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Natural genetic transformation of *Pseudomonas stutzeri* by sand-adsorbed DNA

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Abstract. In a soil/sediment model system we have shown recently that a gram-positive bacterium with natural competence (*Bacillus subtilis*) can take up transforming DNA adsorbed to sand minerals. Here we examined whether also a naturally transformable soil bacterium of the gram-negative pseudomonad (*Pseudomonas stutzeri*) can be transformed by mineral-associated DNA. For these studies the transformation protocol of this species was further improved and characterized. The peak of competence during growth of *P. stutzeri* was determined to occur at the beginning of the stationary phase. The competence state was conserved during shock freezing and thawing of cells in 10% glycerol. Kinetic experiments showed that transformant formation after addition of DNA to competent cells proceeded for more than 2 h with DNA adsorption to cells being the rate limiting step. By means of the defined protocol *P. stutzeri* was shown to be transformed by sand-adsorbed DNA. Transformation by adsorbed or dissolved DNA occurred between 16° and 44°C. Efficiency and DNaseI-sensitivity of transformation by DNA adsorbed to sand or in liquid were comparable. It is concluded that uptake of particle-bound DNA by *P. stutzeri* in soil is possible. This finding adds evidence to the view that transformation occurs in natural environments where DNA is assumed to be significantly associated with mineral/particulate material and thereby is protected against enzymatic degradation.

Key words: Competence – DNA uptake – Transformation – DNA-sand complex – Genetically engineered microorganisms – Gene transfer – *Pseudomonas stutzeri*

The ability of bacteria to take up high molecular weight DNA and express its genetic information as a part of the normal cellular physiology has been termed natural genetic transformation (for review see Stewart and Carlson 1986). Among the bacteria living in soil and sediments many species were shown to be naturally transformable (Lorenz and Wackernagel 1988). Because particulate material is the main constituent of soil and other sedimentary habitats and because bacteria can attach to solid surfaces, gene transfer by transformation may occur on minerals and organic particles. For such a gene transfer to occur in natural habitats the following steps are required: (i) release of DNA from viable or dead bacterial cells, (ii) persistence of free DNA in the environment and (iii) competence development by recipient bacteria followed by uptake of DNA. Extracellular DNA appears in cultures of soil bacteria including *Bacillus subtilis* (Takahashi 1962; Ephrati 1968; Borenstein and Ephrati-Elizur 1969; Sinha and Iyer 1971; Crabb et al. 1977), *Micrococcus*, *Alcaligenes*, *Flavobacterium* (Catlin 1956), *Pseudomonas aeruginosa* (Hara and Ueda 1981) and the cyanobacteria *Anacystis nidulans* (Herdman and Carr 1971) and *Synechocystis* sp. (Lorenz unpublished result). A way for the persistence of extracellular DNA in natural environments was suggested by the finding that DNA adsorbed to mineral surfaces and thereby became more resistant to enzymatic degradation (Lorenz et al. 1981; Aardema et al. 1983; Lorenz and Wackernagel 1987). In fact, it has been shown that DNA adsorbed to soil and sediment particles (Ogram et al. 1988) and high molecular weight DNA has been extracted from sediment (Ogram et al. 1987). Moreover, clay-adsorbed DNA persisted in unsterile soil for several days (Greaves and Wilson 1970). Finally, it has been demonstrated that competent cells of *B. subtilis* were transformed by sand-adsorbed DNA with high efficiency (Lorenz et al. 1988).

We asked whether other soil bacteria developing natural competence are also capable of taking up particle-adsorbed DNA. *P. stutzeri*, a soil inhabiting pseudomonad, has been shown to be naturally transformable (Carlson et al. 1983). The aim of the present study was to examine the natural transformation of this species by sand-adsorbed DNA. For this purpose the natural transformation of *P. stutzeri* had to be further characterized with emphasis on cell-DNA interactions. This

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study together with an earlier one (Lorenz et al. 1988) shows that members of the gram-negative and the gram-positive soil bacteria can take up mineral-adsorbed DNA during transformation.

Methods and materials

Strains, media and DNA extraction

Pseudomonas stutzeri JM302 (*his*-1) and JM375 (his^+, *str*-5, *rif*-12) were kindly provided by Dr. J. L. Ingraham, University of California. LB-glucose medium (Carlson et al. 1983) was used for competence induction. Transformants were selected on minimal succinate agar (MS) as described by Carlson et al. (1983). Total viable counts were determined on MS supplemented with 40 µg histidine/ml. DNA was extracted from JM375 according to Marmur (1961). The DNA preparation used throughout the experiments had a mean M_r of 60 kb as determined by electrophoresis in a 0.75% agarose gel.

Transformation

Generally a competent culture (0.18 ml) either fresh or thawed from a frozen stock plus 5 h of aeration was incubated with 0.02 ml DNA solution (in 0.1 M NaCl, 10 mM Tris-HCl, pH 7.0, final DNA concentration generally 1 µg/ml) at 37° C for 90 min unless stated otherwise. Then DNaseI solution (2 µl of 1 mg/ml in 5% [v/v] glycerol) was added to a final concentration of 10 µg/ml and incubation was continued at 37° C for 10 min (DNaseI treatment). Aliquots of the culture were spread on MS (transformants) and MS plus histidine (viable counts), respectively, and incubated for 2 days at 37° C. Transformation frequencies are expressed as transformants per viable recipient cell.

Transformation by sand-adsorbed DNA

Jacketed glass columns (5 × 70 mm) filled with sand (glowed and washed with acid; purchased from Merck, Darmstadt, FRG) were loaded with 0.2 ml of a JM375 DNA solution (2.25 µg/ml in 0.1 M NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.0 buffer) as described previously (Lorenz et al. 1988). Prior to loading with DNA, the sand was incubated with buffer in the columns for 15 to 20 min. A competent culture for the subsequently described transformations was prepared in the following way. A culture from the peak of competence (Fig. 1) was shock-frozen (liquid N_2) in the presence of 10% (v/v) glycerol. A thawed aliquot (4 ml) was shaken in a waterbath at 37° C for 5 h. Then 2 ml of the culture were centrifuged in a Biofuge A centrifuge (Heraeus) for 10 min. A portion (0.1 ml) of the supernatant (having 0.03% of the initial cell concentration) was mixed with a portion (0.1 ml) of the competent culture which had not been centrifuged. This 1 : 2 diluted culture was applied to a DNA-loaded sand column as described (Lorenz et al. 1988). The content was forced out of the column into a glass tube by means of a silicone suction nozzle put on top of the column. The glass tube was incubated for 90 min in a water bath at 30° C unless otherwise stated. Thereafter, 0.8 ml LB-glucose and 10 µl of DNaseI solution (1 mg/ml) were added. This gave a total liquid volume of 1 ml and a final DNaseI concentration of 10 µg/ml. The tube was vigorously vortexed and incubated for another 10 min, before aliquots of the suspension were plated on MS and MS plus histidine, respectively. In comparison experiments in liquid culture, a sample (0.18 ml) of the 1 : 2 diluted culture (see above) was incubated with DNA (0.02 ml of 1.3 µg/ml) for 90 min at 30° C unless otherwise stated. Further uptake of DNA was stopped by DNaseI treatment. Aliquots were spread on MS and MS plus histidine, respectively.

Additional experiments were done to determine the number of transformants resulting from uptake of DNA released from sand into the interstitial solution of the sand bed. For this purpose DNA-loaded sand was incubated with the supernatant of the competent culture (see above) in glass columns for 90 min at 16°, 23° or 37° C. Then 0.2 ml LB-glucose were layered on top of the sand bed and the outflow of the column was opened. The effluent (interstitial solution) was collected and 0.1 ml were mixed with 0.1 ml undiluted competent culture. After 90 min at 16°, 23° or 37° C the transformation mixture was subjected to DNaseI treatment and samples were plated on MS and MS plus histidine, respectively.

Determination of sand-adsorbed DNA

To a sand-filled column 0.2 ml of a DNA solution (2.25 µg/ml in 0.1 M NaCl, 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.0 buffer) were added. After 2 h incubation at 23° C the column was eluted with 5 ml buffer at 0.2 ml/min. DNA was quantified in 1 ml fractions of the effluent with Hoechst H33258 according to Labarca and Paigen (1980). The amount of DNA retained on the sand was the difference between the amount of DNA applied to the column and the amount determined in the 5 ml of the effluent. The amount adsorbed is independent of the temperatures used in these experiments (Lorenz and Wackernagel 1987).

DNA binding to competent cells

Binding of DNA to competent cells was studied by the following procedure. A competent thawed JM302 culture (2 ml) was aerated at 37° C for 5 h. Then DNA (2 µg) was added and incubation continued in a 100 ml Erlenmeyer flask. At various times samples were withdrawn and the DNA was degraded by DNaseI treatment or the DNA was removed by filtration or centrifugation.

Filtration

Samples (0.2 ml) from the transformation mixture were filtered together with 5 ml of LB-glucose through a cellulose acetate filter (0.45 µm, Sartorius). The filter was placed on a prewarmed LB glucose agar plate and incubated at 37° C for 90 min minus the time elapsed between the start of transformation and withdrawal of the sample. Then the filters were submerged in 5 ml LB-glucose and shaken for 15 min to resuspend cells. The cells were sedimented by centrifugation and resuspended in 0.2 ml of LB-glucose for determination of transformants and viable count.

Percoll gradient centrifugation

Percoll (Pharmacia) was made iso-osmotically by mixing 9 vol with 1 vol of tenfold concentrated MS. The solution was further diluted to 80% (v/v) iso-osmotic Percoll with MS and a gradient was formed by centrifugation at 30.000 *g* for 15 min at 4° C in a fixed angle rotor. Samples (0.2 ml) were layered on top of ice-cold gradients (10 ml) and centrifuged at 400 *g* for 20 min at 4° C. In the gradient a single band of cells formed which was carefully removed with a Pasteur pipette and plated for the determination of transformants and viable count.

Results

Development of a defined transformation protocol

Carlson et al. (1983) have shown that after adding DNA to a growing broth culture of *Pseudomonas stutzeri* trans-

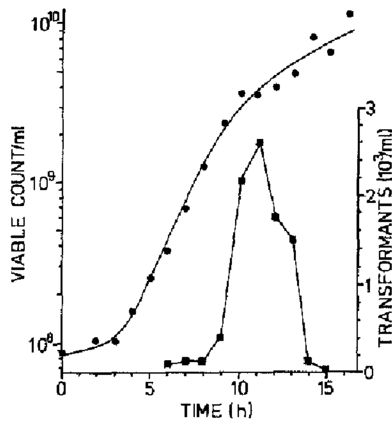

Fig. 1. Growth (●) and transformant formation (■) of JM 302 in LB-glucose. The culture was shaken at 37°C. Samples were taken at the times indicated and incubated with DNA for 60 min before plating for determination of transformants and viable counts

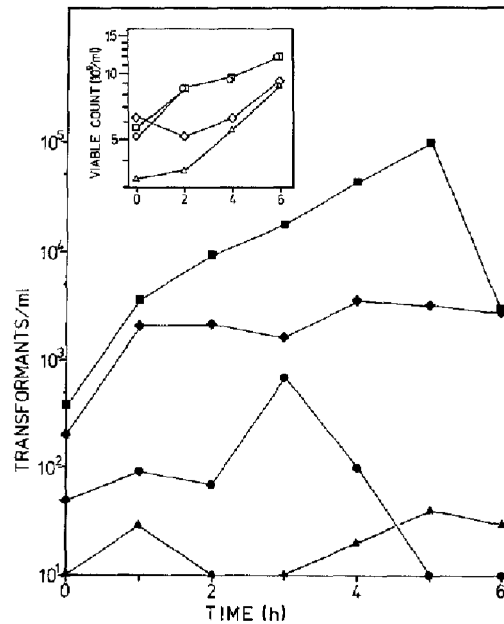

Fig. 2. Influence of shock-freezing and glycerol on competence (*closed symbols*) and growth (*open symbols*, see *inset*). A culture was grown to the competence point (see Fig. 1) and divided into four portions: untreated culture (●; ○), culture with 10% glycerol (■; □), culture shock-frozen and thawed (▲; △), culture shock-frozen in the presence of 10% glycerol and thawed (◆; ◇). For transformation samples of the cultures were withdrawn at the intervals indicated, incubated with DNA for 90 min at 37°C, and treated with DNaseI before plating for determination of transformants and viable counts

formants increased in number during several hours of growth before decreasing. For studies with sand-adsorbed DNA it was desirable to perform the transformation within a limited time period and with high reproducibility.

Therefore we first looked for the actual time course of competence development in a growing culture by determination of the transformation frequency at sequential points of a growth curve. Stationary cells of *P. stutzeri* JM302 were aerated in fresh LB-glucose medium (8×10^7 cells/ml) at 37°C. After onset of growth cells were collected at hourly intervals and incubated with DNA for 60 min before plating on MS medium for selection of His^+ transformants (Fig. 1). A peak of competence was observed in repeated experiments ($2-5 \times 10^{-7}$ transformants/viable cell) at the beginning of the stationary phase (Fig. 1).

Secondly, we sought for a way to conserve the competence of cells. When a culture from the peak of competence was shock-frozen in liquid nitrogen in the presence of 10% glycerol, competence and viability were unchanged upon thawing, even after several months of storage at −80°C. During further incubation after thawing (37°C, 5 h) the transformation frequency increased about tenfold and growth resumed after 4 h (Fig. 2). The increased competence was probably due to the presence of glycerol during incubation, since even a fresh competent culture yielded more transformants when incubated with glycerol (Fig. 2). The experiments suggest that the improvement of competence by glycerol depends at least partly on its metabolism. Recently it was observed that glycerol is an energy and carbon source for *P. stutzeri* and stimulates competence development (Lorenz and Wackernagel, manuscript in preparation). The experiment in Fig. 2 shows that transformation with fresh cells is superior to thawed cells, but for convenience all of the following experiments were done with cells of a frozen stock culture which was thawed and further incubated (5 h) to increase competence. The increase of competence during incubation of thawed cells with glycerol was eliminated in the presence of chloramphenicol (250 µg/ml), indicating the necessity of protein synthesis for competence improvement.

Cell-DNA interactions

Because we wanted to study cell-DNA interactions at the solid/liquid interphase, we first characterized the competent cells from a frozen stock culture with respect to several cell-DNA interactions in liquid.

In the standard transformation assay the number of transformants increased linearly with the amount of DNA up to a concentration of about 1 µg/ml (not shown). This is in accordance with earlier results (Carlson et al. 1983) and shows that the cells from the frozen stock are comparable to the competent cultures previously employed.

It has been suggested that *P. stutzeri* selectively takes up homologous DNA by surface receptors (Carlson et al. 1983). This conclusion was based on competition studies including heterologous and non-transforming homologous DNA. We confirmed this with our system (0.2 µg his^+ DNA/ml) in competition experiments using a tenfold excess of heterologous DNA (*Escherichia coli*). The transformation frequency was reduced from 3.2×10^{-7} (untreated culture) to 2.3×10^{-7} (i.e. 78%) in the presence of heterologous DNA. When the heterologous DNA was incubated with the culture 30 min prior to adding transforming homologous DNA a further small decline of the

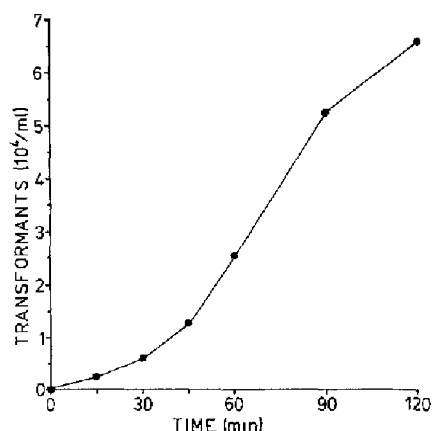

Fig. 3. Kinetics of transformation. A competent culture was incubated with DNA and samples were withdrawn at the intervals as indicated. Samples were treated with DNaseI before determination of transformants

transformation frequency was observed (0.9×10^{-7}). We conclude that competition for uptake was small, if there was any. Heat denatured DNA had no transforming activity.

For the examination of transformation by mineral-adsorbed DNA in a flow-through system previously employed with *B. subtilis* it was necessary to know how rapidly transformants form during cell-DNA interactions in the transformation assay. A kinetic experiment (Fig. 3), in which transformation was terminated after various times by addition of DNaseI, showed that His^+ transformants formed continuously for at least 2 h during incubation of competent cells with DNA. We wanted to determine whether the adsorption of DNA to cells or the uptake of DNA into cells was the rate limiting step. Therefore, non-adsorbed DNA was gently removed from a transformation mixture at various times and the transformants quantified (Table 1). In a parallel transformation mixture DNA was degraded after various times by addition of DNaseI. The kinetics of appearance of transformants were similarly slow whether DNA adsorption and uptake were stopped by DNaseI or non-adsorbed DNA was removed by either filtration or Percoll gradient centrifugation of cells (Table 1). Results similar to those reported with rich medium (Table 1) were also obtained in MS medium (data not shown). It is concluded that DNA adsorption to cells is rate limiting for transformation. Once DNA is adsorbed it is rapidly taken up into a DNaseI-resistant state. Addition of monovalent and divalent cations (including Na^+, K^+, Ca^{2+}, Mg^{2+}, and Mn^{2+} at concentrations between 5 and 200 mM) or polyamines (including spermidine and protaminesulfate at 50 to 150 mM) did not improve transformation either in rich or minimal medium (not shown).

Transformation on sand

The flow-through column system previously described (Lorenz et al. 1988) was initially used to examine transformation of *P. stutzeri* on sand. In this system DNA is adsorbed to sand in a glass column. After non-adsorbed DNA is removed by elution, a competent culture is applied to the column, incubated for 2 min and then is slowly pumped through with fresh aerated medium. However, no transformants were observed in the sand bed or in the effluent. Contrary to earlier experiments with *B. subtilis*, less than 0.8% of the *P. stutzeri* cells were retained on the sand with or without adsorbed DNA. Time for cell-DNA interactions during the flow of the culture through the column was probably too short for transformant formation (see Fig. 3).

Therefore the experimental procedure was modified. Competent cells (0.2 ml) were applied to a column filled with sand to which DNA (0.13 μg) was adsorbed (giving a non-saturating DNA concentration of 0.72 μg/ml in the interstitial volume of 0.18 ml). In order to supply cells with oxygen during the following transformation, the content of the column was subsequently transferred to a tube and incubated for 90 min at the temperatures indicated. Then the frequency of transformation was determined (Table 2, first entry). The transformants obtained resulted from DNA at sand plus DNA released from sand during the 90 min of incubation. For comparison, twelve- to fourtyfold lower numbers of transformants were produced from DNA released during 90 min from sand into the interstitial solution of the sand column (Table 2, second entry) filled with the supernatant

Table 1. Kinetics of His^+ transformant formation determined by removal of DNA from transformation mixtures[a]

Culture treatment	Transformation frequency [$\times 10^7$]				
	0.5 min	15 min	30 min	60 min	90 min
Experiment 1					
DNaseI	<0.03	4.7	19.7	N.D.[b]	N.D.[b]
Filtration	0.16	5.2	15.8	N.D.[b]	N.D.[b]
Experiment 2					
DNaseI	<0.03	N.D.[b]	1.5	7.3	15.1
Centrifugation	<0.1	N.D.[b]	1.3	17.6	6.5

[a] At the times indicated DNA was removed by DNaseI treatment or filtration or Percoll gradient centrifugation of cells (for details see Methods)

[b] N.D. = not determined

Table 2. Transformation at various temperatures by sand-adsorbed and desorbed DNA

Transformation by	Transformation frequency[a] [$\times 10^7$] at		
	16°C	23°C	37°C
Total DNA in sand	1.3	13.4	10.7
(adsorbed and desorbed)[b]	(1.3; 1.3)	(8.3; 13.1; 19.2)	(13.3; 10.7; 8.2)
DNA desorbed from sand[c]	<0.03	1.05	0.40
		(0.9; 1.2)	(0.18; 0.41; 0.6)

[a] The numbers in brackets give values of independent determinations

[b] Competent cells (0.2 ml) were applied to 0.7 g sand with 0.13 μg DNA adsorbed, incubated at the temperatures indicated for 90 min, and treated with DNaseI (for details see Methods)

[c] The supernatant of a competent culture (0.2 ml) was applied to 0.7 g sand with 0.13 μg DNA adsorbed. After 90 min at the temperatures indicated the interstitial solution was removed from the sand and incubated with a competent culture at the temperatures indicated (for details see Methods)

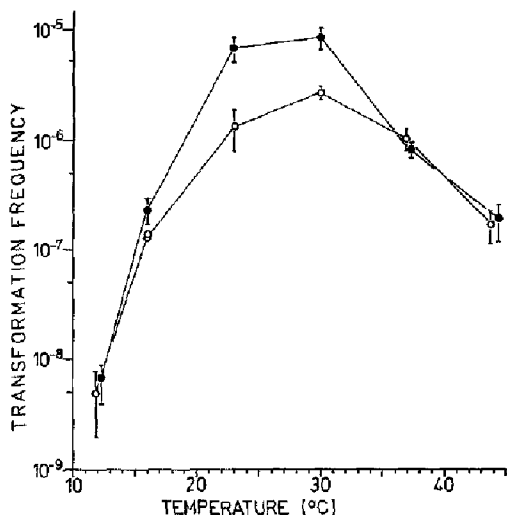

Fig. 4. Influence of temperature on transformation in liquid culture (●) and in sand (○). *Bars* indicate the standard deviation of the mean ($n \geq 3$). Transformation frequency is defined as His^+ transformants/viable cell $\times$ 0.13 μg DNA (per 0.7 g sand or 0.2 ml culture)

of a competent culture. The data (Table 2) show that *P. stutzeri* is transformed by sand-adsorbed DNA and that this transformation occurs at various temperatures. In one experiment (Table 2, 37°C) the amount of DNA remaining on sand after treatment with the supernatant of the competent culture was determined by transformation. A competent culture applied to the column gave 55% of the transformation frequency obtained in a parallel column without supernatant treatment. This indicates that the supernatant treatment eliminates only a minor fraction of transforming activity. Transformation on sand and in liquid culture was observed between 16° and 44°C (Fig. 4). In the optimum range (23° to 30°C) transformation on sand was slightly less efficient than in liquid culture.

With *B. subtilis* transformation on sand was much more resistant to DNaseI than in liquid culture, possibly because of a shielding effect of cells at the mineral surface (Lorenz et al. 1988). With *P. stutzeri* DNaseI concentrations higher than 50 ng/ml of transformation mixture eliminated transformation both on sand and in liquid culture. A DNaseI concentration of 10 ng/ml reduced transformation to 11% in liquid culture and to 9% on sand compared to parallel transformations without DNaseI. The DNaseI sensitivity of transformation equal in liquid medium and on sand is consistent with the observation that *P. stutzeri* does not adhere to DNA-sand complexes so that no shielding is possible.

Discussion

The fact that duplex linear DNA adsorbs to sand minerals present in bacterial habitats such as soil and sediment raises the question as to whether adsorbed DNA has the option of returning into bacterial cells by transformation. Recently, a gram-positive soil bacterium with natural competence, *Bacillus subtilis*, was shown to take up DNA adsorbed to sand (Lorenz et al. 1988).

A similar examination with a gram-negative soil bacterium, *Pseudomonas stutzeri*, presented here required the improvement of a published transformation protocol (Carlson et al. 1983) allowing reproducible quantitative assays within a limited time period for cell-DNA interactions. The improved transformation procedure employed cells from a frozen competent culture. These cells were similar to fresh cells with respect to transformation efficiency, level of DNA saturation and weak inhibition of transformation by heterologous DNA. Kinetic experiments showed that the rate of *P. stutzeri* transformation is limited by the slow adsorption of DNA to competent cells (Table 1). The reason for slow adsorption may be repulsion of negatively charged DNA from the negatively charged cell surface. However, the presence of monovalent and divalent cations or polyamines at concentrations that should reduce repulsive forces between negatively charged molecules and surfaces did not increase the number of transformants. It is possible that the high motility of the cells hindered effective binding of DNA to transformation-related components of the cell envelope.

The main result of this work is the observation that *P. stutzeri* took up sand-adsorbed DNA (Table 2; Fig. 4). The efficiency of transformation by sand-adsorbed DNA and by dissolved DNA was similar over the range of temperatures tested (Fig. 4). Furthermore, transformation was sensitive to low concentrations of DNaseI,

irrespective of the DNA being either adsorbed to sand or dissolved. In contrast, transformation of *B. subtilis* on sand was much more efficient and DNaseI-resistant (Lorenz et al. 1988) and proceeded at much lower temperatures than transformation in liquid (Lorenz and Wackernagel 1988). The absence of increased transformation efficiency of *P. stutzeri* on sand can be explained by the slow adsorption of DNA to the cells and by the inefficient binding of the cells to minerals (without or with DNA).

The results obtained so far have ecological significance for a possible gene transfer in *P. stutzeri* in natural environments: transformation is hardly disturbed by heterologous DNA and proceeds over a wide range of temperatures. In addition, it has been shown recently that transformation of *P. stutzeri* is stimulated by nutrient limitation (Lorenz and Wackernagel, manuscript in preparation) a condition that is presumably frequently encountered by bacteria in natural habitats. Uptake of mineral-adsorbed DNA may actually facilitate gene transfer in nature. DNA efficiently adsorbs to mineral surfaces and thereby is protected from enzymatic degradation (Lorenz and Wackernagel 1987). We assume that mineral surfaces are sites in natural environments where DNA accumulates. The studies presented here with *P. stutzeri* as well as previous observations with *B. subtilis* (Lorenz et al. 1988) suggest that DNA representing the bacterial extracellular gene pool remains available for transformation when the DNA is adsorbed to mineral particles. Gene transfer by natural transformation should be considered in the risk assessment for the release of genetically engineered microorganisms to the environment.

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