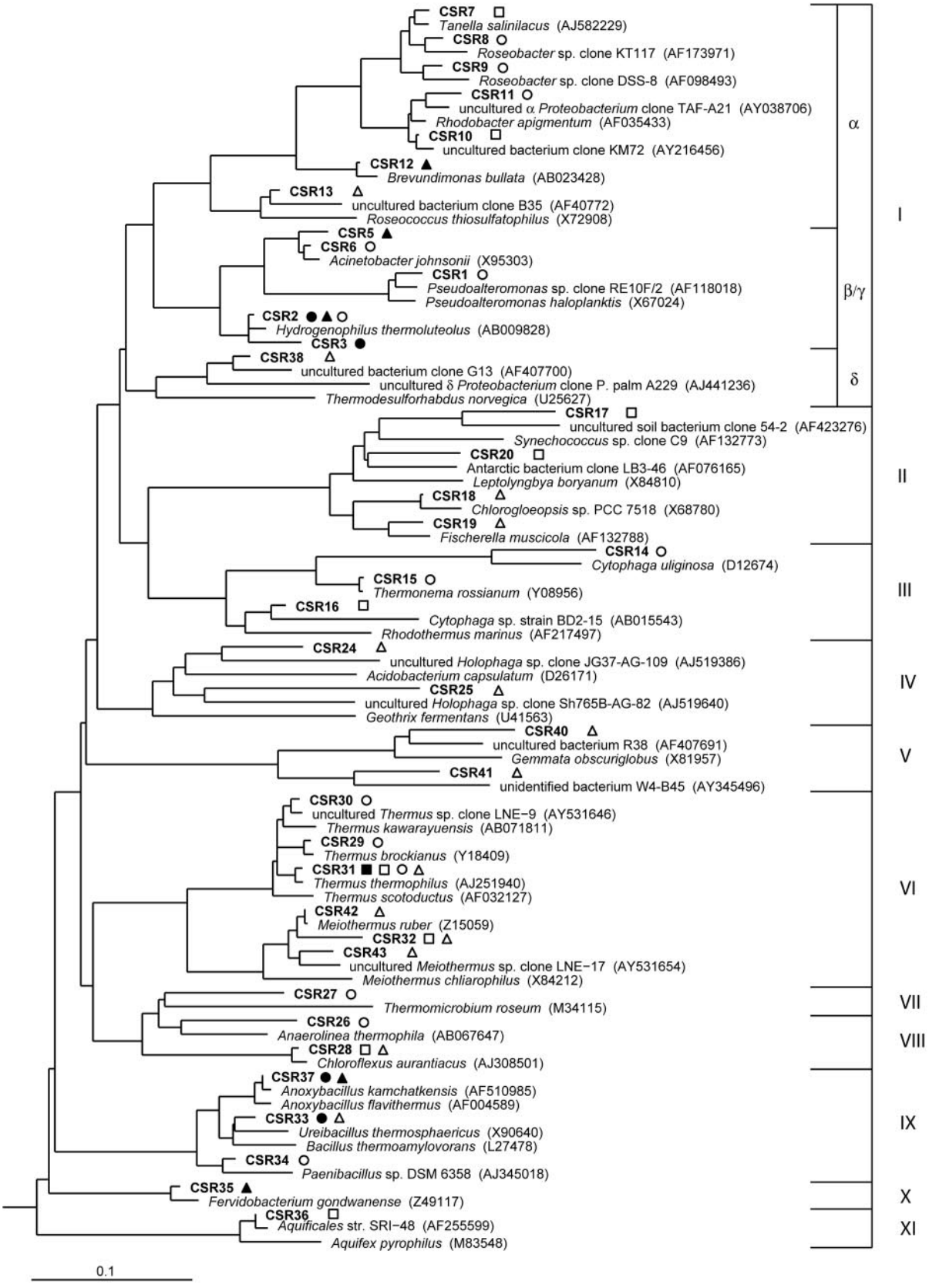

FIG. 2. Evolutionary maximum-likelihood phylogenetic dendrogram of the 16S rRNA sequences detected in the in situ enrichments and biomass samples from the Hveravik intertidal freshwater hot springs (designated CSR) and their closest relatives. *Sulfolobus acidocaldarius* was used as an outgroup. The scale bar represents the expected number of substitutions per nucleotide position. I, proteobacteria; II, cyanobacteria; III, bacteroidetes; IV, acidobacteria; V, planctomycetes; VI, *Deinococcus-Thermus*; VII, thermomicrobia; VIII, chloroflexi; IX, *Firmicutes*; X, thermotogae; XI, aquificae. ■, IE102; ●, IE103; ▲, IE105; □, AS106; ○, AS107; △, B110.

species composition similar to those of common terrestrial mats despite the periodic exposure to cold seawater for 8 to 12 h daily. Interestingly, no sequences related to *Rhodothermus marinus*, originally isolated from this site (1), or sequences belonging to the *Archaea* were observed in any samples. Those species may have been present but in quantities too low to be detected with PCR-based molecular analysis.

Comparison of the species composition of respective 16S rRNA clone libraries from samples IE102 (low diversity) and AS106 (high diversity) clearly indicated that chitin significantly affected the species diversity in the enrichments. The low diversity observed in the two other enrichment samples IE103 and IE105 also corroborated the inferred selective or inhibiting effect of chitin, allowing the growth of only a few microorganisms in the enrichments. The overwhelming dominance of *Thermus* 16S rRNA sequence in the clone library of sample IE102 may have resulted from the intrinsic PCR bias of the method (20, 22), since the chitinase screening results suggest that a more complex microbial community was promoted in this enrichment.

Molecular analysis of chitinase gene diversity. About 100 microbial amino acid sequences of chitinases from the glycoside hydrolase family 18 (4) were retrieved from GenBank and Pfam (18) and used by multiple alignments as template for the design of degenerate PCR probes as described by Hobel et al. (7). All chitinases belonged to group A of the catalytic domains from family 18 of the glycoside hydrolases, which appear to be most prevalent in nature (13). Two overlapping forward priming sites (ChiA_F1 and ChiA_F2) were chosen at the active site, where a [FY]DGDDWEYP motif was found highly conserved (23). Two overlapping reverse priming sites (ChiA_R1 and ChiA_R2) were designed using the MYDxxG[WF] motif. Both targeted sites were 90 to 100 amino acids apart, on the basis of comparison with reference sequences. The predicted size of the amplicons was then approximately 270 bp to 300 bp. The sequences of the chitinase screening primers were as follows: ChiA_F1, 5′-ACG GCG TGG ACA TCG AYT GGG ART-3′; ChiA_F2, 5′-CGT GGA CAT CGA CTG GGA RTW YCC-3′; ChiA_R1, 5′-CCC AGG CGC CGT AGA RRT CRT AYS-3′; and ChiA_R2, 5′-CCC AGG CGC CGT AGA RRT CRT ARS WCA-5′. Amplification reactions were carried out using the two pairs of primers ChiA_F1/ChiA_R1 and ChiA_F2/ChiA_R2. The PCR amplifications and the subsequent phylogenetic analysis of the chitinases were performed as previously described (7).

Chitinase gene diversity was studied by the creation of six chitinase gene libraries obtained from the screening of all enrichments and reference samples (see Table S2 in the supplemental material). Unique PCR bands of predicted size were produced and cloned for all samples. A total of 650 clones were obtained, and 467 clones thereof were successfully sequenced. For all samples, protein groups were created using pairwise similarity calculation, with an 85% amino acid identity cutoff. All 467 clones were identified as chitinase genes by BLASTX searches, and 36 groups of chitinase sequences were built (ChiCSR sequence types; Fig. 3). As controls, oligonucleotides published previously for the detection of chitinase genes from several biotopes (3, 13, 14, 24) were assayed with all samples, and the resulting bands were cloned as described above. No additional sequences were hereby detected by using either specialized (e.g., streptomycetes) or universal probes (data not shown).

Twenty-five ChiCSR clones showed the best database matches with chitinase sequences belonging to the four homogeneous bacterial *Firmicutes* groups, β-proteobacteria, γ-proteobacteria, and actinobacteria. Six ChiCSR sequence types showed highest identity to chitinase sequences obtained from uncultured microbes from pasture soil (13). Four clones were related to eukaryal chitinase sequences from fungi, the nematode *Heterodera glycines*, and the shrimp *Penaeus monodon*, and one sequence type had the best database match with a viral chitinase from *Paramecium bursaria* chlorella virus. Low identity scores (≤40% to 50%) were found for 12 sequences, and therefore identification within the glycoside hydrolase family 18 was sometimes uncertain, yet it suggested a high degree of novelty for the respective enzymes.

The chitin enrichments enabled the growth of a great diversity of putative chitinolytic microbes and showed the highest chitinase gene diversity. The relatively high chitinase gene yield in AS107 was most likely related to the multiplicity of microbial species attached to the support (thermophiles and psychrophiles and terrestrial and marine species), whereas AS106 and B110 showed high dominance of typical terrestrial thermophilic species and were hence less likely to harbor the targeted genes.

About 45% of the chitinase genes identified in the enrichments were not detected in the biomass samples, and 86% of all chitinase genes showed high novelty, based on amino acid identity. The chitinases detected here were mainly related to chitinases from the proteobacteria, actinomycetes, and *Firmicutes* as well as a few eukaryote-related chitinases. However, it is difficult to assign an exact phylogenetic origin to the ChiCSR sequence types and link them to the CSR bacterial OTUs, especially since the extent of lateral gene transfer between species within microbial communities is undetermined (15). Little is known about the distribution and prominence of chitinases among thermophiles in marine and terrestrial environments, but our results, notably from samples IE102, AS106, and AS107, bring substantial information suggesting that a still-higher diversity of chitinases remains to be discovered. It is interesting that no ChiCSR sequences were found related to chitinase genes from uncultivated psychrophilic or mesophilic marine microorganisms previously detected by molecular screening (3). Additionally, the use of control oligonucleotide probes reportedly specifically targeted at particular phylogenetic groups (streptomycetes [24] or proteobacteria [3]) proved unable to increase the number of genes amplified in this experiment and to specifically amplify sequences targeted by the group-specific probes, although detected with our probes.

Implications of the methodology. The coastal hot spring field of Hveravik is a unique geothermal area because the hot springs are submerged during high tide, therefore creating a highly dynamic environment subject to constant periodic disturbances with steep gradients of temperature and salinity between the hot alkaline freshwater fluid and the cold seawater. This environment also provides attractive conditions for the study of extremophilic heterotrophs due to profusion of algal vegetation and crustacean shells around the hot springs. However, the influence of the tides increases significantly the com-

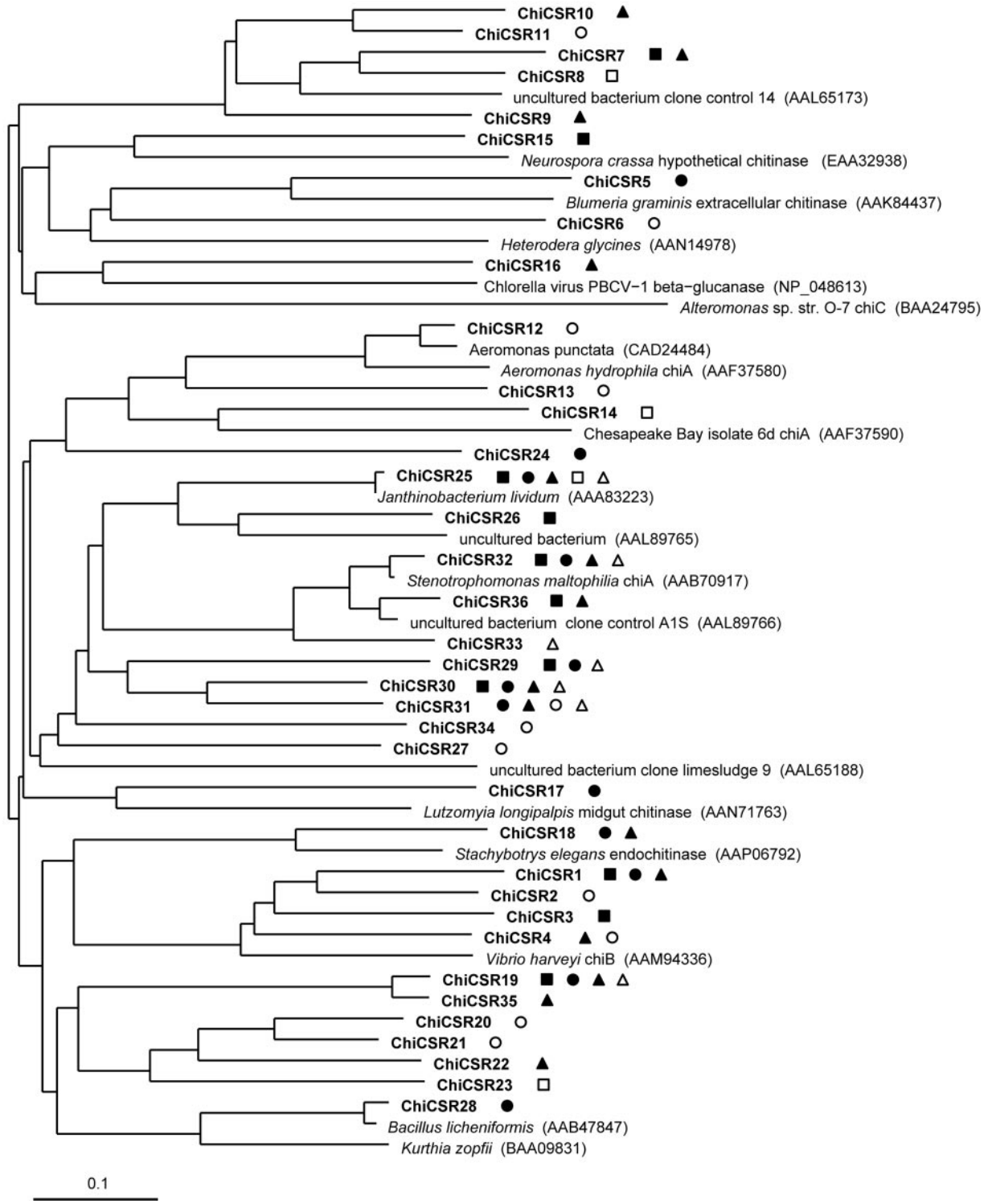

FIG. 3. Unrooted neighbor-joining phylogenetic dendrogram of the chitinase clone sequences detected in the Hveravik intertidal freshwater hot springs (designated ChiCSR). The scale bar represents the expected number of substitutions per amino acid position. ■, IE102; ●, IE103; ▲, IE105; □, AS106; ○, AS107; △, B110.

plexity of studying this particular site, as only a little biomass is readily accessible.

Diversity analysis methods using sequence-based strategies in environmental samples are highly dependent on the spatial distribution and temporal occurrence of specific populations and also on the quantity, quality, and composition of the DNA recovered from the samples. It is therefore necessary to obtain sufficient quantities of targeted biomass to ensure a compre-

hensive assessment of the biodiversity. Although large quantities of biomass can generally be sampled in nature, the species diversity of microbial communities is governed by the environmental biochemical conditions and such communities are typically characterized by low diversity of a few dominating organisms (17). Also, the direct amplification of specific genes from biomass samples is limited by low gene copy numbers, and little diversity is frequently obtained in such samples alone (7). However, we have shown that the combined approach of in situ enrichment and in situ colonization yielded larger quantities of microbes from a highly complex environment, and by allowing the increase of gene copy number we gained better access to a high diversity of novel and valuable genes.

Nucleotide sequence accession numbers. All chitinase gene sequences (ChiCSR) were deposited in the GenBank database under accession numbers AY699325 to AY699360, and all 16S rRNA genes (CSR) were deposited under accession numbers AY699361 to AY699398.

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