ORIGINAL ARTICLE

Silica deposition and phenotypic changes to *Thermus thermophilus* cultivated in the presence of supersaturated silicic acid

Satoru Iwai¹, Katsumi Doi², Yasuhiro Fujino², Takeo Nakazono¹, Kosai Fukuda¹, Yoshinobu Motomura³ and Seiya Ogata¹

¹Department of Applied Microbial Technology, Faculty of Biotechnology and Life Science, Sojo University, Kumamoto, Japan; ²Department of Genetic Resources Technology, Faculty of Agriculture, Kyushu University, Higashi-ku, Fukuoka, Japan and ³Department of Earth and Planetary Science, Faculty of Science, Kyushu University, Higashi-ku, Fukuoka, Japan

***Thermus thermophilus* cells formed siliceous deposits in the presence of supersaturated silicic acid (600 p.p.m SiO₂). The supersaturated silicic acid promoted interaction between cells and the inside walls of glass culture bottles, leading to the development of cell aggregates or biofilms. Electron probe microanalysis showed that within the aggregates most of the cell surfaces were covered with silica. Under these conditions, there was remarkable production of silica-induced protein (Sip), a solute-binding component of the Fe³⁺-binding ABC transporter. Furthermore, supersaturated silica enhanced resistance to the peptide antibiotics bacitracin, colistin and polymyxin B, which all act on the cell envelope. By contrast, supersaturated silica did not induce resistance to ampicillin, chloramphenicol, kanamycin and tetracycline, which inhibit peptide synthesis. Although strong expression of Sip was detected in liquid cultures of *T. thermophilus* in the presence of supersaturated silica and colistin, upregulated transcription of putative efflux pump and multidrug resistance ABC transporter genes were not detected by quantitative real-time PCR analysis. These findings suggest Sip promotes silica deposition on the surfaces of cells, after which the silicified outer membrane may serve as a 'suit-of-armor,' conferring resistance to peptide antibiotics.**

The ISME Journal (2010) 4, 809–816; doi:10.1038/ismej.2010.12; published online 11 March 2010

Subject Category: geomicrobiology and microbial contributions to geochemical cycles

Keywords: *Thermus thermophilus*; silicic acid; antibiotic resistance; cell adhesion

Introduction

Silica deposition is an ongoing and often problematic process in many modern hot spring systems. For example, the siliceous deposits (called silica scale) formed on the surface of the equipment and in the pipelines at geothermal power plants have a significant negative economic impact related to energy loss and maintenance costs (Inagaki *et al.*, 2003). Microscopic observation of these siliceous deposits reveals numerous microbe-like structures, and it has been suggested that these fossils represent archaic microorganisms that grew in the hot, supersaturated fluids (Cook and Stakes, 1995; Yee *et al.*, 2003; Handley *et al.*, 2008). There have been a number of studies characterizing the physical changes associated with various bacteria during silicification (Toporski *et al.*, 2002; Phoenix *et al.*,

2003; Lalonde *et al.*, 2005; Orange *et al.*, 2009); however, the molecular mechanisms underlying microbial silicification remain poorly understood.

We previously observed numerous bacteria within the silica scale at a geothermal power plant, and after examining their population and its characteristics, we suggested that it is the extremely thermophilic bacteria that participate in forming the siliceous deposits (Inagaki *et al.*, 1997, 1998, 2001). One of these bacteria, *Thermus thermophilus* TMY, was isolated from the silica scale (Fujino *et al.*, 2008) and was found to induce precipitation of supersaturated amorphous silica (>400 p.p.m SiO₂) during its log-growth phase (Inagaki *et al.*, 1998). Moreover, a protein of ~35 kDa, called silica-induced protein (Sip), was induced in the presence of supersaturated silica and was isolated from the cell envelope fraction of the TMY cells (Doi *et al.*, 2009). The amino-acid sequence of Sip is similar to that of the predicted solute-binding component of the Fe³⁺-binding ABC transporter in *T. thermophilus* HB8. What's more, *sip* gene is situated as a component of the Fbp-type ABC transporter operon, and a Sip-like protein was also induced in strain

Correspondence: S Ogata, Department of Applied Microbial Technology, Faculty of Biotechnology and Life Science, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan.
E-mail: ogata28@bio.sojo-u.ac.jp

Received 30 November 2009; revised 14 January 2010; accepted 17 January 2010; published online 11 March 2010

HB8 in the presence of supersaturated silicic acid. Only very small amounts of Sip are detected in cells cultivated without silica, but marked Sip production is initiated within 1 h after addition of supersaturated silicic acid (Doi *et al.*, 2009). Notably, silica-induced changes in protein expression were also recently observed in *Anabaena* sp. (Konhauser *et al.*, 2008), suggesting that silica stress may induce protein expression in a variety of thermophiles.

There have been many reports focusing on the function and/or gene expression of Fe³⁺-binding ABC transporters and on similar membrane transporters and secretion systems in gram-negative pathogenic bacteria such as *Pseudomonas* and *Salmonella* (Fath and Kolter, 1993; Higgins, 1995). It is known that some of these transporters have key functions in mediating surface adherence (Costerton *et al.*, 1999; Drenkard, 2003; Gooderham *et al.*, 2008) and/or antibiotic resistance (Gunn *et al.*, 1998; Zhou *et al.*, 2001; Lee *et al.*, 2004; Nishino *et al.*, 2006). For thermophilic microorganisms, surface adherence seems to actively contribute to the rapid formation of large siliceous deposits, suggesting that the deposits are important for the maintenance of their position and survival within the limited niches of geothermal environments. Indeed, Phoenix and Konhauser (2008) suggested that bacterial biomineralization acts as a protective screen against UV and dehydration and provide a source of essential nutrients for growth and protection against toxins—that is biomineralization acts as a ‘suit-of-armor,’ protecting the organism and creating a more favorable growth environment.

With that as background, our work has focused on characterizing the physiological changes that *Thermus* species undergo during *in vitro* biosilicification. In this study, we examined the development of cell adherence and drug resistance along with silica deposition during the growth of *T. thermophilus* in the presence of supersaturated silicic acid.

Materials and methods

Bacterial strains and cultural conditions

T. thermophilus TMY (JCM 10668) and *T. thermophilus* HB8 (ATCC 33923) were used in this work. The former was isolated from silica scale formed in geothermal water at a geothermal power plant in Japan (Fujino *et al.*, 2008). The optimum temperature for cultivation was 75 °C for strain TMY and 70 °C for strain HB8. These strains were routinely grown under strong aeration (200 r.p.m) in TM liquid medium (Fujino *et al.*, 2008). Prior to use, the pH of the medium was adjusted to 7.2 with HCl, after which it was autoclaved. A stock solution of silicic acid was prepared by dissolving sodium metasilicate in 10 mM NaOH, as described earlier. In this work, the concentration of silicic acid in the TM medium was 600 p.p.m, which is the average

concentration in geothermal water at geothermal power plants (Inagaki *et al.*, 1998).

Turbidity measurement

The optical density (OD) of cultures was measured at 660 nm using a photoelectric colorimeter (TAITEC MiniPhoto 518R; Taitec Co. Ltd, Tokyo, Japan).

Scanning electron microscopy and electron probe microanalysis

Cell aggregates on the glass surface of culture bottles were examined using a JEOL JCXA-733 electron microprobe analyzer (JEOL Ltd., Tokyo, Japan) to identify silicon and other metallic minerals (relative qualitative analysis) (Inagaki *et al.*, 2001).

Antibiotic sensitivity

The antibiotics used in this study were ampicillin (Nacalai Tesque, Kyoto, Japan), bacitracin (Sigma-Aldrich Co., St Louis, MO, USA), chloramphenicol (Wako Pure Chemical Industries Ltd., Osaka, Japan), colistin (Wako), kanamycin (Sigma-Aldrich), polymixin B (Wako), streptomycin (Meiji Seika Kaisha Ltd., Tokyo, Japan), tetracycline (Sigma-Aldrich) and thiostrepton (Sigma-Aldrich). Antibiotic sensitivity was preliminarily tested using the dilution method on solid medium containing 2.0% Gellan Gum (Wako), with or without silicic acid. The minimum inhibitory concentration of each antibiotic was determined and expressed as µg per ml. Cells were also exposed to antibiotics in TM broth with or without 600 p.p.m silicic acid, and the OD₆₆₀ of the cultures was monitored for 12 h.

RNA preparation

After exposure to 5 µg ml⁻¹ colistin for 1 h, with or without 600 p.p.m silica, *T. thermophilus* HB8 cells were placed in two volumes of RNeasy Protect Bacteria Reagent (QIAGEN GmbH, Hilden, Germany) for 10 min. The cells were then collected, and the crude RNA was extracted by the addition of 1.4 ml of solution containing 5 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.25% SDS and 50% water-saturated phenol. This mixture was incubated at 65 °C for 5 min, chilled on ice for 5 min, and then centrifuged at 4 °C. Thereafter, 750 µl of TRIzol LS (Life Technologies Corporation, Carlsbad, CA, USA) was added to 0.2 ml of the aqueous phase and after incubation for 5 min at room temperature, the RNA was extracted using 0.2 ml of chloroform. The extraction was then repeated with 0.5 ml of chloroform, after which the crude RNA was purified using an RNeasy mini kit (QIAGEN) according to the manufacturer's instructions, which included digestion with DNase I to remove any contaminating DNA. RNA concentrations were calculated using NanoDrop 1000 (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Primer design and quantitative real-time PCR

The primers designed for quantitative real-time PCR were 19–21 bases long, had a G/C content >50% and a T_m of about 60 °C. The length of the PCR products ranged from 90 to 160 bp (Table 1). Primer3 design software (<http://primer3.sourceforge.net/>) was used to select the primer sequences. Quantitative real-time PCR were performed using a One Step SYBR PrimeScript RT-PCR Kit II (TAKARA BIO Inc., Otsu, Japan) in a LightCycler 2.0 (Roche Diagnostics GmbH, Basel, Switzerland) according to the manufacturer's instructions. The reverse transcription step was carried out at 42 °C (5 min) and was followed by denaturation of the reverse transcriptase by heating at 95 °C for 10 s. The amplification protocol entailed 40 cycles of 95 °C for 5 s and 60 °C for 20 s. After the amplification, melting curve analysis was carried out at temperatures ranging from 65 to 95 °C. A standard curve was generated for the relative quantification of all genes, and a control reaction lacking reverse transcriptase was performed for every RNA sample. The copy number of each sample was calculated from the means of triplicate PCR sample. The gyrase subunit B (*gyrB*) gene was used as a housekeeping gene to normalize the amount of RNA in each reaction.

Results and discussion

Surface adherence of cells during growth in the presence of supersaturated silicic acid

T. thermophilus strains TMY and HB8 form yellow or yellowish orange colonies, reflecting the presence of carotenoid pigments. During growth in the presence of supersaturated silicic acid, the cells adhered to the glass surface of the culture bottles (Figure 1). Thanks to the yellow or orange coloration, adhesion of the cells was easily observable where the edge of the liquid cultures (Figures 1b and c). The cell adhesion was first visually detectable after 7–8 h of culture, and with further cultivation developed into adherent cell aggregates resembling biofilm. This aggregation of adherent cells reached a maximum after about 36 h and persisted for an additional 12 h with the limited nutrition available in a bottle culture. We previously observed that in cultures containing 600 p.p.m silicic acid, *in vitro*

silica deposition with considerable Sip production was detectable after 6–7 h (mid-log phase) (Doi *et al.*, 2009). The levels of Sip production peaked after 8–10 h, and the level of silica deposition peaked after 30–36 h. The progression of the cell adhesion could be closely related to Sip production and silica deposition, as there was little cell adhesion in the presence of a lower concentration of silicic acid (<400 p.p.m) (Figure 1a). There was no deposition of silica in cell-free cultures containing 600 p.p.m silicic acid, even after 120 h (data not shown).

To obtain information about the bacterial cell aggregates that formed on the glass surface of the culture bottles and the Si distribution, we next examined the cells using electron probe microanalysis (Figure 2). Scanning electron microscopy examination of TMY cell aggregates formed in silica-free medium showed clumps of individual rod-shaped cells (Figure 2a), whereas Si mapping revealed that very little Si was present (Figure 2b). By contrast, when cell aggregates formed in the presence of supersaturated silicic acid, cells embedded in silica particles were observed within the aggregates (Figure 2c), and strong Si signals were detected (Figure 2d). On the other hand, there were no differences between Si-free and supersaturated Si cultures with respect to other elements, including Na, K, Ca, Mg and Fe. The same results were obtained with HB8 cell aggregates. We therefore suggest that the *T. thermophilus* cells strongly interact with silica, and that the observed deposition of silica on the glass surface of culture bottles was not caused by evaporation. In addition, these results suggest that silica is an important component supporting the formation of cell aggregates and biofilms on the surface of inorganic materials.

Responses of strains TMY and HB8 to antibiotics in supersaturated silicic acid

When we examined the ability of antibiotics to inhibit the growth of *T. thermophilus* strain TMY on TM agar plates, we found that the minimum inhibitory concentrations were 0.1, 0.5, 1, 5, 5, 1, 1 and 0.5 µg ml⁻¹ for ampicillin, bacitracin, chloramphenicol, colistin, kanamycin, polymyxin B, streptomycin, tetracycline and thiostrepton, respectively. Nearly the same effects were obtained

Table 1 Primers used in this study

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Annotation for product
TTHA0263	CTCGCCCTCAACCTTTCCA	CACGACCCCGTAGAAGACCA	Multidrug efflux transporter
TTHA0351	CACCAACGACCTCTTCTCCA	GGAGTAGCGGAAACCAGAA	Efflux protein (fosmidomycin resistance)
TTHA0554	TCCCGAGGTCTACGTCTTCT	ACGAGGAGGTTCCCAAGAG	Small multidrug export protein
TTHA0729	GCGTTTTGGGCTTCACCTAC	GCGGTAGCGGTCAATGTTTT	AcrB/AcrD/AcrF family efflux transporter
TTHA1004	GCCTTTCCGAAAACCTCTCC	GTTCCTCTCGCTTGAGTT	Outer membrane efflux protein
TTHA1315	AGTTTCTGCCCTCCCTCCTC	CAGCCCGTGTAGACCAAGAA	Putative integral membrane efflux protein
TTHA1341	GGAAGAAGAGGGCGAACCA	AAAGGGAGCGGGAGGAGAAG	ABC-type multidrug transport system
sip	ACCCGCTTCTCACCTACCT	GTCCAGCTTGGGGCTTTTC	Silica-induced protein (iron ABC transporter)

Figure 1 Photographs showing the adhesion of cells and cell aggregates to the glass surface of culture bottles in the presence and absence of supersaturated silicic acid. (**a** and **b**) *T. thermophilus* TMY culture grown for 36 h at 75 °C in the absence (**a**) and presence (**b**) of supersaturated silicic acid (600 p.p.m). (**c**) *T. thermophilus* HB8 culture grown for 36 h at 70 °C in supersaturated silicic acid (600 p.p.m).

Figure 2 Scanning electron micrographs and silicon distribution images of cell aggregates obtained using electron probe microanalysis. (**a** and **b**) Scanning electron microscopy images of *T. thermophilus* TMY cell aggregates in the absence (**a**) and presence (**b**) of supersaturated silicic acid. (**c** and **d**) Silicon mapping of cell aggregates grown in the absence (**c**) and presence (**d**) of supersaturated silicic acid. Bars indicate 1 μ m.

with strain HB8. For some of the antibiotics, however, we noticed a difference in sensitivity in the presence and absence of supersaturated silicic acid. Both strains appeared to be rendered resistant to bacitracin, colistin and polymyxin B in the presence of supersaturated silicic acid in the solid medium (data not shown).

To confirm this observation, we also tested the sensitivity to antibiotics of strains TMY and HB8 in liquid cultures. The growth tendencies and OD_{660} of the liquid cultures of both strains were nearly the same in the absence and presence of supersaturated silicic acid (600 p.p.m). On the other hand, with the addition by supersaturated silicic acid, the bacteria exhibited three patterns of sensitivity to the antibiotics tested (Figures 3 and 4). Their sensitivity to ampicillin, chloramphenicol, kanamycin, streptomycin and thiostrepton was nearly the same in the

presence and absence of supersaturated silica—that is the growth tendencies and the OD_{660} of the cultures were about the same (Figure 3). The susceptibility to tetracycline increased somewhat in the presence of supersaturated silica (Figure 3), though the pattern remained consistent with the aforementioned antibiotics in that supersaturated silicic acid did not confer resistance to the antibiotic. With the peptide antibiotics bacitracin, colistin and polymyxin B, however, we observed greater growth of both strains in the presence of supersaturated silicic acid than in its absence (Figure 4). This means that both strains are highly susceptible to these peptide antibiotics in the absence of supersaturated silicic acid, but their sensitivity is diminished by the presence of supersaturated silicic acid. Moreover, when antibiotics were added after 4–7 h of culture with 600 p.p.m

silicic acid, the antibiotic resistance was more pronounced than after 0–2 h; little growth inhibition was seen, with or without silicic acid, when antibiotics were added after 8 h (data not shown).

Bacitracin acts by interfering with the dephosphorylation of C₅₅-isoprenyl pyrophosphate, a

molecule that carries the building blocks of the peptidoglycan cell wall outside the inner membrane (Stone and Strominger, 1971). Colistin is a cyclic cationic peptide that kills gram-negative bacteria by interacting with and disrupting the outer membrane (Sun *et al.*, 2009). Polymyxin B is a polycationic peptide that disrupts the bacterial cell membrane by interacting with its phospholipids (Roes *et al.*, 2005). That these antibiotics all attack the cell envelope suggests supersaturated silicic acid induces phenotypic changes in the *Thermus* cell envelope that lead to an increase in peptide antibiotic resistance. As the other antibiotics tested in this study inhibit protein or peptide synthesis, phenotypic changes to the cell envelope induced by supersaturated silicic acid had little or no effect on the bacteria's resistance to these drugs.

Bacterial growth in the presence of iron ion or other metal ions reportedly results in modification of surface substances (for example lipopolysaccharide) that renders the organisms resistant to antibiotics such as polymyxin B. In *Pseudomonas* and *Salmonella*, for example, iron ion or Mg²⁺ affects the PmrA/PmrB system, which consists of an efflux pump (PmrA) and a sensor protein (PmrB) that regulates some surface modifications, including modification of lipopolysaccharide, rendering the organisms resistant to polymyxin B (Groisman *et al.*, 1997; Mcphee *et al.*, 2003; Lee *et al.*, 2004; Gooderham *et al.*, 2008). In this study, however, supersaturated silicic acid induced expression of the ABC transporter component Sip, which rendered the cells resistant to peptide antibiotics like those produced by the soil bacterium *Bacillus subtilis* and related species. In that regard, Wösten *et al.* (2000) proposed that resistance to polymyxin B would be important for the natural habitation of bacteria. Moreover, the observed increase in

Figure 3 Effect of six representative antibiotics on the growth of *T. thermophilus* TMY in the presence and absence of supersaturated silica. Open symbols show the growth curve in the absence of supersaturated silicic acid. Filled symbols show the growth curve in the presence of supersaturated silicic acid (600 p.p.m.). ○ ●, no antibiotics; □ ■, chloramphenicol (1 µg ml⁻¹); △ ▲, kanamycin (5 µg ml⁻¹); ◇ ◆, ampicillin (0.1 µg ml⁻¹); ▽ ▴, tetracycline (0.5 µg ml⁻¹). The data represent the average of five or more independent experiments. Standard deviations for each experiment were not greater than ± 5% of the value shown. Strain HB8 shows nearly the same growth patterns as strain TMY.

Figure 4 Effect of three peptide antibiotics on the growth of *T. thermophilus* strains TMY and HB8 in the presence and absence of supersaturated silica. (a) Strain TMY; (b) strain HB8. Open symbols show growth curves in the absence of supersaturated silicic acid. Closed symbols show growth curves in the presence of supersaturated silicic acid (600 p.p.m.). ○ ●, no antibiotics; ◇ ◆, bacitracin (0.5 µg ml⁻¹); □ ■, colistin (5 µg ml⁻¹); △ ▲, polymyxin (b) (5 µg ml⁻¹). The data represent the averages of five or more independent experiments. Standard deviations for each experiment were not greater than ± 5% of the value shown.

susceptibility to tetracycline can also be explained by the modification of surface transporters due to Sip production.

Some ABC transporters and efflux pumps support the secretion of soluble factors and materials needed for colonization and biofilm formation, and can function as either importers or exporters with diverse substrates (Fath and Kolter, 1993; Linton and Higgins, 1998; Mah and O'toole, 2001; Vallet *et al.*, 2001). Moreover, there have been numerous reports of biofilm-associated resistance to antibiotics and its possible mechanisms in gram-negative bacteria (Costerton *et al.*, 1999; Drenkard, 2003; Lai *et al.*, 2009). In *Pseudomonas* and *Salmonella*, for example, the phenomenon seems to be similar to silica-induced resistance in *Thermus* cells, in that certain antibiotics such as β -lactams, aminoglycosides and fluoroquinolones are unaffected by biofilm-associated resistance (Mulcahy *et al.*, 2008). In addition, other studies have shown that extracellular polymeric substances often have key functions in biomineralization (Karthikeyan and Beveridge, 2002; Omoike *et al.*, 2004; Orange *et al.*, 2009), and that the S layer is involved in biomineralization in the natural environment (Schultze-Lam *et al.*, 1992). We are presently investigating these issues in *Thermus* cells by carrying out a metabolome analysis of cell aggregates in the presence of supersaturated silica.

Analysis of the expression of the sip, multidrug resistance ABC transporter and efflux pump genes in the presence of supersaturated silica and antibiotics
As described above and in earlier papers (Inagaki *et al.*, 2003; Doi *et al.*, 2009), *T. thermophilus* strains TMY and HB8 promote silica deposition, cell adhesion and resistance to peptide antibiotics in the presence of supersaturated silica, all of which may be related with Sip production. Sip, the multidrug resistant ABC transporter and/or the efflux pump system in *Thermus* may participate in interactions between the cell surface and silicic acid and between the cells and inorganic materials for cell adhesion. In addition, expression of these genes may contribute to the secretion of soluble factors and materials that support colonization or biofilm formation. To investigate these ideas, we analyzed expression of the *sip*, ABC transporter and efflux pump genes in strain HB8, with or without colistin and supersaturated silicic acid. Colistin was selected as the antibiotic for this experiment because the largest change in tolerance induced by supersaturated silicic acid was observed with colistin (Figure 3). Completion of the strain HB8 genome project (http://www.thermus.org/e_index.htm) enabled the design of specific primers for real-time PCR analysis of the genes in question. As shown in Table 1, *sip* gene, eight efflux pump genes and two multidrug resistance ABC transporter genes have been annotated in the HB8 genome.

We previously detected expression of *sip* using SDS-PAGE (Doi *et al.*, 2009). We could also confirm the expression of *sip* by quantitative real-time PCR. Although remarkable transcription (>15-folds) of *sip* gene was observed in cultures containing both colistin and supersaturated silica or containing only supersaturated silica, sufficient *sip* transcripts was detected in cells grown in TM broth containing only colistin (Figure 5). Gene expression of ABC transporters and efflux pumps was not inducible under any conditions (Figure 5). We therefore suggest their expression would not be stimulated by either supersaturated silica or a peptide antibiotic such as colistin. This suggests that the resistance to colistin conferred by supersaturated silica is not mediated by a drug export system involving transporters or pumps, but by increasing the robustness of the cell surface through embedding in silica. Consistent with that idea, the silica coating or silica embedded in the cell envelope apparently does not have efficacy against antibiotics that act in the cell cytoplasm such as ampicillin and so on.

Thermus species living in high-temperature zones in hot reservoirs attach to silica deposits or other materials to maintain their position within appropriate niches, which leads to the formation of biofilms. These bacteria can also survive exposure to highly siliceous solutions (>600 p.p.m), but under such conditions they are exposed to the menaces of antibacterial substances produced by other microorganisms. Bacitracin, colistin and polymyxin B are produced by *Bacillus* species, which grow in a variety of habitats. The resistance to antibiotics induced by supersaturated silica would be profitable for *Thermus* species living in natural geothermal water and soil environments; as Konhauser *et al.* (2008) suggested, *Thermus* species can use the mineral 'suit-of-armor' to their advantage.

Figure 5 Relative gene expression levels (fold change, mean $\pm$ s.d.) encoding putative efflux pumps, multidrug resistance ABC transporters and *sip*. Quantitation of these genes was determined relative to *gyrB* as a housekeeping gene by quantitative real-time PCR. Changes in gene expression in cells exposed to silica, colistin and silica/colistin are shown relative to that in cells grown in TM broth. Results shown are the mean of three independent RNA isolations.

Although the molecular mechanisms by which Sip relates to silicification and drug resistance remain unclear, it might help *Thermus* don its 'armor.' As Sip is annotated as a solute-binding component of the Fe³⁺-binding ABC transporter, it remains uncertain whether this protein is located on the cell surface and whether it interacts directly with silica. We have succeeded in cloning *sip* and expressing it in *Escherichia coli* and are now investigating its abilities. The information obtained should provide clues to the significance and utilization of silica for the maintenance of the position and survival of *Thermus* species within limited natural niches. Moreover, it may shed light on how to control the formation of silica scale in geothermal power plants.

Acknowledgements

We are grateful to Dr Fumio Inagaki (Kochi Institute for Core Sample Research, JAMSTEC, Japan) for helpful advice. This research was supported in part by Kumamoto Technology and Industry Foundation (2005, 2006), by Kyushu University Interdisciplinary Programs in Education and Projects in Research Development (2008, 2009) and by JSPS KAKENHI No. 21580097.

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