Transformation is a Mechanism of Gene Transfer in Soil

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토양에서 Transformation에 의한 유전자 전이

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ABSTRACT: The survival and transfer of chromosomal genes coding for the synthesis of amino acids (threonine, tryptophan, histidine, leucine, methionine) and of plasmid-borne genes coding for resistance to antibiotics (chloramphenicol, kanamycin, erythromycin) by transformation in sterile and nonsterile soil (the soil was amended to 12% vol/vol with the clay mineral, montmorillonite) was studied. In pure culture, the numbers of vegetative cells of the *Bacillus subtilis* strains decreased by 1 to 1.5 orders of magnitude within one week, but spores of each strain showed lesser decreases. In sterile soil, the populations of vegetative cells and spores decreased by 1.5 to 3 orders of magnitude within 2 to 4 days and then showed little additional decreased. The transformation frequencies (number of transformants/numbers of donors and recipients) of individual amino acid-genes *in vitro* ranged from $1.3 \pm 0.6 \times 10^{-6}$ to $6.0 \pm 2.3 \times 10^{-6}$, of two amino acid-genes from $8.5 \pm 0.7 \times 10^{-8}$ to $3.1 \pm 0.6 \times 10^{-7}$, and of the antibiotic-resistance genes from $1.5 \pm 0.2 \times 10^{-7}$ to $1.4 \pm 0.4 \times 10^{-5}$. In sterile soil, the frequencies of transfer of individual amino acid-genes ranged from 2.0×10^{-7} to 2.0×10^{-5} and of the antibiotic-resistance genes from 2.0×10^{-7} to $9.4 \pm 4.7 \times 10^{-6}$. The transfer of two amino acid-genes in sterile soil was detected at a frequency of 2.0×10^{-6} to 4.5×10^{-6}, but only in three instances. The transformation frequencies of antibiotic-resistance genes in nonsterile soil were essentially similar to those in sterile soil. However, to detect transformants in nonsterile soil, higher concentrations of antibiotics were needed, as the result of the large numbers of indigenous soil bacteria resistant to the concentration of antibiotics used in the sterile soil and *in vitro* studies. The results of these studies show that genes can be transferred by transformation in soil and that this mechanism of transfer must be considered in risk assessment of the release of genetically engineered microorganisms to the environment.

KEYWORDS □ Survival, transformation frequency, *Bacillus subtilis*, nonsterile soil, transformant.

Concern has been expressed about the potential risks associated with the release of genetically engineered microorganisms (GEMs) into natural environments (Curtiss, 1976; Rissler, 1984; Sharples, 1983), including the possible transrer of the novel genetic information to indigenous soil microbes. Transformation occurs in many bacteria, and it was the first mechanism of genetic exchange to be elucidated. However, there is little informaiton on the transfer of genetic information between bacteria by transformation in soil and other natural habitats, although such transfer has been demonstrated in a broad spectrum of gram-negative and gram-positive species in pure culture (Stewart and Carlson, 1986; Stotzky and Babich, 1986; Stotzky, 1989). The possible survival and uptake of transforming DNA in microcolonies within microhabitats, the adsorption of DNA on clay minerals and other surfaces, which may protect the DNA against degradation, the ability of some species to incorporate heterospecific as well as

homospecific DNA, and the routine use of transformation to incorporate genetic information, including engineered plasmids, into bacteria *in vitro* suggest that this mechanism of transfer could be important *in situ*.

Transformation in *Bacillus subtilis* in sterile soil was demonstrated by Graham and Istock (1978, 1979, 1981), and the frequency of transformation of *B. subtilis* by DNA in a simulated marine system was reduced by the presence of sea sand (Aardema *et al.*, 1983; Lorenz, 1986). However, calf thymus DNA adsorbed on this marine sediment was protected against degradation by DNase (Lorenz *et al.*, 1981). Other studies, however, has indicated that although DNA and RNA are adsorbed on clay minerals (Blanton and Barnett, 1969; Filip, 1973; Goring and Bartholomew, 1952; Greaves and Wilson, 1969, 1970, 1973; Ivarson *et al.*, 1982; Stotzky, 1986, 1989), they are still susceptible to microbial degradation (Greaves and Wilson, 1970). Soil is a structured environment with a high solid: water ratio, and microbes in soil are restricted to microhabitats that have a continual supply of available water, i.e., microhabitats that contain clay minerals. Consequently, the discontinuous nature of soil mitigates against the same frequencies of transformation that are obtained in a continuous aqueous system (e.g., in a flask, a lake, or ocean) or on an agar plate. This study examined the survival and transfer of chromosomal genes coding for the synthesis of amino acids (threonine, tryptophan, histidine, leucine, and methionine) and of plasmid-borne genes coding for resistance to antibiotics (chloramphenicol, kanamycin, and erythromycin) by transformation in sterile and nonsterile soil (the soil was amended to 12% vol/vol with the clay mineral, montmorillonite).

MATERIALS AND METHODS

Bacterial strains

The strains of *Bacillus subtilis* used are listed in Table 1, along with some of their characteristics. Bacterial stocks were maintained at room temperature and transferred every two weeks to slants of Tryptose Blood Agar Base (TBAB, Difco) (1% Bacto-tryptose, 0.3% Bacto-beef extract, 0.5% sodium chloride, and 1.5% Bacto-agar) supplemented with the appropriate antibiotics (100 μg/m*l*) and 5 m*l* of 1 M $MgSO_4 \cdot 7H_2O$ and 0.2 m*l* of 0.1 M $MnCl_2 \cdot 4H_2O$ per liter (Anagnostopoulos and Spizizen, 1961). The phenotype (i.e., antibiotics resistance and auxotrophy) of the strains was checked regularly.

Table 1. *Some characteristics of the* ***Bacillus subtilis*** *strains.*

Strain	Genotype or relevant characteristics*	Plasmid
BD431	trp^- thr^-	pE194 (erythromycin-resistance)
BD364	trp^- thr^-	pC194 (chloramphenicol-resistance)
BD366	trp^- thr^-	pUB110 (kanamycin-resistance)
BD170	trp^- thr^-	
BD1512	his^- leu^- met^- chl^r	
BD54	ile^- leu^- met^-	

*trp: tryptophan; thr: threonine; his: histidine; leu: leucine; met: methionine; ile: isoleucine; chl: chloramphenicol.

Soil

Soil from the Kitchawan Research Laboratory of the Brooklyn Botanic Garden, Ossining, New York, was used in these studies. The soil was amended with 12% (vol/vol) montmorillonite (Volclay, Panther Creek-Aberdeen, American Colloid Co.). The physicochemical characteristics of this soil-clay mixture have been described (Babich and Stotzky, 1977). The soil was sieved (1-mm screen), adjusted to its -33 kPa water potential, and 2-g aliquots were placed into screw-cap test tubes, which were equilibrated for 24 h at 4 °C. When the soil was sterilized, additional water was added to compensate for that lost during autoclaving (30 min at 121 °C), and the sterile soils were cured of toxic materials produced during autoclaving by incubation for one week in a high humidity chamber before inoculation. After inoculation, the soils were incubated in a high humidty chamber maintained under fluctuating temperatures (23 to 27 °C) to prevent desiccation (Stotzky, 1973).

Measurement of bacterial survival

Cultures were grown overnight (18 h) at 37 °C in 25 m*l* of Spizizen Salts (SS) (0.6% KH_2PO_4, 1.4% K_2HPO_4, 0.2% $(NH_4)_2SO_4$, and 0.1% sodium citrate) (Spizizen, 1958) supplemented with the necessary amino acids (10 μg/m*l*), appropriate antibiotics (10 μg/m*l*), and 1 m*l* of 50% glucose and 0.25 m*l* of 1 M $MgCl_2$ per 100 m*l* of SS. The glucose was autoclaved separately and added aseptically, and the amino acids and antibiotics were sterilized by filtration (0.2-μm filter; gelman.).

To determine the survival of each *Bacillus* strain

as both vegetative cells and spores *in vitro*, the cultures were incubated in appropriately supplemented SS for 14 days in a water bath shaker at 25 °C and periodically enumerated by plating on the appropriate media. TBAB was used for the enumeration of the initial populations of BD54 and BD170, and strains containing plasmids that coded for antibiotic resistance were enumerated on TBAB containing 10 μg/m*l* chloramphenicol (BD364, BD1512), kanamycin (BD366), or erythromycin (BD431). To determine the number of spores present, a 1-m*l* aliquot of each culture was added to 9 m*l* of 0.9% NaCl containing 10 mM phosphate buffer (pH 7.2), heated at 80 °C for 10 min, and plated on the appropriate media.

To determine the survival of each strain in soil, 0.2 m*l* of an overnight culture, prepared as for the *in vitro* studies, was inoculated into test tubes containing 2g soil at the -33 kPa water tension. The soil samples were maintained at their -33 kPa water tension with sterile distilled water (sdH_2O) on the basis of periodic weight measurements. The soil tubes were incubated at 25 ± 2 °C. At each sampling time, duplicate soil tubes were sacrificed, 18 m*l* sdH_2O was added to each soil tube, serial decade dilutions were made with sterile saline, and the numbers of vegetative cells and spores were enumerated on the appropriate media.

The experiments were conducted at least twice. The means ± the standard error of the means (x ± SEM) were calculated for each sampling time and expressed as $\log_{10}$ colony-forming units (CFU)/g oven-dried soil. In some instances, transformation was detected in only one of the duplicate tubes and in only one experiment, and therefore, it was not possible to calculate the SEM.

***In vitro* transformation**

Strains were grown individually overnight to a density of approximately 10^8 to 10^9 CFU/m*l* in 125-m*l* Erlenmeyer flasks containing 25 m*l* of supplemented SS at 37 °C in a water bath shaker (120 rpm). The cells of each strain were harvested (10 min at 15,000g) and washed twice in 10 m*l* of saline containing 0.01% Nutrient Browth (NB). The cells were resuspended in 3 m*l* of SS. One milliliter of aliquots were taken from each suspension and mixed with 4 m*l* of SS. The resulting 5-m*l* aliquots of each strain were mixed in 125-m*l* Erlenmeyer flasks and incubated at 25 °C in a shaking water bath, and transformants were counted periodically on the appropriate selective media (Table 2). When used, antibiotics were added to each medium individually to a concentration of 10 μg/m*l*. The frequency of transformation was calculated as follows:

Frequency of transformation = No. of transformants/No. of donors + No. of recipients added initially.

Table 2. *Media used to study transformation of amino acid- and antibiotic resistance-genes.*

Amino acid media*	Antibiotic media
MA	MA + met + ile + leu + chloramphenicol
MA + trp	MA + met + leu + kanamycin
MA + thr	MA + met + ile + leu + erythromycin
MA + chl	
MA + leu + chl	
MA + met + chl	
MA + his + chl	
MA + chl	
MA + leu + chl	
MA + met + chl	
MA + his + chl	
MA + leu + met + chl	
MA + leu + his + chl	
MA + met + his + chl	

*MA:minimal agar; see Table 1 for other abbreviations.

Transformation in sterile and nonsterile soil

Each strain was grown overnight to a density of approximately 10^8 to 10^9 CFU/m*l* in 125-m*l* Erlenmeyer flasks, and 0.1 m*l* was inoculated, without washing, into each soil tube. After inoculation, the tubes were incubated in a humidified incubator at 25 °C. Duplicate soil tubes were periodically sacrificed, diluted with 18 m*l* sdH_2O, and serial decimal dilutions of this initial 1:10 dilution were made with sterile saline (Devanas *et al.*, 1986) and plated on appropriate selective media, incubated at 37 °C for 1 to 2 days, and the results scored.

For transformation studies in nonsterile soil, the auxotrophic amino-acid markers could not be used as selectors as in sterile soil, as the result of the indigenous soil microbiota, and only antibiotic-resistance was used as markers. However, the auxotrophic markers were used to confirm the transformation of antibiotic-resistance genes. Moreover, 200 μg/m*l* of cycloheximide was added to the selective media to inhibit the growth of fungi.

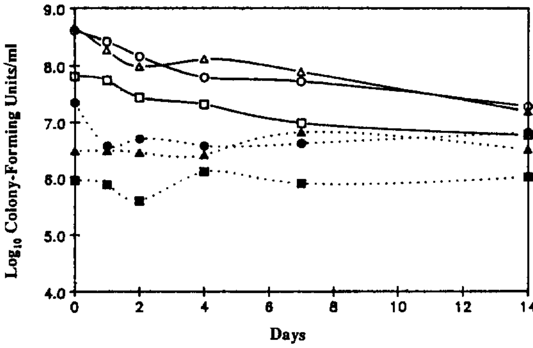

Fig. 1a. *Survival of Bacillus subtilis strains in vitro.* (o——o vegetative cells of BD364, •------• Spores of BD364, Δ——Δ Vegetative cells of BD366, ▲------▲ Spores of BD366, □——□ Vegetative cells of BD431, ■------■ Spores of BD431).

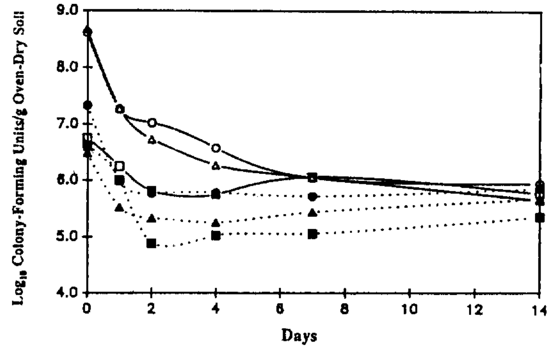

Fig. 2a. *Survival of Bacillus subtilis strains in sterile soil.* (o——o vegetative cells of BD364, •------• Spores of BD364, Δ——Δ Vegetative cells of BD366, ▲------▲ Spores of BD366, □——□ Vegetative cells of BD431, ■------■ Spores of BD431).

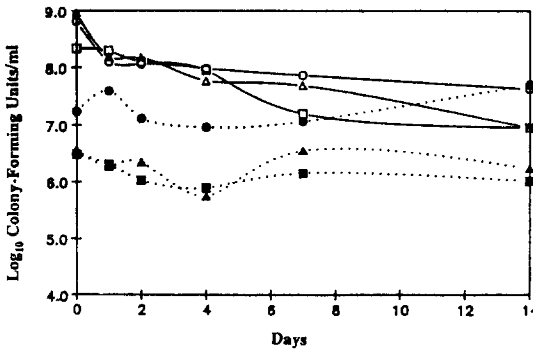

Fig. 1b. *Survival of Bacillus subtilis strains in vitro.* (o——o Vegetative cells of BD54, •------• Spores of BD54, Δ——Δ Vegetative cells of BD170, ▲------▲ Spores of BD170, □——□ Vegetative cells of BD1512, ■------■ Spores of BD1512).

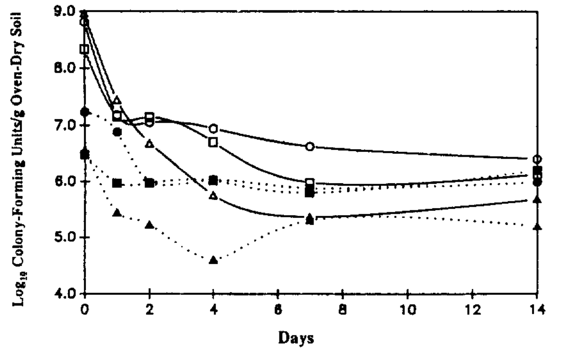

Fig. 2b. *Survival of Bacillus subtilis strains in sterile soil.* (o——o Vegetative cells of BD54, •------• Spores of BD54, Δ——Δ Vegetative cells of BD170, ▲------▲ Spores of BD170, □——□ Vegetative cells of BD1512, ■------■ Spores of BD1512).

RESULTS

The populations of vegetative cells of the *Bacillus* strains decreased by approximately by 1 to 1.5 orders of magnitude during a 14-day incubation *in vitro*. However, the number of spores of each strain decreased less, and in some strains (BD54, BD366, and BD431), there was no apparent decreased, although some fluctuations in numbers occurred (Figs. 1a and 1b). The numbers of spores were about 0.5 to 1.5 orders of magnitude lower than the numbers of vegetative cells throughout the experiment. When the strains were incubated in sterile soil, there was a greater decrease (1.5 to 3 orders of magnitude) in vegetative cells in the initial 2 to 6 days than *in vitro*, followed by either a slight decrease (BD54, BD364, BD366) or no further decrease (BD431, BD170, GD1512). The numbers of spores in sterile soil also decreased more within the first 4 days than *in vitro*, followed by essentially constant numbers (Figs. 2a and 2b). When pairs of *Bacillus* strains were incubated together in sterile soil, the numbers of vegetative cells of each strain decreased approximately 1.0 to 2.0 orders of magnitude within 2 to 4 days, but changes in the numbers of spores were similar to changes that occurred when the strains were incubated alone in sterile soil and *in vitro* (Fig. 3).

The transformation frequency (number of transformants/numbers of donors and recipients) during 7 days *in vitro* of individual amino acid-genes was $1.3 \pm 0.6 \times 10^{-6}$ for threonine (thr), $4.7 \pm 0.4 \times 10^{-6}$ for tryptophan (trp), $2.7 \pm 1.0 \times 10^{-6}$ for histidine (his), $3.7 \pm 0.6 \times 10^{-6}$ for methionine (met), and $6.0 \pm 2.3 \times 10^{-6}$ for leucine (leu). The transformation

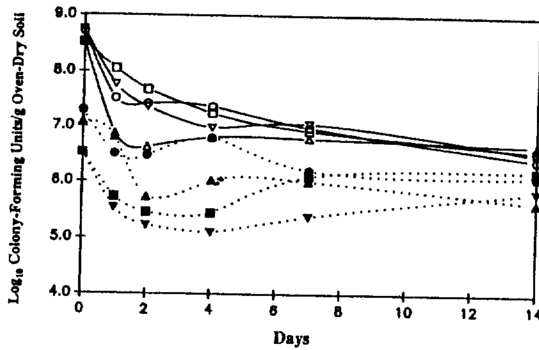

Fig. 3. *Survival of mixed Bacillus subtilis strains in sterile soil.*
(o——o Vegetative cells of BD54 & BD364, •······• Spores of BD54 & BD364, △——△ Vegetative cells of BD54 & BD366, ▲······▲ Spores of BD54 BD366, □——□ Vegetative cells of BD54 & BD431, ■······■ Spores of BD54 & BD431, ▽——▽ Vegetative cells of BD170 & BD1512, ▼······▼ Spores of BD170 & BD1512).

Table 3. *Transformation frequency of amino acid auxotrophs in vitro.*

Amino acid	Days 1	4	7
Threonine (Thr)	$1.3 \pm 0.6 \times 10^{-6}$	—	—
Tryptophan (Trp)	$4.7 \pm 0.4 \times 10^{-6}$	—	—
Histidine (His)	$2.7 \pm 1.0 \times 10^{-6}$	—	—
Methionine (Met)	$3.7 \pm 0.6 \times 10^{-6}$	—	—
Leucine (Lau)	$6.0 \pm 2.3 \times 10^{-6}$	—	—
Trp + Thr	$2.7 \pm 0.6 \times 10^{-6}$	—	—
His + Met	$2.0 \pm 0.9 \pm 10^{-6}$	$1.4 \pm 0.7 \times 10^{-7}$	—
His + Leu	$3.1 \pm 1.0 \times 10^{-6}$	$3.1 \pm 0.6 \times 10^{-7}$	—
Met + Leu	$8.5 \pm 0.7 \times 10^{-8}$	$2.9 \pm 0.2 \times 10^{-7}$	—

frequency of two amino acid-genes *in vitro* ranged from $1.4 \pm 0.9 \times 10^{-7}$ to $2.0 \pm 0.9 \times 10^{-6}$ for his + met, from $3.1 \pm 0.6 \times 10^{-7}$ to $3.1 \pm 1.0 \times 10^{-6}$ for his + leu, from $8.5 \pm 0.7 \times 10^{-8}$ to $2.9 \pm 0.2 \times 10^{-7}$ for met + leu, and $2.7 \pm 0.6 \times 10^{-6}$ for trp + thr (Table 3). The transfer of individual amino acid-genes was detected only after the first day of incubation. However, transformants of two amino acid-genes were detected on both days 1 and 4, although the frequencies were lower, with the exception of met + leu, on day 4.

The transformation frequency of antibiotic resistance-genes *in vitro* ranged from $2.7 \pm 0.2 \times 10^{-7}$ to $8.3 \pm 1.2 \times 10^{-7}$ (donor BD364, recipient BD54) and from 1.3×10^{-6} to $5.3 \pm 1.2 \times 10^{-6}$ (donor BD1512, recipient BD170) for chloramphenicol, $1.5 \pm 0.2 \times 10^{-7}$ to $1.5 \pm 0.5 \times 10^{-6}$ for kanamycin, and $4.1 \pm 0.6 \times 10^{-7}$ to $1.4 \pm 0.4 \times 10^{-5}$ for erythromycin (Table 4). The transformation of chloramphenicol-resistance of BD54 from DB364, kanamycin-resistance to BD54 from BD366, and erythromycin-resistance to BD54 from BD431 was detected during 7 days. The transformation frequencies of the genes for chloramphenicol-resistance (from BD364), kanamycin-resistance, and erythromycin-resistance increased during the incubation and were highest after 2 or 4 days and then decreased. In contrast, the transformation of the chromosomal chloramphenicol-resistance gene from BD1512 to BD170 was detected only on days 1 and 2.

In sterile soil, the transformation frequencies during 7 days of individual amino acid-genes ranged from $1.6 \pm 0.6 \times 10^{-6}$ to $4.2 \pm 1.4 \times 10^{-6}$ for thr, from $1.1 \pm 0.9 \times 10^{-6}$ to 2.0×10^{-6} for trp, from $3.6 \pm 1.7 \times 10^{-6}$ to $5.5 \pm 2.2 \times 10^{-6}$ for his, and from 2.0×10^{-7} to 2.0×10^{-5} for leu (Table 5). No transformation of the gene for met was detected in sterile soil. The transfer of two amino acid-genes in sterile soil was detected at a frequency of 4.5×10^{-6} for trp + thr, 2.0×10^{-6} for his + met, and 4.0×10^{-6} for met + leu, but each transfer was detected only on one of the 4 days tested, and no transfer of his + leu was detected. The transformation of antibiotic resistance-genes ranged from 4.7×10^{-7} to $9.4 \pm 4.7 \times 10^{-6}$ (to

Table 4. *Transformation frequency of plasmid-borne antibiotics resistance in vitro.*

Antibiotic resistance	Days 1	2	4	7
Chloramphenicol[1]	$4.7 \pm 0.7 \times 10^{-7}$	$8.2 \pm 2.0 \times 10^{-7}$	$8.3 \pm 1.2 \times 10^{-7}$	$2.7 \pm 0.2 \times 10^{-7}$
Chloramphenicol[2]	$5.3 \pm 1.2 \times 10^{-6}$	1.3×10^{-6}	—	—
Kanamycin	$1.5 \pm 0.2 \times 10^{-7}$	$1.2 \pm 0.6 \times 10^{-6}$	$1.5 \pm 0.5 \times 10^{-6}$	$9.5 \pm 3.5 \times 10^{-7}$
Erythromycin	$4.1 \pm 0.6 \times 10^{-7}$	$1.4 \pm 0.4 \times 10^{-5}$	$1.3 \pm 0.1 \times 10^{-5}$	$5.6 \pm 1.8 \times 10^{-6}$

[1] transformants of BD54 (recipient) and BD364 (chl^r, donor).
[2] transformants of BD170 (recipient) and BD1512 (chl^r, donor).

Table 5. *Transformantion frequency of amino acid auxotrophs in sterile soil.*

Amino acid	Days			
	1	2	4	7
Threonine (Thr)	$4.2 \pm 1.4 \times 10^{-6}$	$1.6 \pm 0.6 \times 10^{-6}$	—	2.0×10^{-6}
Tryptophan (Trp)	$1.1 \pm 0.9 \times 10^{-6}$	$1.4 \pm 0.4 \times 10^{-6}$	2.0×10^{-6}	—
Histidine (His)	$3.6 \pm 1.7 \times 10^{-6}$	$4.5 \pm 1.4 \times 10^{-6}$	$5.5 \pm 2.2 \times 10^{-6}$	4.0×10^{-6}
Leucine (Leu)	2.0×10^{-7}	$4.3 \pm 2.2 \times 10^{-6}$	$1.3 \pm 0.1 \times 10^{-5}$	2.0×10^{-5}
Methionine (Met)	—	—	—	—
Trp + Thr	4.5×10^{-6}	—	—	—
His + Met	—	—	—	2.0×10^{-6}
His + Leu	—	—	—	—
Met + Leu	—	2.0×10^{-6}	—	—

Table 6. *Transformation frequency of plasmid-borne antibiotic resistance in sterile soil.*

Antibiotic resistance	Days			
	1	2	4	7
Chloramphenicol[1]	2.8×10^{-7}	$9.4 \pm 4.7 \times 10^{-6}$	4.7×10^{-7}	9.4×10^{-7}
Chloramphenicol[2]	2.0×10^{-7}	$8.7 \pm 0.7 \times 10^{-7}$	2.0×10^{-7}	8.0×10^{-7}
Kanamycin	6.0×10^{-6}	$1.6 \pm 0.7 \times 10^{-6}$	$1.8 \pm 0.6 \times 10^{-6}$	1.8×10^{-6}
Erythromycin	6.4×10^{-7}	—	—	—

[1]transformants of BD54(recipient) and BD364 (chl^r, donor).
[2]transformants of BD170(recipient) and BD1512 (chl^r, donor).

Table 7. *Transformation frequency of plasmid-borne antibiotic resistance in nonsterile soil.*

Antibiotic resistance	Days			
	1	2	4	7
Chloramphenicol[1]	—	—	—	—
Chloramphenicol[2]	—	—	—	—
Kanamycin	7.0×10^{-7}	$1.9 \pm 0.4 \times 10^{-6}$	7.0×10^{-7}	—
Erythromycin	$3.4 \pm 0.4 \times 10^{-6}$	$8.2 \pm 2.2 \times 10^{-6}$	$3.1 \pm 0.4 \times 10^{-6}$	—

[1]transformants ob BD54 (recipient) and BD364 (chl^r, donor).
[2]transformants of BD170 (recipient) and BD1512 (chl^r, donor).

BD54 from BD364) and from 2.0×10^{-7} to $8.7 \pm 0.7 \times 10^{-7}$ (to BD170 from BD1512) for chloramphenicol, from $1.6 \pm 0.7 \times 10^{-6}$ to 6.0×10^{-6} for kanamycin, and 6.4×10^{-7} for erythromycin (Table 6).

Transformants of both chromosomal and plasmid-borne chloramphenicol-resistance and of plasmid-borne kanamycin-resistance was detected for 7 days in sterile soil. the transformation frequency of chloramphenicol-resistance, whether chromosomal or plasmid-borne, was greatest on day 2 and of kanamycin-resistance on days 1. Transformation of erythromycin-resistance was detected only on day 1.

In nonsterile soil, the transformation frequency of kanamycin-resistance ranged from 7.0×10^{-7} to $1.9 \pm 0.4 \times 10^{-6}$ and from $3.1 \pm 0.4 \times 10^{-6}$ to $8.2 \pm 2.2 \times 10^{-6}$ for erythromycin-resistance. These transformants were detected only during the first 4 days, and no chloramphenicol-resistant transformants were detected (Table 7). The transformation frequencies for kanamycin- and erythromycin-resistance in nonsterile soil were essentially similar to those in sterle soil. However, the detection of transformants in nonsterile soil was more difficult than in sterile soil, as the result of the large numbers of indigenous soil bacteria that were resistant to the concentrations of antibiotics used initially in the selective media. Indigenous soil bacteria and fungi were de-

tected in negative controls (i.e., nonsterile soil to which no *Bacillus* strains had been added) when low concentrations of antibiotics (10 μg/m*l*) were added to the media. However, when 100 μg/m*l* of chloramphenicol, 200 μg/m*l* of erythromycin, and 300 μg/m*l* of kanamycin were added, no soil bacteria were detected. The addition of 200 μg/m*l* of cycloheximide inhibited the growth of fungi on the selective media.

The transformation frequencies detected *in vitro* or in soil were the result of transformation and not of reversions or mutations, as no growth was obtained on the selective media when *in vitro* or soil systems inoculated with only the recipient strain were plated.

DISCUSSION

Several reviews of the literature on the survival of GEMs and gene transfer among bacteria in natural environments have been published (Betz *et al.*, 1983; Devanas *et al.*, 1986; Reanney *et al.*, 1983; Stewart, 1989; Stotzky, 1989; Stotzky and Babich, 1985, 1986; Stotzky and Krasovsky, 1981; Trevors *et al.*, 1987). Studies on the survival of plasmid-containing strains of *E. coli* in soil have provided some indications of survival of GEMs in natural habitats (Devanas *et al.*, 1986). In general, the survival was significantly greater in sterile than in nonsterile soil, and the relative survival in soil of *E. coli* cells that retained the plasmid and of cells that apparently lost the plasmid did not differ significantly. In the present studies, the survival of *Bacillus* strains in sterile soil was usually lower than *in vitro*, but there was no significant difference is survival between plasmid-containing and plasmidless strains. During the first 1 to 4 days, the numbers of *Bacillus* decreased by 1.5 to 3 orders of magnitude in soil, but thereafter, all strains showed a rate of decrease that was slower and similar to that *in vitro*. The more rapid and greater decline in *Bacillus* populations in sterile soil than *in vitro* was probably the result, in part, of the starvation of laboratory-cultured cells in the nutrient-limited soil.

The steps involved in the uptake of DNA during transformation of *B. subtilis* have been elucidated (Dubnau and Cirigliano, 1972; Levine and Strauss, 1965; Morrison, 1971; Young and Spizizen, 1961, 1963), and a common mechanism for early events in transformation and transfection has been postulated for *B. subtilis* (Spizizen *et al.*, 1966). When recipient cells acquire the ability to take up DNA (i.e., become competent), transformation results. Competence is the property of transformable bacteria that renders them capable of binding and taking up DNA in a form resistant to exogenous DNase (Venema, 1979). Competence is induced only when the culture reaches a density of about 10^8 cells/m*l*, because an effective concentrations of a competence factor must accumulate (Stewart, 1989; Stewart and Carlson, 1986). In soil systems, the competence factor may not accumulate or, if it does, it may bind on soil particulates (e.g., clays) and not attain an effective concentration on or near all cells to make them competent.

The transformation of individual amino acid-genes was detected *in vitro* only on day 1, and there were some differences in transformation frequencies between the individual amino acid-genes. This may have been the result of the probability of penetration of different segments of DNA (e.g., the indole locus may enter a cell at a later time than other gens) (Young and Spizizen, 1963). The transformation frequencies of two amino acid-genes was probably a function of the distance between the genes on the donor chromosome. The distance between the loci of his and leu is shorter than between the other two gene loci (i.e., between his and met or between met and leu) (Hener and Hoch, 1980; Piggot and Hoch, 1985), and the transformation frequency of his + leu was somewhat higher than of the other combinations. However, on day 4, The transformation frequency was similar for all combinations.

In sterile soil, transformation of individual amino acid-genes were detected for 7 days after introduction. This higher frequency in soil than *in vitro* may have been the result of the physicochemical character of the soil environment, which may have protected the transforming DNA from degradation by DNase (Van Elsas *et al.*, 1987). However, more research is needed to elucidate why some transformants were detected in soil for only a few days and others longer.

The transformation of plasmid-borne kanamycin and chloramphenicol-resistance *in vitro* and in sterile soil was detected during 7 days, whereas transformation of chromosomal chloramphenicol-resistance was detected for only 2 days *in vitro* but for 7 days in sterile soil. The transformation of plasmid-borne erythromycin-resistance was detected for 7 days *in vitro* but for only 1 day in sterile soil. In nonsterile soil, kanamycin- and erythromycin-resistant transformants were detected for 4 days, but no chloramphenicol-resistant transformants were detected, even on day 1. It is not knwon why there was no transfer of chloramphenicol-resistance in nonsterile soil and why the apparent transfer of erythromycin-resistance was higher in nonsterile than in sterile soil. More

research is needed to elucidate these aspects and to determine whether transformation occurs only immediately after inoculation of the donors and recipients (i.e., within 24 h) or whether it occurs throughout the incubation.

The results of this study show that bacteria introduced into soil can persist in relatively high numbers for extended periods and can transfer genes, both chromosomal and plasmid-borne, in this environment by transformation. Consequently, the potential for the transformation of novel genes from GEMs to indigenous bacteria exists and should be considered in risk assessment of the release of GEMs to the environment.

적 요

*Bacillus subtilis*를 재료로 하여 아미노산 합성에 관련된 유전자와 항생제 내성에 관련된 유전자를 갖는 균주들 간의 토양환경에서의 생존기간과 유전자전이 빈도를 측정하였다. 손수배양시 각 균주의 vegetative cell의 수는 일주일 내에 $10^{-1}-10^{-1.5}$배로 감소하였으나 각 포자의 수는 이 보다 적게 감소하였다. 멸균된 토양에서는 각 균주의 vegetative cell과 포자의 감소는 2-4일 이내에 최초 접종한 균체수의 $10^{-1.5}-10^{-3}$배 수준까지는 감소하였으나 그 이후로는 뚜렷한 감소 경향을 나타내지 않았다. *In vitro*에서 각 아미노산 합성에 관련된 두 개의 아미노산에 관련된 유전자들의 transformation frequency(형질전환빈도)는 각각 $1.3\pm0.6\times10^{-6}-6.0\pm2.3\times10^{-6}$, $8.5\pm0.7\times10^{-8}-3.1\pm0.6\times10^{-7}$의 범주에서 변화하였으며, 항생제 내성에 관련된 유전자들의 형질전환 빈도는 $1.5\pm0.2\times10^{-7}-1.4\pm0.4\times10^{-5}$ 범주에서 변화하였다. 멸균된 토양에서는 각 아미노산 합성에 관련된 유전자들과 항생제 내성에 관련된 유전자들의 형질전환 빈도는 각각 $2.0\times10^{-7}-2.0\times10^{-5}$,$2.0\times10^{-7}-9.4\pm4.7\times10^{-6}$이었다. 한편 두 개의 아미노산에 관련된 유전자들의 형질전환 빈도는 $2.0\times10^{-6}-4.5\times10^{-6}$의 범주에서 측정되었다. 멸균되지 않는 토양에서의 항생제 내성에 관련된 유전자들의 형질변환 빈도는 멸균된 토양에서와 유사하였다.

이상의 결과로 토양에서의 유전자 전이는 transformation에 의해 이루어질 수 있음을 보여준다.

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