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Research Article

Earthquakes Promote Bacterial Genetic Exchange in Serpentinite Crevices

Naoto Yoshida and Nori Fujiura

Abstract

We report the results of our efforts to study the effects of seismic shaking on simulated biofilms within serpentinite fissures. A colloidal solution consisting of recipient bacterial cells (*Pseudomonas* sp. or *Bacillus subtilis*), donor plasmid DNA encoded for antibiotic resistance, and chrysotile (an acicular clay mineral that forms in crevices of serpentinite layers) were placed onto an elastic body made from gellan gum, which acted as the biofilm matrix. Silica beads, as rock analogues (*i.e.*, chemically inert mechanical serpentinite), were placed on the gellan surface, which was coated with the colloidal solution. A rolling vibration similar to vibrations generated by earthquakes was applied, and the silica beads moved randomly across the surface of the gellan. This resulted in the recipient cells' acquiring plasmid DNA and thus becoming genetically transformed to demonstrate marked antibiotic resistance. Neither *Pseudomonas* sp. nor *B. subtilis* were transformed by plasmid DNA when chrysotile was substituted for by kaolinite or bentonite in the colloidal solution. Tough gellan (1.0%) promoted the introduction of plasmid DNA into *Pseudomonas* sp., but soft gellan (0.3%) had no such effect. Genetic transformation of bacteria on the surface of gellan by exposure to exogenous plasmid DNA required seismic shaking and exposure to the acicular clay mineral chrysotile. These experimental results suggest that bacterial genetic exchange readily occurs when biofilms that form in crevices of serpentinite are exposed to seismic shaking. Seismic activity may be a key factor in bacterial evolution along with the formation of biofilms within crevices of serpentinite. Key Words: Biofilm—Chrysotile—Evolution—*Pseudomonas* sp.—Seismic shaking. Astrobiology 9, 289–295.

Introduction

Chrysotile forms along anastomosing non-penetrating shear surfaces of schistose serpentinite (Hemley *et al.*, 1977; Karkanas, 1995; Andreani *et al.*, 2005). Chrysotile, $Mg_6Si_4O_{10}(OH)_8$, like kaolinite, is a phyllosilicate that forms clay-sized crystals. Chrysotile is a known carcinogen. Though amphiboles are more potent than chrysotile with regard to the induction of fibrotic lung disease and associated lung cancers, chrysotile, which accounts for over 90% of the world's production of asbestos, is the most common fibrous serpentine. A number of toxicologists have investigated the effect of chrysotile on mammalian cells in an attempt to determine the mechanism by which chrysotile behaves as a mutagen that promotes carcinogenesis (Appel *et al.*, 1988).

Earthquake activity, common on Earth, is concentrated along plate boundaries and occurs beneath volcanoes (Cayol *et al.*, 2000; Ito *et al.*, 2007). Earthquakes occur when rock fails in sliding friction (Vidale and Li, 2003). Accelerations of 100 Gal are common at the surface in earthquake-prone areas. Earthquake activity closely corresponds to geophysical stimulation at sites of marked volcanic activity but also within the deeper fissures of plates (Johnson *et al.*, 2000; Abercrombie and Ekstrom, 2001). Geophysical stimulation induces fault motions, such as shearing and upheaval, which result in rock fissures as a result of sliding friction (Tullis, 1996).

We report here how a serpentinite layer model system was used to investigate the effect of seismic sliding friction at the interface between a biofilm and serpentinite rock under laboratory conditions. The roles of mineral particulates such as chrysotile, bentonite, and kaolinite, and the degree of bacterial genetic exchange were also investigated. Genetic exchange was studied in Gram-positive (*Bacillus subtilis*) and Gram-negative (*Pseudomonas* sp.) bacteria. The extracellular production of gellan gum polysaccharide by *Pseudomonas*

Department of Biochemistry and Applied Biosciences, University of Miyazaki, Miyazaki-shi, Japan.

elodea (Martins and Sá-Correia, 1991; Kanari *et al.*, 2002) was used to model an elastic biofilm.

To investigate the effect of seismic sliding friction on bacterial genetic exchange, we designed an experiment in which a colloidal solution that contained recipient bacterial cells and a chrysotile-plasmid complex were placed on the surface of a biofilm (gellan gum), after which sliding friction between silica beads and the biofilm was created by rolling vibration. Detection of bacterial transformation with plasmid DNA under laboratory conditions provides evidence that seismic shaking might enable genetic exchange on the surface of biofilms within the fissures of serpentinite rock.

Clay Minerals and Bacterial Genetic Exchange

It is well known that bacteria exist in harsh environments, which include clay mineral deposits and rock layers. Anaerobic, thermophilic, and fermenting bacteria have been identified in deep borehole drills that reached a depth of 3500 m in weathered granite rock samples (Szewzyk *et al.*, 1994; Guerrero, 1998). Fukunaga *et al.* (2005) reported on the microbiological characteristics of bacteria in shallow (1 m depth) bentonite deposits and found bacterial cell counts that ranged from 10^2 to 10^6 cells/g dry weight of core sample. A microbial community dominated by a bacterial species affiliated with *Desulfotomaculum* spp. and an archaeal species related to *Methanobacterium* spp. was identified in quartzite fractures 3.3 km below the surface (Moser *et al.*, 2005).

A relationship between the presence of sand or clay minerals and bacterial genetic exchange has been reported. Transformation of chemically competent *Bacillus subtilis* (Lorenz *et al.*, 1988) and *Pseudomonas stutzeri* (Lorenz and Wackernagel, 1990) by sand-adsorbed DNA has been demonstrated. Lorenz and Wackernagel (1990) reported that the transformation frequencies obtained with sand were 25- to 50-fold greater than those observed with the use of a standard liquid culture procedure. Moreover, sand-adsorbed DNA is 100 times more resistant against DNase I than free DNA in solution (Romanowski *et al.*, 1991). In other studies, binding of DNA from *Bacillus subtilis* to the clay mineral montmorillonite with subsequent transformation of competent *Bacillus subtilis* cells with the bound DNA has been observed (Khanna and Stotzky, 1992). Similarly, kaolinite has been observed to increase the transformation frequency of competent cells substantially in experiments with *Bacillus subtilis* cells and chromosomal DNA (Lotareva and Prozorov, 2000). Such experiments support the hypothesis that DNA adsorbed to soil components is resistant to degradation by nuclease (Gallori *et al.*, 1994; Demaneche *et al.*, 2001) and that cryptic genes bind to clay minerals and retain the ability to transform competent cells in natural settings.

Kaolinite and bentonite, which are commonly found in soil, quartzite, and granitic rock, are not known to be especially potent genomic mutagens. However, as described above, clay minerals behave as vehicles for free DNA. We hypothesize that the subsequent uptake of mineral-adsorbed DNA may facilitate a background level of bacterial genomic exchange within natural habitats.

Endolithic Biofilms

At the interface between lithic substrates and the atmosphere, microorganisms accumulate in an organized fashion to form biofilms. Biofilms are found at almost every interface, provided they are exposed to water for at least a short period of time. The biological components of each biofilm grow, reproduce, and produce extracellular polymers, which frequently extend from the cell in a tangled matrix and thus form a structural appendage. A characteristic of all biofilms is that microorganisms are embedded in an elastic body composed of an extracellular polymer matrix (Wingender *et al.*, 1999; Branda *et al.*, 2005). Thousands of species of microorganisms can live and interact within biofilms to form a symbiotic microbial community. Bacteria living in symbiosis with other bacterial species are more common than isolated bacteria in natural settings. If fissures and cavities of rocks are exposed to water (*i.e.*, rainwater, meltwater, or groundwater), these interfaces will likely be occupied by biofilms. For example, de los Ríos *et al.* (2003) emphasized that the fissures and cavities of Antarctic rocks formed of granite are occupied by microbial communities on endolithic biofilms.

Laboratory Simulation of Seismic Shaking

Figure 1A shows a diagrammatic representation of a biofilm formed within a serpentinite near-surface fissure zone or uppermost layer of serpentine soil. Because the serpentinite fissure zone is where acicular serpentinite (chrysotile) forms, a small amount of chrysotile fiber likely accumulates during the process of biofilm formation at both the surface and interior of the biofilm within the serpentinite fissure zone. Indeed, bacterial cells and nucleic acid from autolysed cells remain embedded within biofilms.

The application of rolling vibration is depicted in Fig. 1A. During an earthquake, sliding friction likely occurs at the interface between the serpentinite and the biofilm. Figure 1B depicts the experimental application of seismic sliding friction between the serpentinite and biofilm. The elastic body in the disposable plate represents an artificial biofilm formed with 1% gellan gum, an extracellular polysaccharide produced by *Pseudomonas elodea* (Martins and Sá-Correia, 1991; Kanari *et al.*, 2002; West, 2002). Bacterial cells growing on the surfaces of agar- or gellan-solidified media have been recognized as useful tools for the study of biofilms (Branda *et al.*, 2005). Serpentinite has a greasy or silky feel, especially on smooth flat surfaces. Cut surfaces have a highly polished waxlike slickness, and the main component of serpentinite is silicates. Silica beads were used as a chemically inert mechanical analogue in this experiment.

Pseudomonas sp. was isolated as a heavy metal–resistant strain that was acquired from the undersurface of rock in main. This bacterium could grow fully in nutrient broth containing 2 m*M* of cadmium chloride or 5 m*M* of zinc chloride. *Bacillus subtilis* ISW1214 (Sadaie and Kada, 1983) was purchased from TAKARA BIO Co. for use as the plasmid recipient strain. pHSG298 (Takeshita *et al.*, 1987) and pHY300PLK (Ishiwa and Tsuchida, 1984) were used for the donor plasmids. *Pseudomonas* sp. was transformed with pHSG298 to express kanamycin resistance, since pHSG298 encodes a kanamycin resistance gene. *Bacillus subtilis* was transformed with pHY300PLK to express tetracycline resistance, since pHY300PLK encodes a tetracycline resistance gene. The recipient strains *Pseudomonas* sp. and *Bacillus subtilis* were inoculated on a Luria-Bertani (LB) plate and cultured at 30°C for 18 h to induce colony formation.

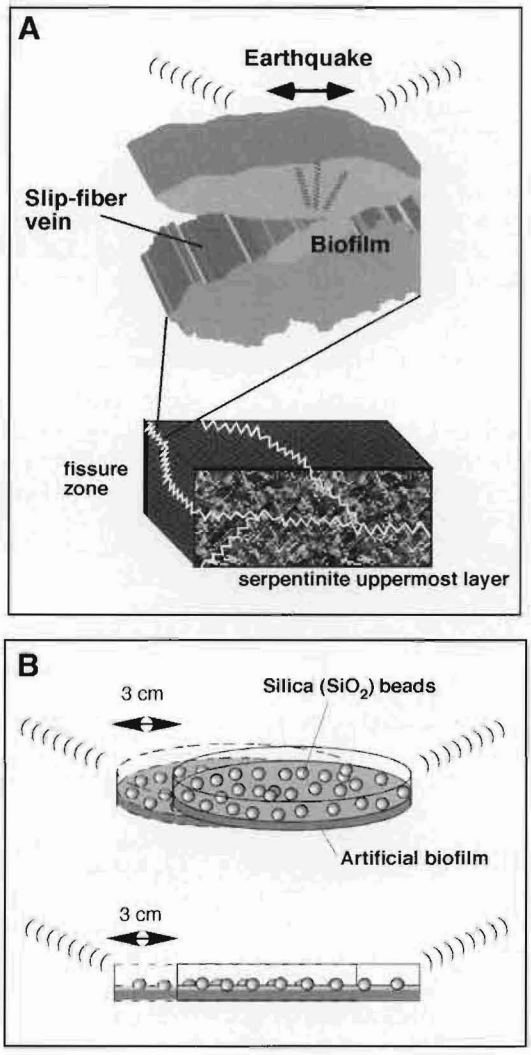

FIG. 1. Schematic representation of a biofilm formed in the near-surface fissure zone of a serpentinite layer. When rolling vibration (seismic friction) occurs, friction might be generated between the serpentinite layer and the biofilm. The slip-fiber vein of chrysotile is depicted by the striped pattern in the cross section of the fissure (**A**). Experimental reproduction in the laboratory (**B**). LB medium solidified with gellan gum provided an artificial biofilm. Silica beads were used to mimic serpentinite rock, of which the principal component is SiO_2. Silica beads were rolled on the moist artificial biofilm to match the intensity of seismic vibration. This seismic shaking provided sliding friction at the interface between the silica beads and the artificial biofilm. Color images available online at www.liebertonline.com/ast.

Gellan gum (Pan *et al.*, 2006) of 1.0% (weight per volume) dissolved in LB broth (pH 7.0) (Sambrook *et al.*, 1989) was autoclaved at 121°C for 20 min. After autoclaving, kanamycin for *Pseudomonas* sp. and tetracycline for *Bacillus subtilis* were added to Gellan-LB broth at final concentrations of 50 μg/ml and 15 μg/ml, respectively. Gellan-LB broth supplemented with each antibiotic was solidified on disposable plates (8.5 cm in diameter) and used as the artificial biofilm.

Chrysotile whiskers purchased from Waco Chemical Co. (Japan) were washed with sterilized distilled water and dried at 60°C for 12 h (Pele and Calvert, 1983). The oven-dried chrysotile was ground to the finest particles (see below) possible with a grinder (Wonder Blender WB-1, Osaka Chemical Co., Japan). The chrysotile powder was then dissolved in 50 μg/ml of sterilized saline and used as the chrysotile colloidal solution (pH 7.0). Scanning electron microscopy revealed that 95% of the chrysotile particles in colloidal solution were between 0.05 and 0.12 μm in diameter and 0.34 and 2.03 μm in length. Kaolinite and bentonite powders purchased from Waco Chemical Co. (Japan) were washed with sterilized distilled water and dried at 60°C for 12 h. These powders were then dissolved in 50 μg/ml of sterilized saline and used as kaolinite and bentonite colloidal solutions (pH 7.0).

Colonies of recipient bacteria were suspended at 2×10^8 cells/ml in 500 μl of chrysotile colloidal solution containing 50–400 ng of donor plasmid DNA (chrysotile-cell colloidal solution). The surfaces of each gellan gum artificial biofilm were dried rapidly in a clean room to remove all visible water before application of the chrysotile-cell colloidal solution. A 50 μl aliquot of each chrysotile-cell colloidal solution was dropped onto the artificial biofilm, after which the surface of the artificial biofilm was rubbed with 25–50 silica (glass) beads (diameter 4 mm, weight 82 mg) by rolling vibration for 60 s (Fig. 1B). The amplitude and intensity of artificial seismic activity was 1.5 cm and 52–133 Gal, respectively. After seismic shaking was applied, the silica beads were immediately removed from the artificial biofilm, after which the biofilm was incubated for 18–20 h at 30°C. The colonies transformed with antibiotic resistance were counted on the artificial biofilm. Transformation efficiency was expressed as the number of transformants per microgram of donor plasmid DNA.

Bacterial Genetic Exchange Mediated by Seismic Shaking

Following the application of a chrysotile-cell colloidal solution, silica beads were placed onto the surface of each artificial biofilm, after which artificial seismic shaking was applied. Silica beads slid across the surface of each artificial biofilm and provided sliding friction at the interface of the silica beads and each biofilm (Fig. 1B). The contact surface area and pressure of a single silica bead on the artificial biofilm was approximately 0.031 cm^2 and 2.7 gf/cm^2, respectively. The contact surface area as a proportion of the total biofilm surface for 50 and 25 silica beads was 3.1% and 1.5%, respectively. Immediately following the application of seismic shaking, moisture from the chrysotile-cell colloidal solution on the surface of the artificial biofilm functioned as a lubricant. Thus, minimal sliding friction was experienced at the interface between the silica beads and the artificial biofilm. However, as soon as this moisture soaked into the artificial biofilm, increased sliding friction force was experienced at the interface between the silica beads and the biofilm.

Pseudomonas sp. was situated between the silica beads and the artificial biofilm and subjected to rolling vibration, after which visible colonies formed. This indicates that *Pseudomonas* sp. was transformed with plasmid pHSG298 and resulted in kanamycin resistance. Agarose gel electorophoresis showed autonomous replication of pHSG298 without integration into genomic DNA in recipient *Pseudomonas* sp. (data not shown). Figure 2A shows the effect of rolling vibration intensity and silica-bead contact surface area on genetic exchange of *Pseudomonas* sp. The use of 50 silica beads resulted

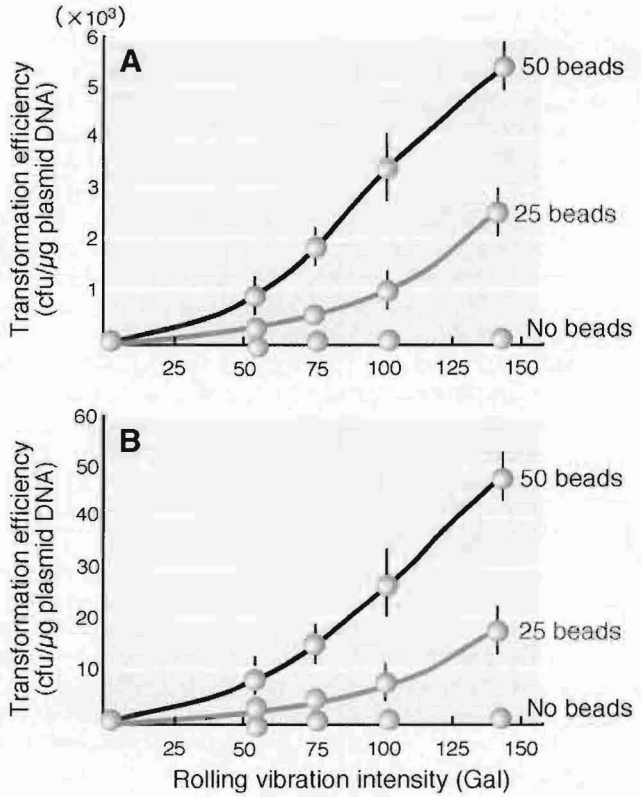

FIG. 2. Relationship between rolling vibration intensity and transformation efficiency of *Pseudomonas* sp. by plasmid pHSG298 after treatment of cells with seismic friction at the artificial biofilm interface with a chrysotile concentration of 50 μg/ml in colloidal solution (**A**). Relationship between rolling vibration intensity and transformation efficiency of *Bacillus subtilis* by plasmid pHY300PLK with a chrysotile concentration of 1.0 mg/ml in colloidal solution (**B**). Values indicate the means ± standard deviation of three independent experiments.

in greater genetic exchange than 25 beads at a rolling vibration intensity of 150 Gal. The number of genetic exchange events was found to be directly proportional to the rolling vibration intensity. In turn, the rolling vibration intensity was found to be closely related to the integral value of the contact surface of the silica beads. Both rolling vibration intensity and duration of seismic sliding friction might increase the chance of bacterial genetic exchange. These experiments indicate that seismic sliding friction between a gellan gum surface and silica beads promotes *in situ* genetic exchange of *Pseudomonas* sp. by exogenous plasmid DNA.

When *Bacillus subtilis* was subjected to rolling vibration, it acquired plasmid pHY300PLK and tetracycline resistance, after which visible colonies formed on the artificial biofilm. A chrysotile concentration of 1.0 mg/ml within the chrysotile-cell colloidal solution enabled genetic transformation of *B. subtilis*, but a concentration of 50 μg/ml did not. Agarose gel electorophoresis demonstrated autonomous replication of pHY300PLK without integration into genomic DNA in *B. subtilis* (data not shown). Figure 2B shows the effects of rolling vibration intensity and silica-bead contact surface area on genetic exchange of *B. subtilis*. The use of 50 silica beads resulted in greater genetic exchange than 25 beads at a rolling vibration intensity of 150 Gal. The number of genetic exchange events was found to be directly proportional to the rolling vibration intensity. These experiments indicate that seismic sliding friction between a gellan gum surface and silica beads promotes *in situ* genetic exchange of bacteria by exogenous plasmid DNA. Genetic exchange occurred with colloidal solutions containing chrysotile and also with solutions containing other acicular minerals, such as crocidolite. No genetic exchange was observed when colloidal solution without chrysotile was subjected to rolling vibration.

Elastic Strength of the Artificial Biofilm

In this experiment, LB medium containing 1.0% gellan gum was used to model a biofilm. Varying the concentration of gellan altered the elastic strength of the artificial biofilm. When rolling vibration was applied to the interface between the artificial biofilm and silica beads, the intensity of sliding friction force between the silica beads and artificial biofilm was likely influenced by the elastic strength of the artificial biofilm. To investigate the relationship between elastic strength of the artificial biofilm and frequency of bacterial transformation, rolling vibration was applied to the chrysotile-cell colloidal solution with the use of 50 silica beads on a LB plate containing 0.3–1.5% gellan gum at 133 Gal for 60 s. Donor plasmid and recipient cells were pHSG298 and *Pseudomonas* sp., respectively. After incubation at 30°C for 24 h, transformed *Pseudomonas* sp. colonies that demonstrated kanamycin resistance were counted. As shown in Fig. 3, the genetic transformation efficiency was high and on the order of 10^3 colony-forming units per microgram (cfu/μg) plasmid DNA for 1.0–1.5% gellan gum. An efficiency of less than 10^3 cfu/μg plasmid DNA was observed when less than 1.0% gellan gum was used. Transformed cells were not observed when 0.3% gellan gum was used. Hard biofilms had a greater effect on transformation than soft biofilms. Soft biofilms contain more moisture and therefore exert a greater lubricating effect, which reduces the sliding friction force. Hard biofilms contain less moisture and therefore generate greater sliding friction force at the interface between the biofilm and silica beads. These results suggest that seismic sliding fiction in the presence of chrysotile at the

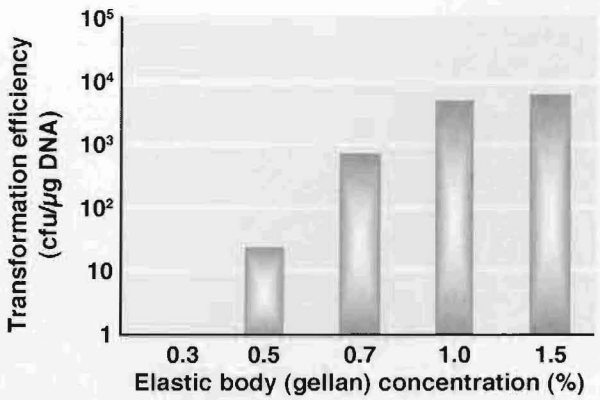

FIG. 3. Relationship between gellan gum concentration of the artificial biofilm and transformation efficiency of *Pseudomonas* sp. by plasmid pHSG298. Values indicate the means of three independent experiments.

biofilm interface mediates bacterial genetic exchange by exogenous DNA.

Chrysotile Penetrates Bacterial Cells

A mixture containing recipient cells, plasmid DNA, and chrysotile (a chrysotile-cell colloidal solution) was sandwiched between a gellan gum surface and silica beads, followed by exposure to rolling vibration similar to that experienced during an earthquake. Subsequently, recipient cells were observed to contain exogenous plasmid DNA. When kaolinite or bentonite was used instead of chrysotile in the mixture, neither *Pseudomonas* sp. nor *Bacillus subtilis* acquired plasmid DNA. The obvious difference between chrysotile and kaolinite and bentonite is in the crystal form. Chrysotile is formed as an acicular crystal with a diameter of 20–50 nm, whereas kaolinite and bentonite are formed as platy crystals. The chrysotile-cell colloidal solutions were plated onto gellan gum, after which sliding friction was applied to the interface between the gellan gum and silica beads. During this process, moisture from the chrysotile-cell colloidal solution soaked into the gellan gum. Thus, large aggregates of chrysotile fiber were observed as increased concentrations of chrysotile remained on the surface (Yoshida and Saeki, 2004). In the chrysotile-cell colloidal solution, plasmid DNA is in a state that binds to chrysotile. The needle-like structure of chrysotile is thin enough to penetrate bacterial cells. Chrysotile aggregates to which donor plasmid bind are thought to penetrate the cell membrane of bacterial cells and thereby enable bacterial cells to acquire plasmid DNA. Sliding friction results in the penetration of bacteria by chrysotile, such as that generated between the gellan gum surface and silica beads. Chrysotile is thought to behave as a vehicle for naked DNA in the serpentinite fissure zone. Transformation of the Gram-positive bacteria *Bacillus subtilis* by pHY300PLK DNA was detected with low transformation efficiency, most likely due to the thicker cell wall of this Gram-positive bacteria, which may have minimized penetration by chrysotile fibers. Increased chrysotile concentrations (1.0 mg/ml) in the chrysotile-cell colloidal solution enabled *B. subtilis* to acquire pHY300PLK DNA, which further supports this theory.

The transformation efficiency of *Pseudomonas* sp. by pHSG298 DNA was examined at varying gellan gum concentrations. When low percentages of gellan gum were used (0.1–0.3%), transformation of *Pseudomonas* sp. was not detectable. The gellan gum content obviously affected the sliding friction force generated at the interface between the gellan gum and silica beads. Soft gellan contains more moisture and therefore has reduced sliding friction force, whereas hard gellan contains less moisture and generates more sliding friction force. As such, an optimal gellan source (biofilm) might promote bacterial transformation by exogenous plasmid DNA by seismic shaking.

Within the natural environment, three mechanisms are thought to be responsible for gene uptake by bacteria (Snyder and Champness, 1997): conjugation, transformation, and transduction. Bacterial genetic transformation is a mechanism by which bacteria acquire naked DNA (de Vries and Wackernagel, 2002; Sikorski *et al.*, 2002; Wang *et al.*, 2002). However, numerous reports indicate that gene transfer events may be a rare occurrence in nature. To achieve transformation of bacteria by plasmid DNA in the laboratory, competent cells have to be chemically treated before use. In our experiments, plasmid DNA acquired by *Pseudomonas* sp. and *B. subtilis* occurs via natural bacterial genetic transformation when the cells are not competent cells treated by divalent cation exposure.

Earthquakes Promote Bacterial Genetic Exchange

Recently, strong motion body waves generated by the Chi-Chi, Taiwan, earthquake in 1999 were found to cause dynamic triggering in the form of high-frequency bursts on band-pass filtered acceleration records (Chen *et al.*, 2006). These bursts appeared to be small, triggered events located in the highly damaged and weathered top few hundred meters of the crust. It is thought that small static stress changes due to permanent fault displacement can alter the likelihood of, or trigger, earthquakes on nearby faults (King *et al.*, 1994). Triggering of earthquakes by dynamic stress fluctuations during the passage of surface waves is well documented (Pankow *et al.*, 2004). There is also evidence that tidal stress fluctuation can trigger local seismicity (Cochran *et al.*, 2004). Both surface waves and tides appear to trigger seismicity down to stresses on the order of 0.005 MPa (51 gf/cm^2). Rate-dependent and state-dependent friction may be a viable mechanism for triggering of the very shallow events (Dieterich, 1987). In our experiment, the pressure between glass beads and artificial biofilm was 2.7 gf/cm^2, thus corresponding to the pressures expected for surface waves and tidal stress. Our experiments are therefore considered analogues for events that likely occur at shallow depths due to earthquake activity.

If biofilms form in acicular clay mineral-bearing environments (*e.g.*, the shallow fissure zones of serpentinite) which are known to host suspended free nano-acicular crystals of chrysotile that form due to weathering processes (*i.e.*, Kashansky *et al.*, 2001), then nutrients (Hodgson, 1979), bacterial cells, and free plasmid DNA may coexist at both the surface and interior of these biofilms. Earthquake-induced vibration (Rubinstein and Beroza, 2004; Bonilla *et al.*, 2005) within shallow serpentinite deposits would create sliding friction between serpentinite surfaces and attached biofilms. Our experimental results indicate that the uptake of chrysotile-bound DNA by *Pseudomonas* sp. and *B. subtilis* on gellan, which is mediated by sliding friction, facilitates bacterial genetic exchange. The ability to incorporate extracellular DNA into bacterial chromosomes is an important mechanism in the horizontal exchange of genetic information.

Serpentinization processes provide important niches for subsurface microbial communities (McCollom, 2007), and they may have been important in facilitating the survival of early and possibly extraterrestrial life (Schulte *et al.*, 2006). The role of earthquake activity in providing a renewable source of nutrients for subsurface communities has recently been discussed by Sleep and Zoback (2007). We hypothesize on the basis of our experimental work that earthquake activity in serpentinite deposits may be an important, yet previously unrecognized, mechanism for increasing the environmental fitness and range of ecological niches available to subsurface microbial communities.

Abbreviations

cfu, colony-forming units; LB, Luria-Bertani.

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Address reprint requests to:
Naoto Yoshida
Department of Biochemistry and Applied Biosciences
University of Miyazaki
1-1 Gakuen, Kibanadainishi
Miyazaki-shi 889-2192, Japan

E-mail: a04109u@cc.miyazaki-u.ac.jp