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Effect of Thermophilic Bacteria on the Siliceous Deposition and Phylogenetic Analysis of the Bacterial Diversity in Silica Scale

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Silica scale, one of the major problems for geothermal power development, was formed on the copper test plates in geothermal hot water (85±2 °C, pH 7.2). Numerous bacterial shaped structures were observed and mixed bacterial population of genomic DNA was stably extracted from the silica scale. The amount of silica scale and extracted DNA increased exponentially with the time of incubation in the geothermal hot water. The molecular phyogenetic survey of extremely thermophilic bacterial diversities in silica scale was carried out by using the PCR–mediated small subunit rRNA gene (rDNA) sequencing. The community was composed mainly of three phylogenetic types in domain *Bacteria*. Cluster I was affiliated with the Aquificales and cluster II was closely related to the genus *Thermus*. Cluster III was homologus with Gram–positive anaerobic thermophilic bacteria. These bacterial communities may possibly contribute to the rapid aggregation of silica.

INTRODUCTION

Silicon is the second most abundant element in the earth's crust, which interacts with living organisms in geothermal water environments. The microbial presence of biogeological edifices coated with amorphous silica has been observed in geothermal and hydrothermal environments (Cook *et al.*,1995; Ferris *et al.*, 1986; Zuerenberg *et al.*, 1990). The type of microorganism involved in the mechanisms of the siliceous biomineralization, however, have remained poorly understood.

We show here the microbial participation in the formation of silica scale in Otake–geothermal power plant, Oita Prefecture, Japan, and its molecular view of extremely thermophilic bacterial diversity. The silica scale formed on the surface of equipment and in pipelines of geothermal power plants presents serious economic problems, related to efficient of energy production (Fig. 1) (Yanagase *et al.*, 1970). The main objective of the present study was to analysis the microbial diversity based on the small subunit rRNA gene (rDNA) (Oyaizu 1992). Recent molecular phylogenetic study using the rDNA have indicated that the microbial diversity in variety of environments is much greater than previously assumed by the standard cultivation and isolation methods (Barns *et al.*, 1996). This research may contribute not only to the clarification of siliceous biomineralization in thermal systems but also to the regulation of silica scale formation in

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Fig. 1. Occurrence of silica scale deposited on the surface in a pipeline.

geothermal electric power plants.

MATERIALS AND METHOD

Sampling of silica scale

Research on silica scale formation was carried out at the Otake–geothermal power plant, Kusu country, in Oita Prefecture, Kyushu, Japan (Yokoyama *et al.*, 1993). Thin copper plates were immersed in the aging tank (85±2°C, pH 7,2), from September to December, 1995. The hot water was injected into the aging tank at the flow rate of 350 tons per hour and was passed through the aging tank for 1 h. The silica scale formed on the surface of copper plates were gently removed from the tank, and then were frozen immediately in liquid nitrogen prior to storage at −20°C.

Microscopy

Scanning electron microscopy (SEM) was carried out using an electron probe microscopical analyzer (EPMA; JEOL JXA–733). Silica scales were freeze–dried overnight (freeze dryer FDU–810; Tokyo Ricakiki Co., Tokyo, Japan) and then viewed with EPMA. For transmission electron microscopy (TEM), approximately 1 g of frozen silica scale was suspended directly in 1 ml of deionized distilled water. This suspension was negative–stained and observed using TEM (JEM 2000EX).

Extraction and purification of DNA from silica scale

Genomic DNA was extracted from the frozen silica scale using the lysozyme and freeze–thaw method (Tsai *et al.*, 1991), but with the following modifications: 1 g (wet weight) of frozen silica scale was gently raked off the copper plate and crushed using a sterilized porcelain mortar. Five cycles of freezing in a −80°C deep freezer for 30 min and

thawing in a 65 °C water bath for 15 min were run to release genomic DNA from bacterial cells present in the silica scale. After phenol–chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989), pellets of DNA were suspended in 1 ml of TE buffer (10 mM Tris–HCl, 1.0 mM EDTA, pH 8.0) and then purified with QIAGEN–tip (DIAGEN GmbH, QIAGEN Inc.) following the manufacturer's instructions.

PCR amplification and cloning of 16S rDNA.

Partial 16S rDNA segments were amplified by PCR, using primers which correspond to nucleotide positions 1101 to 1115 of *Escherichia coli* 16S rRNA (forward primer: 5'– AACGAGCGMRACCC –3') and to the complement of positions 1392 to 1407 (reverse primer: 5'– GACGGGCGGTGTGTRC –3') (where R represent A or G and M represents A or C) (Oyaizu 1992).

The DNA mainly used for this examination was extracted from deposits which formed while the plate was in the tank for 40 days. The 100 μl of amplification reaction mixture contained 0.1 μg of DNA, 10 μl of 10X reaction buffer (500 mM KCl; 100 mM Tris–HCl, pH 9.0; 1% Triton X–100), 1.5 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 1 mM of each primer, and 5 U of *Taq* polymerase. The reaction mixture was incubated in a DNA Thermal Cycler (Astec Co.) for 1 min at 96 °C, and then subjected to 25 amplification cycles of 30 s at 96 °C, 15 s at 58 °C and 72 °C for 4 min. The amplified fragments were gel–purified on 1% agarose gels, blunted using a DNA Blunting Kit (Takara Shuzo Co.), and inserted into pUC119 digested with *Sma* I, using a DNA Ligation Kit Ver. 2 (Takara Shuzo Co.). Partial 16S rDNA sequences of randomly selected clones were sequenced using a Sequencing PRO Autosequencer Core Kit (Toyobo Co.) and a DNA sequencer (DSQ–500; Shimadzu Co.) in accordance with the manufacturer's directions.

Phylogenetic analysis

The homologous sequences of 16S rDNA, 275 bp corresponding to position 1116 to 1391 of *E. coli* sequence, were scanned using the data registered in GenBank by the DNASIS program ver.3.5. Comparisons of the sequence with GenBank and EMBL data–bases were made using the BLAST network service (Altschul *et al.*, 1990). A software package. ODEN ver. 1.1.1. (National Institute of Genetics, Mishima, Japan) was used to generate the evolutionary distance and to reconstruct a phylogenetic tree based on the unweighted pair–group method with arithmetic mean (UPGMA) from distance values (Kimura 1980).

Nucleotide sequence accession numbers

The sequences reported herein have been deposited in the EMBL, GenBank and DDBJ databases under accession numbers AB000684–AB000691, AB000692–AB000696 and AB000697–AB000698 corresponding to the sequences of cluster I, II and III respectively.

RESULTS AND DISCUSSION

Chemical properties of geothermal water and silica scale

The concentration of total silicic acid in geothermal water was about 710 ppm at inlet

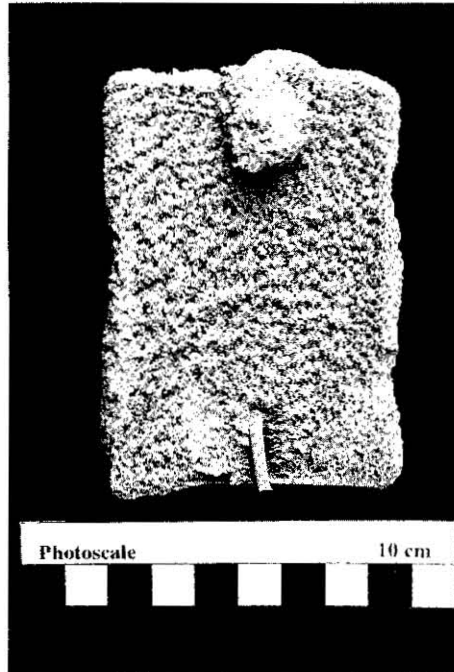

Fig. 2. Photograph of the silica scale formed on the copper plate for 40 days in geothermal hot water at 85±2 °C, pH 7.2.

point in aging tank, which was supersaturated with silicic acid (Iler 1979). Fig. 2 shows the silica scale sample formed on the copper plate for 40 days. Mineralogical analysis of the silica scale showed that the predominant material was 90.3% of amorphous silica and the trace amount of Al (0.75%) and Fe(0.27%) was also detected.

Microscopic observations

The dendritic, tube shaped structures were found in photomicroscopic observations of polished thin section of silica scale, preliminary (Fig. 3A). We assumed that these dendritic structures were derived form the bacteria covered with amorphous silica. The ERMA observations revealed that the silica scale formed on the copper plate was composed of accumulations of spherical amorphous silicate particles and numerous tube-shaped structures (Fig. 3B). The EPMA signals from the silica scale indicated almost total silicon. These tube-shaped materials were 1–10 μm long and 0.5–5 μm wide. The bacteria-like structures are probably formed by the attachment of silica grains to the surface of bacteria. Small spherical particles of amorphous silica were also present in the silica scale.

For purposes of confirmation, TEM observation of negatively stained preparations was also performed. Indeed, rod-shaped bacteria were detected in the supernatant of the

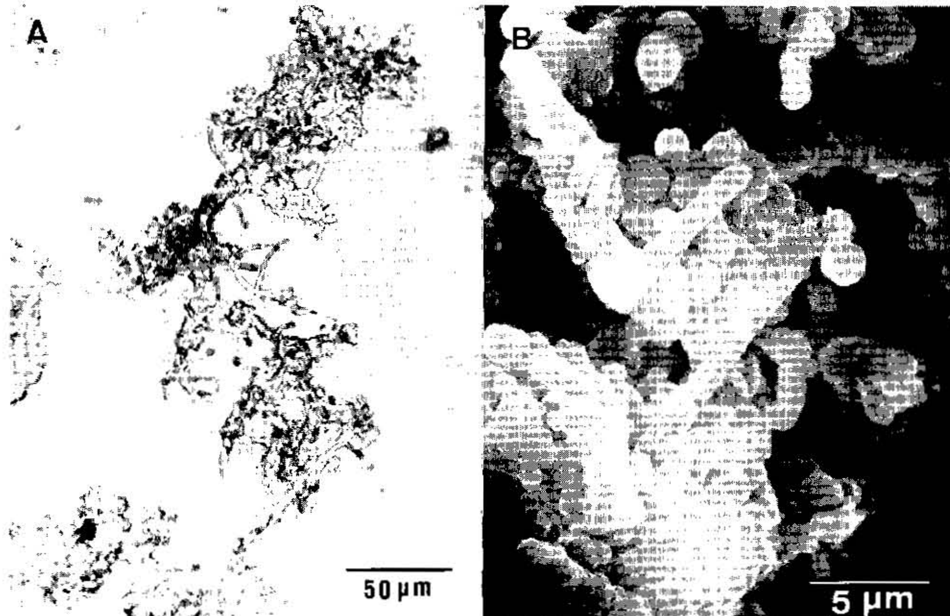

Fig. 3. (A) Photomicrograph of a polished thin section of silica scale. Bacteria-like dendritic structures were observed. (B) EPMA micrograph of silica scale showing rod-shaped bacteria-like structures of amorphous silica.

suspension of silica scale placed in distilled water (data not shown).

Relationship between silica scale formation and extracted DNA amount

Mixed bacterial population of DNA could be constantly and stably extracted form the silica scale, directly. Fig. 4 shows change in volume of silica scale formed on one copper plate and the amount of mixed genomic DNA from the same sample. The volume of silica exponentially increased with the time of incubation. The amounts of extracted DNA paralleled the rate of increase of scale formation.

Scale volume and DNA extracts were decreased by increasing the temperature from 85 °C to 95 °C. At 85 °C, the average concentration of extracted DNA was approximately 7.5 μg per 1 g of silica scale, whereas at 95 °C, it was half or less and the rate of formation of silica scale was also half to one-third lower than at 85 °C. We therefore estimated that the population of living bacteria in the hot water contribute to the rate of silica scale formation.

Characterization of bacterial rDNA compositions recovered from silica scale

Microbial population in the silica scale was composed of three types (clusters I, II and III) in domain *Bacteria* (Woese *et al.*, 1990) by PCR-madiated rDNA composition using universal primers for partial rDNA (Fig. 5). The clones of PCR products from the environmental sample sometime contaminate no environmental DNA (Tanner *et al.*,

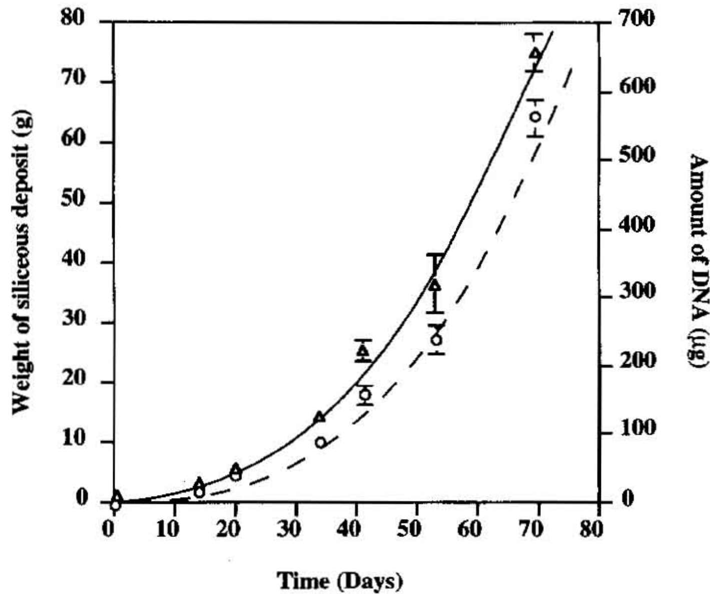

Fig. 4. Weight variation in siliceous deposits (△) formed on one copper plate and the amount of bulk DNA (○) extracted from the deposit. Averages of 3 samples at each incubation time are given with calculations of standard deviations.

1998), but all sequences of clones in this study were derived from extremely thermophilic bacteria. *Archaea* or *Eucarya* were never detected.

Cluster I consisting of 8 clones was closely related to the obligatory chemoautotrophic hydrogen–oxidizing bacterium *Hydrogenobacter thermophilus* isolated form Japan or its close relative *Aquifex pyrophilus* (Pitulle *et al.*, 1994). These clones sequences had a 98–100% similarity

Cluster II, consisting of 15 clones, was the dominant spiecces in the silica scale. These sequences affiliated with the phylogenetic tree of genus *Thermus*. Among *Thermus* isolates, there are geographic grouping in the phylogenetic tree of 16S rDNA analysis (Saul *et al.*, 1993). All sequences were grouped in the group of Japanese isolates and closely related to *T. thermophilus* with 98.7% similarity.

Cluster III was minor composition in the population of silica scale. The cloned sequences of 2 clones were homologous with those of anaerobic thermophilic Gram–positive bacteria. These organisms are extremely thermophilic, non–spore–forming and polysaccharolytic (Rainey *et al.*, 1993). However, the sequences of these clones seem to have little similarity (83%).

Distribution of clusters I, II and III among the 25 clones analyzed was 8:15:2. None of these clones correspond to the sequences previously reported. It was suggested that the bacterial community in the silica scale formed at 85°C was dominantly comprised of autotrophic hydrogen–oxidizing bacteria and heterotrophic bacteria of the genus

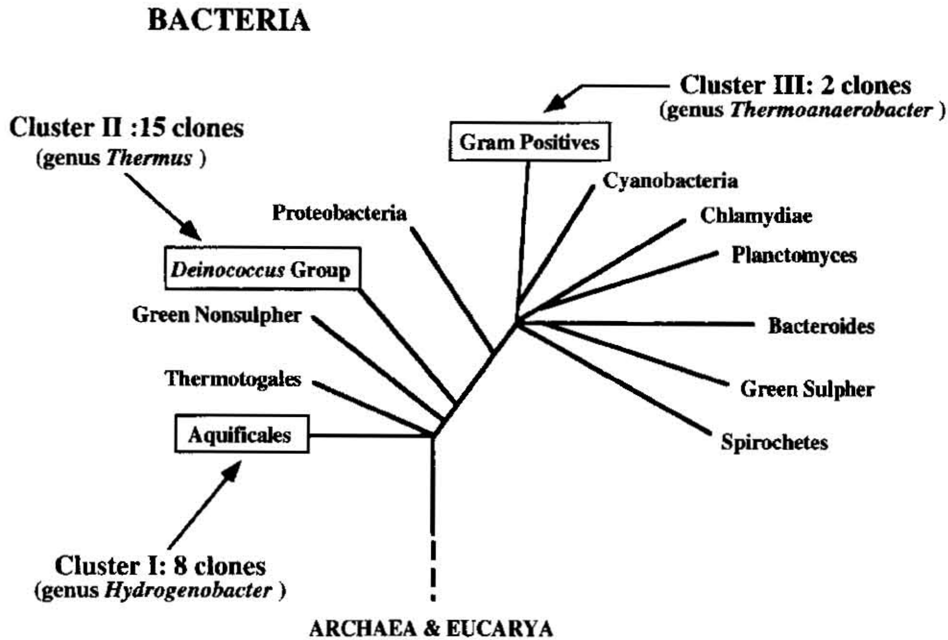

Fig. 5. Phylogenetic tree of domain *Bacteria* and three clusters of thermophilic bacterial community detected from the silica scale by analysis of 16S rDNA sequences.

Thermus. Chemoautotrophic hydrogen–oxidizing bacteria (cluster I) may have a significant role not only as nutrient donors for heterotrophic bacteria as *Thermus* but also as substrate for silica aggregation at the initiation of formation. In many natural and manmade thermal systems, *Thermus* strains (cluster II) are the most common aerobic heterotroph (Stramer *et al.*, 1981; Marteinsson *et al.*, 1995). However, there is no evidence that the ratio of clusters I, II and III indicates exactly those phylotypes in the silica scale.

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