Stimulation of Expression of a Silica-Induced Protein (Sip) in *Thermus thermophilus* by Supersaturated Silicic Acid[▽]

Katsumi Doi,^{1*} Yasuhiro Fujino,¹ Fumio Inagaki,¹ Ryouichi Kawatsu,¹ Miki Tahara,¹
Toshihisa Ohshima,¹ Yoshihiro Okaue,² Takushi Yokoyama,²
Satoru Iwai,³ and Seiya Ogata³

Department of Genetic Resources Technology, Faculty of Agriculture,¹ and Department of Chemistry, Faculty of Sciences,²
Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan, and Department of
Applied Microbial Technology, Faculty of Bioscience, Sojo University, 4-22-1 Ikeda,
Kumamoto 860-0082, Japan³

Received 16 October 2008/Accepted 9 February 2009

The effects of silicic acid on the growth of *Thermus thermophilus* TMY, an extreme thermophile isolated from a siliceous deposit formed from geothermal water at a geothermal power plant in Japan, were examined at 75°C. At concentrations higher than the solubility of amorphous silica (400 to 700 ppm SiO₂), a silica-induced protein (Sip) was isolated from the cell envelope fraction of log-phase TMY cells grown in the presence of supersaturated silicic acid. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the molecular mass and pI of Sip to be about 35 kDa and 9.5, respectively. Induction of Sip expression occurred within 1 h after the addition of a supersaturating concentration of silicic acid to TM broth. Expression of Sip-like proteins was also observed in other thermophiles, including *T. thermophilus* HB8 and *Thermus aquaticus* YT-1. The amino acid sequence of Sip was similar to that of the predicted solute-binding protein of the Fe³⁺ ABC transporter in *T. thermophilus* HB8 (locus tag, TTHA1628; GenBank accession no. NC_006461; GeneID, 3169376). The *sip* gene (987-bp) product showed 87% identity with the TTHA1628 product and the presumed Fe³⁺-binding protein of *T. thermophilus* HB27 (locus tag TTC1264; GenBank accession no. NC_005835; GeneID, 2774619). Within the genome, *sip* is situated as a component of the Fbp-type ABC transporter operon, which contains a palindromic structure immediately downstream of *sip*. This structure is conserved in other *T. thermophilus* genomes and may function as a terminator that causes definitive Sip expression in response to silica stress.

Occurring mainly in the form of silica (SiO₂), silicon (Si) is the second-most abundant element in the earth's crust, accounting for 28.8% of the earth's mass (34). SiO₂ exists as monosilicic acid (Si(OH)₄) in aqueous solution, as represented in the following equation: SiO₂ + 2H₂O · (Si(OH)₄). The solubility of silica greatly depends on temperature, pH (17), and salt concentration, among other parameters (27). As the temperature of a silicic acid solution declines, its concentration can exceed the solubility of amorphous silica. Under those conditions, silicic acid polymerizes to form polysilicic acid, which is relatively stable in aqueous solution because the repulsion between the negative charges on its surface keeps it from readily aggregating and precipitating. In a geothermal reservoir, at high temperature and pressure, the silicic acid concentration at equilibrium shows the solubility of quartz. However, when that geothermal water is discharged to the surface, the silicic acid concentration becomes supersaturated as the water boils, frequently leading to the formation of siliceous deposits called "silica sinter" (11). Microscopic observation of such siliceous deposits reveals many microbe-like structures (20), and it has been suggested that these fossils represent archaean microorganisms that grew in the hot, super-

saturated fluids (26). There have been a number of experimental studies carried out with the aim of characterizing the physical changes associated with various bacteria during silicification (23, 26, 31, 33, 37); however, the effect of silica on the bacterial habitat in geothermal environments and the mechanism by which siliceous deposits are formed remain unexplained.

Recent studies have shown that biosilicification in geothermal areas reflects the activities of various thermophilic microorganisms (15, 23, 29). For instance, geothermal water and the water discharged from hydrothermal vents contain a high concentration of silicic acid, and biogenic textures covered with amorphous silica have been found in areas around both sources (12, 22). Moreover, in a study of experimental silicification, interactions were observed between silica and *Sulfurihydrogenibium azorense*, a representative member of the order *Aquificales* (26). Still, the effect of silica on the bacterial habitat in thermal environments and the molecular properties affecting aggregation and siliceous deposition remain poorly understood.

Our previous studies have focused on the effect of bacteria on the formation of siliceous deposits in geothermal water (18, 21). Siliceous deposits (called silica scale) that form in pipelines and on surface equipment in geothermal power plants cause serious economic problems related to energy loss and to plant maintenance throughout the world (38). We also observed that a *Thermus* strain isolated from silica scale (TMY) was able to efficiently generate and deposit amorphous silica in

* Corresponding author. Mailing address: Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan. Phone and fax: 81 (0)92 642 3059. E-mail: doi@agr.kyushu-u.ac.jp.

[▽] Published ahead of print on 20 February 2009.

vitro, beginning in the latter part of the log growth phase (19). On the basis of its morphological, physiological, and genetic properties, the organism was identified as a strain of *Thermus thermophilus*, and its distinct properties were indicative of the microdiversity of *T. thermophilus* strains (14). *Thermus* strains are the predominant heterotrophs in natural geothermal and hydrothermal habitats, and thus far, the genomes of *T. thermophilus* strains HB8 (GenBank accession no. AP008226) and HB27 (GenBank accession no. AE017221) have been sequenced completely (16). Given its genomic similarity to *T. thermophilus* HB8, we analyzed the genetic information of strain TMY in that context.

Here we report the effect of silicic acid concentration on the growth of *T. thermophilus* TMY, which was isolated from a siliceous deposit formed at a geothermal electric power plant. Notably, supersaturated silicic acid markedly stimulated expression of one cell envelope protein, which we named silica-induced protein (Sip). Induction of Sip expression occurred rapidly after the cells were exposed to supersaturated silicic acid, and the amino acid sequence of Sip showed significant similarity with the Fe^{3+} ABC transporters observed in other *Thermus* strains. These results shed new light on the growth and biosilicification associated with thermophiles in geothermal environments.

MATERIALS AND METHODS

Strains and growth conditions. The bacterial strains used in this study were *Thermus thermophilus* TMY (JCM 10668), which was isolated from silica scale formed at a geothermal power plant (14), *T. thermophilus* HB8, *T. thermophilus* Fij33, A1, *T. thermophilus* AT62, and *Thermus aquaticus* YT-1. These strains were routinely grown at 75°C under strong aeration (200 rpm) in TM liquid medium (24). Prior to use, the pH of the medium was adjusted to 7.2 with HCl, and then the medium was autoclaved. The stock solution of silicic acid was prepared by dissolving sodium metasilicate in 10 mM NaOH.

Measurement of the silica concentration. The total silicic acid concentrations in the culture supernatants were measured using an inductively coupled plasma optical emission spectrometer (Vista-MPX; Seiko Instruments Inc., Chiba, Japan). The concentration of monosilicic acid was measured using the molybdenum-yellow method (17, 38).

Preparation of cell envelope proteins. Cell envelope proteins were prepared by the method of Berenguer et al. (6) with some modifications. Briefly, cells were harvested from 50 ml of late-log-phase culture by centrifugation at $8,000 \times g$ for 10 min. The harvested cells were washed twice with the same volume of 50 mM Tris-HCl (pH 8.0) and then resuspended in 1.5 ml of the same buffer, and then they were broken by sonication for 10 s in an ultrasonic disruptor (UD-201; Tomy Seiko Co. Ltd., Tokyo, Japan) set at output 5 and duty 50. The debris were removed through two centrifugation steps at $10,000 \times g$ for 10 min each, and the supernatant was recovered as the crude protein. Cell envelope proteins were recovered from the crude protein by ultracentrifugation at $279,000 \times g$ for 1 h using an Optima TLX ultracentrifuge (Beckman Coulter, Fullerton, CA). The resultant pellets were suspended in 100 ml of 50 mM Tris-HCl (pH 8.0), and this cell envelope suspension was either used immediately or kept frozen at -20°C until used.

SDS-PAGE profiles. Aliquots (1 ml) of stationary-phase cultures of *Thermus* strains were each transferred to 100 ml of fresh TM broth containing the indicated concentration of silica. Cells cultivated under each condition were subsequently harvested by centrifugation and sonicated, and then cell envelope proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2D-PAGE. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of cell envelope proteins was carried out using an AE-6310 Ag-PAG 2-DE system (Atto Corporation, Tokyo, Japan) by the method of Oh-Ishi and Maeda (28). Agarose isoelectric focusing gels containing 5 M urea and 1 M thiourea were prepared. A glass tube (3.5 by 210 mm) was set up as an electrophoresis unit, and a 600- μg sample of protein solution was applied at the cathodic end of the gel. The first-dimensional electrophoresis was run at 4 mA and 600 V for 20 h at 15°C . The proteins in the gel were then fixed for 1 h at room temperature using

a protein-fixing solution containing 10% trichloroacetic acid and 5% sulfosalicylic acid, and then the gel was washed for 1 h with distilled water, loaded on top of a slab gel, and covered with a 1% agar solution to keep it in place. Thereafter, 1.5 ml of SDS buffer was overlaid on the isoelectric focusing gels, and this was followed by 10% (wt/vol) SDS-PAGE (the second-dimensional electrophoresis) run at 120 mA and 250 V until the end of the run. Finally, the slab gel was stained with Coomassie brilliant blue R250 to visualize the proteins.

Column chromatography. After ultracentrifugation, the cell envelope proteins were solubilized in 0.1% (wt/vol) Tween 20 overnight, and the resultant solution was dialyzed against 0.1 M phosphate buffer (pH 6.8) containing 1 mM dithiothreitol. The dialyzed protein was then purified over a course of three column chromatography steps on a DEAE-Sepharose Fast Flow column, a Q-Sepharose Fast Flow column, and an SP-Sepharose Fast Flow column (GE Healthcare Bio-Sciences, Uppsala, Sweden).

N-terminal and internal amino acid sequences of Sip. To determine the N-terminal amino acid sequence, the purified protein was transferred to a polyvinylidene difluoride membrane (Millipore, Billerica, MA), visualized by staining with Ponceau S (Nacalai Tesque, Inc., Kyoto, Japan), and then analyzed for N-terminal sequences using a PSQ-1 gas-phase sequencer (Shimadzu, Kyoto, Japan) according to the manufacturer's instructions. Deblocking the N-terminal amino acid of Sip was accomplished using an acylamino acid-releasing enzyme (TaKaRa Bio, Inc., Shiga, Japan) and the in-gel method (9). The internal peptide amino acid sequences of Sip were obtained by digesting concentrated samples of purified Sip protein overnight at 37°C with each of three residue-specific endoproteases: *Staphylococcus aureus* protease (V8 protease), trypsin and endoprotease Asp-N (TaKaRa Bio). Proteolytic peptide fragments and undigested Sip were separated by high-performance liquid chromatography (model 600E; Waters Corporation, Milford, MA), and then the amino acid sequences of the peptide fragments were analyzed as described above.

Detection of Sip glycosylation. Glycosylation of Sip was assessed using an immunoblot kit for glycoprotein detection (Bio-Rad Laboratories, Hercules, CA) as instructed by the manufacturer.

Cloning and sequencing of the sip gene. PCR amplification of *sip* and the region adjacent to it was carried out using *Laq* polymerase (TaKaRa Bio) and primers TMY-*sip*-ADF (5'-ACCACGGCAACGCCGAGGAG-3') and TMY-*sip*-ADR (5'-CCCAAGGCTCCGCGAGGCC-3'). These primers were designed based on the conserved sequences up- and downstream of the *sip* gene (see Fig. 5). The amplified fragment was cloned into pTA2 vector (Toyobo, Osaka, Japan) using a T-Target cloning kit (Toyobo), and then the nucleotide sequences were determined using a Thermo Sequenase fluorescently labeled primer cycle sequencing kit with 7-deaza-dGTP and ALF express II DNA sequencer (GE Healthcare Bio-Sciences). Comparisons of the sequence with the GenBank and EMBL databases were made using DNASIS software (Hitachi Software Engineering, Tokyo, Japan). Phylogenetic analysis of the solute-binding proteins of the Fe^{3+} ABC transporter was done with ClustalW version 1.83 using the neighbor-joining algorithm, Kimura's correction, and a bootstrap consisting of 1,000 pseudoreplicates.

Nucleotide sequence accession number. A 2,225-bp fragment from *T. thermophilus* TMY containing the *sip* gene and the region adjacent to it was sequenced and deposited in DDBJ under accession number AB363611.

RESULTS

Relationship between the silica concentration in the culture medium and induction of a cell envelope protein. In our previous study, when cultured *Thermus* species were exposed to silicic acid, deposition of amorphous silica began during the late log growth phase (19). It appeared that *Thermus* cells precipitated all of the supersaturated silicic acid and that some proteins or lipids produced on the bacterial surface might be involved in the silica deposition. In the present study, *T. thermophilus* TMY cells were cultivated in TM medium containing various concentrations of silicic acid, and then the cell envelope proteins were extracted from each culture and subjected to SDS-PAGE. Figure 1 shows that when the cells were cultivated in the presence of 400 ppm (lane 4) to 700 ppm (lane 7) silicic acid (SiO_2), several bands showed an increase in density with increasing concentrations of SiO_2 . In addition, a new band appeared under supersaturated conditions. The molecular

FIG. 1. Relationship between silica concentration and the production of a silica-induced protein in *T. thermophilus* TMY. Cells were cultivated in TM medium containing various concentrations of silicic acid. The cell envelope proteins were extracted from each culture and subjected to 10% SDS-PAGE. The silicic acid concentration in each sample was as follows: 0 ppm (lane 1), 200 ppm (lane 2), 300 ppm (lane 3), 400 ppm (lane 4), 500 ppm (lane 5), 600 ppm (lane 6), and 700 ppm (lane 7). The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel. The presence (+) or absence (-) of siliceous deposition is indicated below the gel. The position of the silica-induced protein (Sip) is indicated by the black arrowhead to the right of the gel.

mass of the new band was approximately 35 kDa, and its density also increased in proportion to the concentration of silicic acid. Production of this new protein and its upregulation were in excellent temporal agreement with the silica precipitation, which was observed at silicic acid concentrations greater than 400 ppm in TM medium at 75°C. We therefore named the new protein silica-induced protein (Sip), though small amounts of a protein with the same molecular mass as Sip were detected in cells cultivated in TM medium containing less than 400 ppm (SiO_2). Our earlier findings suggested that the molecular mass of Sip may affect the aggregation or deposition of supersaturated polysilicic acid (19), or Sip may act as the stress response protein against the adhesion of silicic acid molecules. *T. thermophilus* HB8 and *T. aquaticus* YT-1 also produced Sip-like proteins that were nearly the same as that produced by strain TMY under the same conditions (data not shown).

Time-dependent induction of Sip expression in *Thermus* strains by supersaturated silica. By plotting the growth curve, we determined that *T. thermophilus* TMY grown in TM broth without silicic acid at 75°C with shaking entered early log phase after 2 h of cultivation and reached stationary phase after 10 h (data not shown). In the presence of undersaturated silicic acids, the growth tendencies and the optical density at 600 nm of the cultures were same as those of cultures without silicic acid. In cultures of *T. thermophilus* TMY in TM broth containing 600 ppm silicic acid, which is the average concentration in the geothermal water at geothermal power plants, considerable Sip protein was detected after 6 h (middle of the log phase), and the levels peaked after 8 to 10 h (Fig. 2). After 12 h

FIG. 2. Time-dependent expression of Sip by *T. thermophilus* TMY in the presence of 600 ppm silica. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel. The position of Sip is indicated by the black arrowhead to the right of the gel.

of cultivation or more, the level of Sip production had declined somewhat.

When silicic acid was added (final concentration, 600 ppm) to strain TMY cultures during late log growth phase (8 to 10 h), induction of Sip production was detected within 1 h, and substantial amounts of Sip were produced after 2 h of induction (Fig. 3A). By contrast, induction was not observed in that time frame when silicic acid was added during the early log growth phase (4 to 6 h). Similar induction was observed when preheated fresh TM medium containing 600 ppm silicic acid was added to precipitated bacterial cells from a 9-h culture (Fig. 3B). Induction of Sip by supersaturated silicic acid was also observed with *Thermus* species, such as *T. aquaticus* YT-1, *T. thermophilus* Fij3 A.1 and *T. thermophilus* AT-62 (data not shown). These results suggest that Sip expression is initiated as soon as these bacterial cells are exposed to supersaturated silicic acid, provided they have reached late log phase.

Figure 4 shows the 2D-PAGE profiles of the cell envelope proteins from cultures containing 0 or 600 ppm silicic acid. The molecular mass was found to be about 35 kDa, which confirmed our earlier analysis, and the pI was about 9.5. While small amounts of Sip were produced in the absence of silicic acid (Fig. 4A), Sip expression was markedly upregulated at a silicic acid concentration of 600 ppm (Fig. 4B). The levels of Sip were estimated to be about 10-fold higher in the presence of 600 ppm silicic acid than in its absence.

Purification and examination of glycosylation of the Sip protein. To purify Sip protein, 13.6 mg of the insoluble fraction of the cell envelope proteins from *T. thermophilus* TMY were applied first to a DEAE-Sepharose Fast Flow column. The proteins were then eluted by stepwise addition of sodium chloride from 0 to 2 M and concentrated to 8.8 mg of protein per sample. This protein sample was applied to a Q-Sepharose column and eluted with glycine-NaOH buffer, which yielded 0.96 mg of protein. Finally, 130 μg of protein was recovered after a second DEAE-Sepharose Fast Flow column. Because it

FIG. 3. SDS-PAGE profiles showing induction of Sip in the cell envelope fraction from supersaturated silica-stressed *T. thermophilus* TMY (A) and *T. thermophilus* HB8 (B) cells. Silica was added (+) or not added (–) to the bacterial cells. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gels. The position of Sip is indicated by the black arrowheads to the right of the gels.

was previously reported that diatom frustulins are glycoproteins, we tested whether the purified Sip protein was glycosylated, but we detected no signal indicating glycosylation (data not shown). Thus, while both Sip and frustulins likely interact

with silica, there appears to be little similarity between their structures or the mechanisms by which they interact with silica.

Determination of the amino acid sequence of Sip. To analyze the primary structure of Sip, we first attempted to determine its N-terminal amino acid sequence. When purified Sip was transferred directly to a polyvinylidene difluoride membrane and sequenced, however, no sequence could be determined, most likely because the Sip N terminus is blocked. We therefore next attempted to determine Sip's internal sequence by digesting the protein with three site-specific endoproteases: V8 protease, trypsin, and endoproteinase Asp-N. Using this approach, we found the internal sequence to be S(A)-X-I-X-A-G-E(T)-I-X-L-G-S-X-N-D-Y-A-V-V, which shows significant similarity to the substrate-binding protein in the Fe^{3+} ABC transporter, a periplasmic iron-binding protein from *T. thermophilus* HB8 (locus tag, TTHA1628; GenBank accession no. NC_006461; GeneID, 3169376) and a substrate-binding protein in the bacterial iron ABC transporter of *T. thermophilus* HB27 (locus tag, TTC1264; GenBank accession no. NC_005835; GeneID, 2774619). The molecular mass and pI of these predicted products of HB8 and HB27 were 36.1 kDa and 9.74, respectively, which are very similar to those of Sip (35 kDa, pI 9.5). We therefore suggest that Sip is likely to be a kind of periplasmic iron-binding protein.

Nucleotide sequences of sip and its adjacent region in *T. thermophilus* TMY. Using a primer set designed from the nucleotide sequence of sip in *T. thermophilus* HB8 (TTHA1628), we carried out a PCR that amplified sip and the region adjacent to it in *T. thermophilus* TMY. The reaction amplified a 2,225-bp fragment that was subsequently cloned and sequenced (DDBJ accession no. AB363611). The sequence of the sip open reading frame (987 bp) showed 89.0% and 88.6% similarity to TTHA1628 and TTC1264, respectively. In both *T. thermophilus* strains HB8 and HB27, sip was surrounded by

FIG. 4. 2D-PAGE of the insoluble fraction of cell envelope proteins extracted from *T. thermophilus* TMY cells cultivated in TM medium without silica (A) or with 600 ppm silicic acid (B). The positions of molecular mass markers (lanes M) (in kilodaltons) are indicated to the right of the gels. The positions of Sip on the gels are indicated by the black arrows.

FIG. 5. Gene organization and the intergenic spacer region in the Fe^{3+} ABC transporter gene cluster of *T. thermophilus*. (A) Schematic representation of the Fe^{3+} ABC transporter gene cluster. The white arrow represents *sip* (encoding solute-binding protein in *fbp* operon), whereas the gray and black arrows represent the permease and ATPase genes, respectively. The directions of transcription of the genes are indicated by the arrows. (B) Putative palindromic sequences present in the intergenic spacer regions (indicated by light gray shading). Arrows indicate the inverted repeats. Asterisks indicate the stop codons for the genes encoding solute-binding proteins.

open reading frames encoding a permease and a hypothetical metalloenzyme (Fig. 5A), and a 25-nucleotide palindromic sequence was situated within an intergenic spacer region between *sip* and the permease gene (Fig. 5B).

The computer-estimated molecular mass and pI of the Sip protein was 35,714 Da and 9.89, respectively, and the protein showed significant homology with several substrate-binding proteins in the Fe^{3+} ABC transporter. In addition to those from *Thermus* species, Sip has 46%, 42%, and 40% amino acid identity with the deduced solute-binding proteins from *Salinibacter ruber* DSM 13855 (GenBank accession no. YP_446499), *Rubrobacter xylanophilus* DSM 9941 (GenBank accession no. YP_643953), and *Nodularia spumigena* CCY9414 (GenBank accession no. ZP_01630623), respectively. To visualize Sip's evolutionary relationships, we constructed a phylogenetic tree for various substrate-binding proteins in the Fe^{3+} ABC transporter (Fig. 6). Sip was categorized within a specific cluster of solute-binding proteins of the Fe^{3+} ABC transporter from *T. thermophilus* species and extremely halophilic bacteria.

DISCUSSION

Our in vitro silicification experiments demonstrated that Sip production is induced when the silicic acid concentration in the medium is greater than 400 ppm and that a corresponding siliceous precipitation is produced (Fig. 1) that is very much like the amorphous silica deposits formed in natural geothermal water (19). The solubility of amorphous silica is 340 ppm at 75°C (3), indicating that a supersaturated silicic acid concentration is required for induction of the protein. The negatively charged polysilicic acid (colloidal silica) formed under such supersaturated conditions may affect the bacterial cell, as evidenced by the growth inhibition observed under supersaturated conditions. We suppose that silica stress might initiate a cell response that leads to induction of Sip. Our results from an earlier study suggest that certain proteins or lipids on the cell

surface react with silicic acid, perhaps to reduce silica stress (19). Our finding that small amounts of silica-independent Sip production occur in the *Thermus* cell envelope is in contrast to trehalose metabolic enzymes, which are produced in cytoplasm solely in response to salt stress (32). We therefore suggest that the induction of Sip by supersaturated silicic acid is a distinct phenomenon that selectively occurs in a silica-rich geothermal environment and plays a significant role in *Thermus* ecology. Given that Sip production and biosilicification are synchronized, we suggest they are likely regulated by a common mechanism. Our 2D-PAGE showed that Sip production by *T. thermophilus* TMY was 10-fold greater in the presence of 600 ppm silica than in its absence. This silica-stimulated Sip production was particularly apparent in *T. thermophilus* HB8, suggesting the HB8 *sip* promoter could represent a novel tool with which to abundantly express the heterologous protein in *Thermus* cells.

The predicted Sip showed no similarity to silica-depositing proteins, such as silicatein and silaffin (7, 25), but did show similarity to the substrate-binding proteins in Fe^{3+} ABC transporters. The ABC transporter functions in the process of Fe^{3+} uptake, and detailed investigations have been carried out with pathogenic bacteria, such as *Serratia marcescens* (5), *Actinobacillus actinomycetemcomitans* (35), *Haemophilus influenzae* (4), and *Vibrio cholerae* (36). Genomic sequencing has shown that the genomes of some microorganisms contain several genes encoding ABC transport systems that have been annotated as Fe^{3+} ABC transporters. These Fbp (ferric iron-binding protein) or Sfu (*Serratia* ferric uptake) systems consist of a periplasmic substrate-binding protein, a permease, and an ATPase (2, 5). The genes encoding these systems are usually regulated as an operon, like *fbpABC*, *sfuABC*, or *hitABC* (1), and correlate with the ability of pathogenic bacteria to obtain iron from transferrin or lactoferrin and with their ability to cause disease.

Although homologues of the *fbpABC* operon have been detected in *Thermus* genomes, Sip is produced in *Thermus* cells in response to supersaturated silica stress. In all *Thermus* strains,

FIG. 6. Phylogenetic tree of Sip and its related proteins in Fe³⁺ ABC transporters. The tree was inferred from the amino acid sequences using the neighbor-joining method. The scale bar indicates 0.1 amino acid replacement per site. GenBank accession numbers are given in parentheses.

a 44-bp intergenic region following the *sip* stop codon contains a strong palindromic structure (Fig. 5B) that is predicted to be similarly positioned in the *fbp* operon. Since stem-loop structures can function to stabilize messages, the presence of the palindrome may enable Sip to be expressed at much higher levels than permease or ATPase, which is consistent with our inability to detect proteins corresponding to those two enzymes in silica-stressed *Thermus* membranes. The phylogenetic tree for presumed Fe³⁺-binding proteins showed that Sip from *T. thermophilus* belonged to a specific cluster of proteins that differed from many Fe³⁺ ABC transporters in pathogenic microbes (Fig. 6), suggesting that the

main function of Sip proteins from *T. thermophilus* strains is something other than iron uptake.

Sip is a Fe³⁺-binding protein homologue; however, it is induced by supersaturated silica in *Thermus* species. Sip might interact with supersaturated silica directly to function as a core for silica precipitation. The rapid production of Sip and the corresponding deposition of silica previously seen in response to exposure to supersaturated silica are consistent with that hypothesis (Fig. 1 and 3). It remains unclear, however, whether Sip isolated from the cell envelope fraction of *Thermus* cells is able to bind directly to silica. Expression of Sip was initiated

within 1 h after the addition of silica to late-log-phase cultures, while it took about 6 h to detect Sip production when the bacterial cells were transplanted into a fresh TM culture containing silica (Fig. 2). If silica-induced Sip expression is a response to the need to acquire iron from the medium, such a long period of expression could have an adverse effect on the cell. This behavior is likely to differ from that of cells responding to iron-deficient conditions, and an alternative promoter or σ factor would be required for the response to supersaturated silicic acid. Recently, the relationship among Fe^{3+} uptake protein, quorum sensing, and biofilm formation was examined in *Pseudomonas* species (8, 30). In an earlier study, we observed biofilm-like siliceous deposits when *Thermus* cells were cultivated in TM broth containing supersaturated silica (19). The facts that this phenomenon and Sip induction occur only during the middle of the exponential growth phase suggests they might be relevant to quorum sensing. Both the bacterial surface and colloidal silica are negatively charged, making it unlikely that colloidal silica would be adsorbed onto the cell surface. Instead, Fortin and Beveridge proposed that a cross-linking bond is required for silica to bind to the bacterial surface (13). Metallic ions, such as Fe^{3+} , could mediate the chemical bonding between the silica and the cell surface, and in turn, a silica- Fe^{3+} -cell surface interaction would be formed (10). Unexpectedly, Sip shares homology with the Fe^{3+} ABC transporter. Considering that Sip expression was in phase with siliceous deposition, there is a good possibility that Sip contributes to silica deposition by mediating a Sip-Fe-silica interaction. On the other hand, Phoenix et al. (31) reported that although bacterial systems immobilize more Fe than bacterium-free systems, they do not immobilize more silica than bacterium-free systems. It follows that silica and iron immobilization is dominated by nonbacterially mediated precipitation. In addition, Konhauser et al. suggested that microorganisms living in hot springs play a passive role in silicification and contribute only marginally to the magnitude of silicification (23); beyond that, biosilicification is an inorganically controlled process, the reaction kinetics of which are not strongly influenced by the presence of bacteria (37). Whether the bacteria are able to interact actively with silica remains controversial, but it is an undeniable fact that many microbe-like structures are observed around siliceous deposits in geothermal areas. We suggest that hyperthermophilic *Thermus* species, which are dominant species in hot spring and silica scale, assume a key role in silicification. On the basis of the results of Lalonde et al., thermophilic, biofilm-forming members of the order *Aquificales* produce their biofilms in a manner related to silicification (26). Moreover, it is clear that *Thermus* and members of the *Aquificales* are dominant in silica scales and sinter, which strongly supports the notion that they play a key role in silicification (18). Although silica is not essential for the growth of *Thermus* species, the bacteria effectively precipitate amorphous silica. This characteristic seems to be common among *Thermus* species and appears to be related to their strategy for survival in an extreme environment with limited nutrition. In particular, silica scaling would provide these bacteria with a firm scaffold against the strong current in the pipelines of geothermal power plants. Further investigation will be required to fully understand the mechanisms that control the interactions between supersaturated colloidal silica and bacterial cells.

ACKNOWLEDGMENTS

We are grateful to Seiki Kuramitsu (Graduate School of Science, Osaka University) for analysis of the *T. thermophilus* HB8 genome (<http://www.thermus.org/>).

This research was supported in part by grants from the Iwatani Naoji Foundation, Kyushu University Interdisciplinary Programs in Education and Projects in Research Development, Foundation NAGASE Science Technology Development, and Grants-in-Aid for Scientific Research (B) 11460047.

REFERENCES

- Adhikari, P., S. D. Kirby, A. J. Nowalk, K. L. Veraldi, A. B. Schryvers, and T. A. Mietzner. 1995. Biochemical characterization of a *Haemophilus influenzae* periplasmic iron transport operon. *J. Biol. Chem.* **270**:25142–25149.
- Adhikari, P., S. A. Berish, A. J. Nowalk, K. L. Veraldi, S. A. Morse, and T. A. Mietzner. 1996. The *fbpABC* locus of *Neisseria gonorrhoeae* functions in the periplasm-to-cytosol transport of iron. *J. Bacteriol.* **178**:2145–2149.
- Alexander, G. B., W. M. Heston, and R. K. Iler. 1954. The solubility of amorphous silica in water. *J. Phys. Chem.* **58**:453–455.
- Anderson, D. S., P. Adhikari, K. D. Weaver, A. L. Crumbliss, and T. A. Mietzner. 2007. The *Haemophilus influenzae* hFbpABC Fe^{3+} transporter: analysis of the membrane permease and development of a gallium-based screen for mutants. *J. Bacteriol.* **189**:5130–5141.
- Angerer, A., S. Gaisser, and V. Braun. 1990. Nucleotide sequences of the *sfuA*, *sfuB*, and *sfuC* genes of *Serratia marcescens* suggest a periplasmic binding-protein-dependent iron transport mechanism. *J. Bacteriol.* **172**:572–578.
- Berenguer, J., M. L. Faraldo, and M. A. De Pedro. 1988. Ca^{2+} -stabilized oligomeric protein complexes are major components of the cell envelope of "*Thermus thermophilus*" HB8. *J. Bacteriol.* **170**:2441–2447.
- Cha, J. N., K. Shimizu, Y. Zhou, S. C. Christiansen, B. F. Chmelka, G. D. Stucky, and D. E. Morse. 1999. Silicatein filaments and subunits from a marine sponge direct the polymerization of silica and silicones *in vitro*. *Proc. Natl. Acad. Sci. USA* **96**:361–365.
- Cha, J. Y., J. S. Lee, J. I. Oh, J. W. Choi, and H. S. Baik. 2008. Functional analysis of the role of Fur in the virulence of *Pseudomonas syringae* pv. *tabaci* 11528: Fur controls expression of genes involved in quorum-sensing. *Biochem. Biophys. Res. Commun.* **366**:281–287.
- Coligan, J. E., B. M. Dunn, H. L. Ploegh, D. W. Speicher, and P. T. Wingfield. 1995. Current protocols in protein science. John Wiley & Sons, New York, NY.
- Fein, J. B., S. Scott, and N. Rivera. 2002. The effect of Fe on Si adsorption by *Bacillus subtilis* cell walls: insights into non-metabolic bacterial precipitation of silicate minerals. *Chem. Geol.* **182**:265–273.
- Fernandez-Turiel, J. L., M. Garcia-Valles, D. Gimeno-Torrente, J. Saavedra-Alonso, and S. Martinez-Manent. 2005. The hot spring and geyser sinters of El Tatio, Northern Chile. *Sediment. Geol.* **141**:235–250.
- Ferris, F. G., T. J. Beveridge, and W. S. Fyfe. 1986. Iron-silica crystallite nucleation by bacteria in a geothermal sediment. *Nature* **320**:609–611.
- Fortin, D., and T. J. Beveridge. 1997. Role of the bacterium *Thiobacillus* in the formation of silicates in acidic mine tailings. *Chem. Geol.* **141**:235–250.
- Fujino, Y., R. Kawatsu, F. Inagaki, A. Umeda, T. Yokoyama, Y. Okaue, S. Iwai, S. Ogata, T. Ohshima, and K. Doi. 2008. *Thermus thermophilus* TMY isolated from silica scale taken from a geothermal power plant. *J. Appl. Microbiol.* **104**:70–78.
- Handley, K. M., S. J. Turner, K. A. Campbell, and B. W. Mountain. 2008. Silicifying biofilm exopolymers on a hot-spring microstromatolite: templating nanometer-thick laminae. *Astrobiology* **8**:747–770.
- Henne, A., H. Brueggemann, C. Raasch, A. Wier, T. Hartsch, H. Liesegang, A. Johann, T. Lienard, O. Gohl, R. Martinez-Arias, C. Jacobi, V. Starkuviene, S. Schlenczek, S. Dencker, R. Huber, H. P. Klenk, W. Kramer, R. Merkl, G. Gottschalk, and H. J. Fritz. 2004. The genome sequence of the extreme thermophile *Thermus thermophilus*. *Nat. Biotechnol.* **22**:547–553.
- Iler, R. K. 1979. The chemistry of silica. John Wiley & Sons, New York, NY.
- Inagaki, F., S. Hayashi, K. Doi, Y. Motomura, E. Izawa, and S. Ogata. 1997. Microbial participation in the formation of siliceous deposits from geothermal water and analysis of the extremely thermophilic bacterial community. *FEMS Microbiol. Ecol.* **24**:41–48.
- Inagaki, F., T. Yokoyama, K. Doi, E. Izawa, and S. Ogata. 1998. Bio-deposition of amorphous silica by an extremely thermophilic bacterium *Thermus* spp. *Biosci. Biotechnol. Biochem.* **62**:1271–1272.
- Inagaki, F., Y. Motomura, K. Doi, E. Izawa, S. Taguchi, S., D. R. Lowe, and S. Ogata. 2001. Silicified microbial community at Steep Cone hot spring, Yellowstone National Park. *Microb. Environ.* **16**:125–130.
- Inagaki, F., Y. Motomura, and S. Ogata. 2003. Microbial silica deposition in geothermal hot waters. *Appl. Microbiol. Biotechnol.* **60**:605–611.
- Jones, B., R. W. Renaut, and M. R. Rosen. 1997. Biogenicity of silica precipitation around geysers and hot-spring vents, north island, New Zealand. *J. Sediment. Res.* **67**:88–104.

23. Konhauser, K. O., B. Jones, V. R. Phoenix, G. Ferris, and R. W. Renaut. 2004. The microbial role in hot spring silicification. *Ambio* **33**:552–558.
24. Koyama, Y., T. Hoshino, N. Tomizuka, and K. Furukawa. 1986. Genetic transformation of the extreme thermophile *Thermus thermophilus* and of other *Thermus* spp. *J. Bacteriol.* **166**:338–340.
25. Kröger, N., R. Deutzmann, and M. Sumper. 1999. Polycationic peptides from diatom biosilica that direct silica nanosphere formation. *Science* **286**:1129–1132.
26. Lalonde, S. V., K. O. Konhauser, A.-L. Reysenbach, and F. G. Ferris. 2005. The experimental silicification of *Aquificales* and their role in hot spring sinter formation. *Geobiology* **3**:41–52.
27. Marshall, W. L., and J. M. Warakowski. 1980. Amorphous silica solubilities. II. Effect of aqueous salt solutions at 25°C. *Geochim. Cosmochim. Acta* **44**:915–917.
28. Oh-Ishi, M., and T. Maeda. 2005. 2-D PAGE of high-molecular-mass proteins, p. 145–150. *In* J. M. Walker (ed.), *The proteomics protocols handbook*. Humana Press, Totowa, NJ.
29. Pancost, R. D., S. Pressley, J. M. Coleman, L. G. Benning, and B. W. Mountain. 2005. Lipid biomolecules in silica sinters: indicators of microbial biodiversity. *Environ. Microbiol.* **7**:66–77.
30. Patriquin, G. M., E. Banin, C. Gilmour, R. Tuchman, E. P. Greenberg, and K. Poole. 2008. Influence of quorum sensing and iron on twitching motility and biofilm formation in *Pseudomonas aeruginosa*. *J. Bacteriol.* **190**:662–671.
31. Phoenix, V. R., K. O. Konhauser, and F. G. Ferris. 2003. Experimental study of iron and silica immobilization by bacteria in mixed Fe-Si systems: implications for microbial silicification in hot springs. *Can. J. Earth Sci.* **40**:1669–1678.
32. Silva, Z., S. Alarico, A. Nobre, R. Horlacher, J. Marugg, W. Boos, A. I. Mingote, and M. S. da Costa. 2003. Osmotic adaptation of *Thermus thermophilus* RQ-1: lesson from a mutant deficient in synthesis of trehalose. *J. Bacteriol.* **185**:5943–5952.
33. Toporski, J. K. W., A. Steele, F. Westall, K. L. Thomas-Keppta, and D. S. McKay. 2002. The simulated silicification of bacteria—new clues to the modes and timing of bacterial preservation and implications for the search for extraterrestrial microfossils. *Astrobiology* **2**:1–26.
34. Wedepohl, K. H. 1995. The composition of the continental crust. *Geochim. Cosmochim. Acta* **59**:1217–1232.
35. Willemsen, P. T., I. Vulto, M. Boxem, and J. de Graaff. 1997. Characterization of a periplasmic protein involved in iron utilization of *Actinobacillus actinomycetemcomitans*. *J. Bacteriol.* **179**:4949–4952.
36. Wyckoff, E. E., A. R. Mey, and S. M. Payne. 2007. Iron acquisition in *Vibrio cholerae*. *Biometals* **20**:405–416.
37. Yee, N., V. R. Phoenix, K. O. Konhauser, L. G. Benning, and F. G. Ferris. 2003. The effect of cyanobacteria on silica precipitation at neutral pH: implications for bacterial silicification in geothermal hot springs. *Chem. Geol.* **199**:83–90.
38. Yokoyama, T., Y. Sato, Y. Maeda, T. Tarutani, and R. Maeda. 1993. Siliceous deposits formed from geothermal water. I. The major constituents and the existing states of iron and aluminum. *Geochem. J.* **27**:375–384.