Symbiosis, 25 (1998) 311–322
Balaban, Philadelphia/Rehovot

Interspecific Transformation of *Bacillus subtilis* by Clay-Bound DNA in Non-Sterile Soil

E. GALLORI*, M. FRANCHI, L. RINALDI, and C. VETTORI
Department of Animal Biology and Genetics, University of Florence, via Romana 17, 50125 Florence, Italy. Tel. +39-55-2288241, Fax. +39-55-222565, -2288250, E-mail. gallori@dbag.unifi.it

Received April 13, 1997; Accepted October 9, 1997

Abstract

The interspecific transformation of *Bacillus subtilis* by both free and clay (montmorillonite)-bound DNA, isolated from *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. polymyxa*, *B. pumilus* and *B. thuringiensis*, and from intergenotes obtained in the heterologous exchanges, was investigated in non-sterile soil conditions. Transformation by free heterologous DNA from all the species occurred at a very low frequency, with the exception of DNA from *B. amyloliquefaciens*. Intergenotic DNA was capable of transforming with a higher efficiency than heterologous DNA, similar to that of the homologous DNA. Heterologous transformation by clay-DNA complexes was obtained only with DNA from *B. amyloliquefaciens*, while intergenotic DNA bound on clay transformed at a frequency about one-two orders of magnitude lower than homologous DNA. Transformation frequency in non-sterile soil was similar to that obtained in the absence of non-sterile soil. The genetic distances among the *Bacillus* species and the intergenotes were determined by the random amplified polymorphic DNA (RAPD) fingerprinting technique. The results show that *B. subtilis* is very similar to the intergenotes and *B. amyloliquefaciens* and more distant from the other *Bacillus* species.

Keywords: Heterologous transformation, *Bacillus* genus, clay-DNA complex, non-sterile soil

Presented at the Second International Congress of Symbiosis, April 13–18, 1997, Woods Hole, MA

*The author to whom correspondence should be sent.

0334-5114/98/$05.50 ©1998 Balaban

1. Introduction

Genetic and biochemical studies carried out in recent years on the population structure of soil bacteria have revealed the existence of a panmictic (sexual) structure of genes and genome, which suggests the occurrence of horizontal gene exchange between bacteria in the natural habitat (Maynard Smith et al., 1991; Istock et al., 1992; Smith et al., 1992; Maynard Smith et al., 1993; Roberts and Cohan, 1993; Duncan et al., 1994). One of the three mechanisms of bacterial gene transfer, transformation has recently gained greater credit thanks to the observations that most sexual populations are naturally transformable (Stewart, 1992) and that transforming DNA can persist for long periods in natural habitats as a result of its interaction with soil components (sand and clay particles) which appear to protect DNA against nuclease degradation (Lorenz and Wackernagel, 1987; Romanowski et al., 1991, Khanna and Stotzky, 1992; Paget et al., 1992; Chamier et al., 1993; Gallori et al., 1994). Several studies based on soil bacteria belonging to *Bacillus* spp. and *Pseudomonas* spp., both in sterile and non-sterile soil-mimic conditions ("microcosms"), have suggested that transformation can take place in this habitat (for review see Lorenz and Wakernagel, 1994). Most of these studies were carried out with homologous DNA. Limited information is available on interspecific transformation (Duncan et al., 1989), even though laboratory studies have established that genetic exchange between species can occur (Wilson and Young, 1972; Harford and Mergeay, 1973; Harris-Warrick and Lederberg, 1978; te Riele and Venema, 1982; Cohan et al., 1991; Roberts and Cohan, 1993). The purpose of this work was to investigate the occurrence and frequency of interspecific transformation within the genus *Bacillus* by the use of chromosomal DNA, either free or complexed with clay mineral montmorillonite (M), isolated from six closely related *Bacillus* species: *B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. polymyxa*, *B. pumilus* and *B. thuringiensis* (Priest, 1993).

2. Materials and Methods

Bacterial strains and growth conditions

The strains of *Bacillus* used in these experiments are reported in Table 1. Bacteria were grown in Antibiotic medium No. 3 (Assay Broth, Oxoid) supplemented with the appropriate antibiotics (10 μg ml^{-1}) or in minimal medium (MM) (Davis and Mingioli, 1950) containing the required amino acids at a final concentration of 25 μg ml^{-1}. The strains were maintained on slants of Nutrient Agar (NA, 1.5%, w/v; Difco) supplemented with antibiotics (10 μg

Table 1. Strains of *Bacillus* used.

Strain	Genotype	Source
B. subtilis BD1512	*his*A1 *met*B5 *leu*A8 Cm^r	D. Dubnau
B. subtilis BD170	*thr*-5 *trp*C2	D. Dubnau
B. amyloliquefaciens PB1824	prototroph	A. Galizzi
B. cereus 6A2	prototroph	BGSC
B. licheniformis 5A2	prototroph	BGSC
B. polymyxa PB1829	prototroph	A. Galizzi
B. pumilus PB1822	prototroph	A. Galizzi
B. thuringiensis 4A2	prototroph	BGSC
B. subtilis FB310	*met*B5 *leu*A8 Cm^r	(BD1512) × [PB1824] [a]
B. subtilis FB311	*his*A1 *met*B5 Cm^r	(BD1512) × [PB1824] [a]
B. subtilis FB314	*met*B5 *leu*A8 Cm^r	(BD1512) × [6A2] [a]
B. subtilis FB315	*met*B5 *leu*A8 Cm^r	(BD1512) × [5A2] [a]
B. subtilis FB316	*met*B5 *leu*A8 Cm^r	(BD1512) × [PB1829] [a]
B. subtilis FB317	*met*B5 *leu*A8 Cm^r	(BD1512) × [PB1822] [a]
B. subtilis FB318	*met*B5 *leu*A8 Cm^r	(BD1512) × [4A2] [a]

[a]By transformation, general notation: (recipient cell) × [donor DNA].

ml^{-1}) and 10^{-5} $MnCl_2$ $4H_2O$. The phenotypes of the strains were verified regularly.

Preparation of competent recipient cells

Recipient cells of *B. subtilis* strain BD1512 were made competent by the method described by Khanna and Stotzky (1992).

Preparation of DNA

Chromosomal DNA from donor strains of *Bacillus* was prepared as described by Khanna and Stotzky (1992).

Preparation of homoionic clay

The <2-mm fraction of montmorillonite (Crook County, WY, USA), a 2:1 (Si:Al) swelling clay, was made homoionic to calcium (Ca), as previously described (Harter and Stotzky, 1971; Fusi et al., 1989).

Preparation of clay-DNA complexes

The clay-DNA complexes were prepared by reacting chromosomal DNA (50 μg) with 100 μl of a suspension of Ca-montmorillonite (M) (22 mg ml^{-1} ddH_2O) in 1 ml of DNA buffer (0.01 M Tris-HCl, 10^{-4} M EDTA, 4 mM NaCl, pH 7.5), as previously reported (Khanna and Stotzky, 1992; Gallori et al., 1994). The clay pellets containing tightly bound DNA were used for the transformation of competent cells.

Transformation in liquid culture

Transformation was performed by incubation of competent cells with saturating levels of chromosomal DNA (2 to 10 μg ml^{-1}), as described by Gallori et al. (1994). Transformants and total viable cells were plated on media consisting of MM agar, 0.5% glucose and 25 μg of the appropriate amino acids ml^{-1}. The transformation frequency was expressed as the number of His^+ or Leu^+ transformants/number of total viable cells.

Transformation by clay-bound DNA

The pellets of bound clay-DNA complexes (2.2 mg of montmorillonite containing 8 to 20 μg of chromosomal DNA) were resuspended with 1 ml of competent cells and rotated at 70 rpm at 37°C for 30 min. The cells were plated on the appropriate selective media, as described above. Transformants and total viable cells were enumerated, and transformation frequencies were calculated.

Transformation in non-sterile soil

One g of non-sterile soil, collected from the plant science farm at the Department of Soil Science of the University of Florence (Italy) (Gallori et al., 1994), was added to the pellets of bound clay-DNA complexes (see previous section) contained in centrifuge tubes (polystyrene tubes Ultra Clear™, Beckman, 13 × 51 mm) and then resuspended with 1 ml of competent cells. After incubation at 37°C for 30 min, serial 10-fold dilutions in saline were plated on appropriate selective media, containing cycloheximide (200 μg ml^{-1}, to inhibit the growth of fungi), for the enumeration of transformants and viable cells. Competent cells, not treated with clay-DNA complexes, were plated on the same media as controls. All plates were incubated at 37°C for 36 h.

RAPD conditions

Amplifications were performed with DNA extracted from *Bacillus* strains reported in Table 1; 10-mer random primers were used (Table 2). The reactions were carried out in a 25 µl volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 0.001% (w/v) gelatin, 200 µM of each dNTP, 6.4 µM of primer, 1 µl of template, and 0.625 U of AmpliTaq DNA polymerase (Perkin Elmer, USA). After incubation at 90°C for 90 sec and at 95°C for 90 sec, the reaction mixtures were cycled 45 times through the following temperature profile: 95°C for 30 sec (denaturation), 36°C for 1 min (annealing), and 75°C for 2 min (extension), followed by one extension step at 72°C for 10 min. A Perkin Elmer 9600 thermocycler was used.

Table 2. Oligonucleotides used as primers in the RAPD experiments.

Primer	Sequence	G+C (%)	Reference
1247	5' AAG AGC CCG T	60	Paffetti et al., 1996
1253	5' GGT TCC GCC C	70	Vettori et al., 1996
RF2	5' CGG CCC CTG T	80	Paffetti et al., 1996

Amplification products (10 µl per lane) were analyzed by gel electrophoresis on 2% (w/v) agarose gels (Boehringer, Mannheim, Germany) at 10 V cm^{-1} for 2 h in Tris-acetate-EDTA buffer containing 0.5 µg ml^{-1} (w/v) of ethidium bromide (Sambrook et al., 1989). The gels were photographed with a UVP gel scanner (GDS2000; Ultra Violet Product Ltd., Cambridge, UK).

RAPD pattern analysis

The RAPD patterns were analyzed to assess the genetic distance between different strains. We used the vector of presence and absence of RAPD markers (1 and 0 indicated that a band was present or not on a gel, respectively) for each strain to compute the genetic distance between each pair of strains. The measurement used was the Euclidean metric measurement (E) of Excoffier et al. (1992), defined by Huff et al. (1993) as follows:

$E = \varepsilon^2_{xy} = n\,(1 - 2\,n_{xy}/2\,n)$, where $2\,n_{xy}$ is the number of markers shared by two individuals and n is the total number of polymorphic sites. It was calculated

by combination of the RAPD markers from all the primers used. The relationships among the strains were represented by a dendrogram constructed with the E values and the neighbor-joining (NJ) method (Saitou and Nei, 1987).

Statistics

All experiments were performed in triplicate. Statistical analysis include standard errors of the means (±S.E.M.) and Student's t-test ($P<0.05$) is considered significant.

3. Results and Discussion

Heterologous and intergenotic transformation in liquid culture

Heterologous transformation (Table 3) of *B. subtilis* BD1512 (*his leu met* Cm^r) occurred with a different efficiency according to the donor strain and the selected marker. In crosses involving *B. amyloliquefaciens* as donor strain, the prototrophy to *his* and *leu* was obtained at a frequency about one order of magnitude lower than homologous transformation (Table 3). Transformation with DNA from the other *Bacillus* species (Table 3) occurred at a very low and constant frequency (about 10^{-7} for His^+), while no transformants were recovered for the *leu* marker (data not shown). Nevertheless, it was possible to introduce genes from all the *Bacillus* species into *B. subtilis* BD1512. Since the reversion rates in the experiments were less than 1 colony per 10^9 cells to the *his* marker and no colonies to the *leu* marker, the selection of revertants instead of heterologous transformants was unlikely.

To increase the low efficiency of heterologous transformation, mostly due to sequence non-homology near or within the transformed locus (Wilson and Young, 1972; Harris-Warrick and Lederberg, 1978), DNA from transformants of heterologous crosses, "intergenote" (Wilson and Young, 1972), was used as donor in the intergenotic transformation. Results obtained showed that intergenotic DNA from strains FB310, FB311, FB314, FB315, FB316, FB317 and FB318 (Table 1) was capable of transforming *B. subtilis* BD1512 with an efficiency significantly higher (by about two orders of magnitude) than heterologous transformation (Table 3). In particular, intergenotic DNA from *B. amyloliquefaciens*, strains FB310 and FB311, was even more efficient than homologous DNA for both *his* and *leu* markers (Table 3), as already found for the rifampin marker by Wilson and Young (1972).

Table 3. Interspecific transformation of *B. subtilis* BD1512 by chromosomal DNA bound on montmorillonite in non-sterile soil.

Donor	Selected marker	Transformation frequency		
		Liquid culture [a]	DNA-montmorillonite[b]	DNA-montmorillonite [b] + non-sterile soil
B. subtilis BD170	His$^+$	2.1×10^{-4}	7.8×10^{-5}	1.3×10^{-5}
	Leu$^+$	1.8×10^{-4}	5.2×10^{-6}	7.1×10^{-6}
B. amyloliquefaciens	His$^+$	1.8×10^{-5}	3.8×10^{-6}	8.4×10^{-7}
	Leu$^+$	6.6×10^{-6}	4.5×10^{-8}	5.4×10^{-8}
B. subtilis FB310	His$^+$	3.0×10^{-4}	2.3×10^{-5}	2.7×10^{-6}
B. subtilis FB311	Leu$^+$	2.2×10^{-4}	3.8×10^{-6}	1.3×10^{-6}
B. cereus	His$^+$	2.3×10^{-7}	ND	ND
B. subtilis FB314	His$^+$	3.2×10^{-5}	2.1×10^{-7}	1.4×10^{-7}
B. licheniformis	His$^+$	2.3×10^{-7}	ND	ND
B. subtilis FB315	His$^+$	1.2×10^{-4}	3.3×10^{-6}	7.8×10^{-7}
B. polymyxa	His$^+$	2.4×10^{-7}	ND	ND
B. subtilis FB316	His$^+$	1.0×10^{-5}	5.8×10^{-7}	6.4×10^{-8}
B. pumilus	His$^+$	1.7×10^{-7}	ND	ND
B. subtilis FB317	His$^+$	1.0×10^{-5}	1.8×10^{-7}	3.7×10^{-7}
B. thuringiensis	His$^+$	2.3×10^{-7}	ND	ND
B. subtilis FB318	His$^+$	6.9×10^{-5}	2.7×10^{-6}	2.0×10^{-7}

ND = no detectable transformants. [a] 4 to 10 μg DNA ml^{-1}. [b] 4 to 10 μg DNA mg^{-1} of clay.

Transformation by clay-bound DNA in sterile and non-sterile soil conditions

To provide evidence that interspecies genetic exchange can occur in the soil habitat, transformation experiments were performed with DNA bound on M in the presence of non-sterile soil. Chromosomal DNA from all the *Bacillus* species and intergenotes were bound on M (see Materials and Methods) and used to transform competent cells of *B. subtilis* BD1512. The heterologous transformation was obtained only with clay-DNA from *B. amyloliquefaciens* (Table 3), while clay-intergenotic DNA retained the ability to transform competent cells of *B. subtilis* BD1512, although the transformation frequency

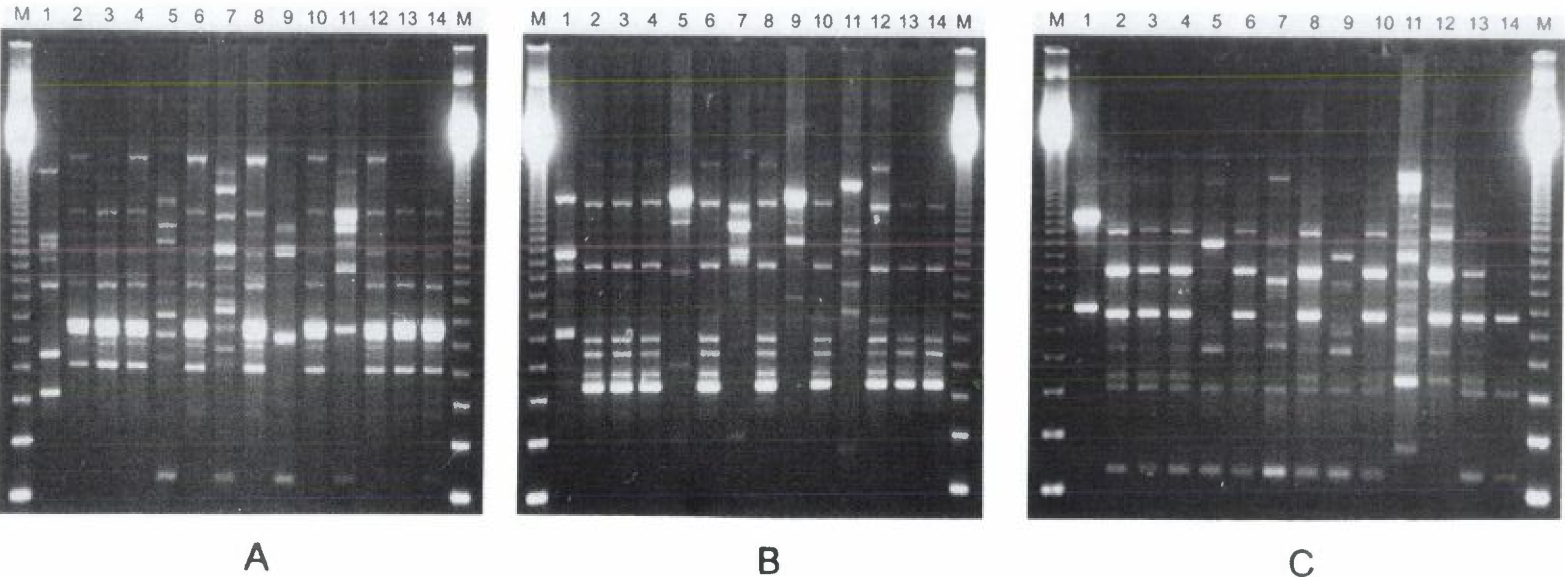

Figure 1. RAPD amplification patterns of different samples obtained with primers 1247 (A), 1253 (B), and RF2 (C). Lane M, molecular weight marker: 123-bp DNA ladder (Gibco BRL, USA); lane 1, *B. licheniformis*; lane 2, *B. subtilis* FB315; lane 3, *B. amyloliquefaciens*; lane 4, *B. subtilis* FB310; lane 5, *B. subtilis cereus*; lane 6, *B. subtilis* FB314; lane 7, *B. pumilus*; lane 8, *B. subtilis* FB317; lane 9, *B. thuringiensis*; lane 10, *B. subtilis* FB318; lane 11, *B. polymyxa*; lane 12, *B. subtilis* FB316; lane 13, *B. subtilis* BD1512; lane 14, *B. subtilis* BD170.

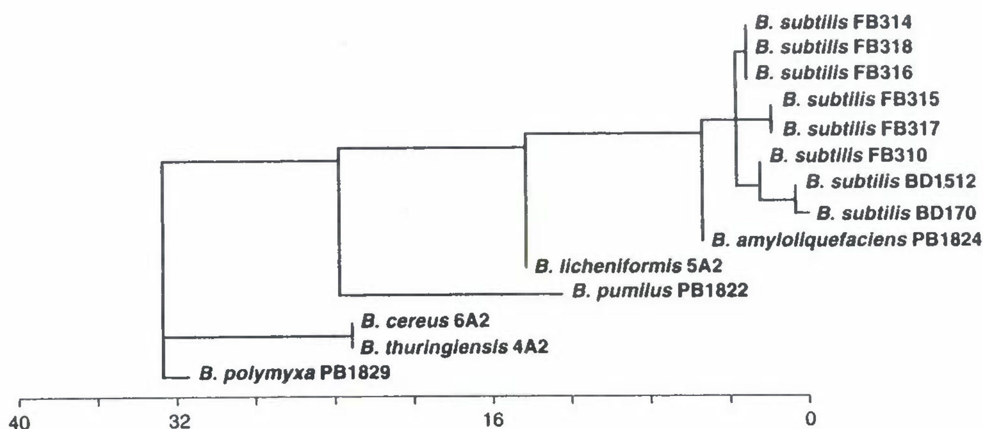

Figure 2. Dendrogram based on NJ clustering of the *E* matrix obtained by joining the data from the RAPD produced with the primers 1247, 1253, and RF2.

was significantly lower (by one-two orders of magnitude) than in liquid culture (Table 3). This could be due to the fact that the adsorption and binding of DNA on clay can reduce its ability to transform, as previously observed (Khanna and Stotzky, 1992; Gallori et al., 1994). The transformation frequency in non-sterile soil was comparable to that obtained in the absence of soil (Table 3).

Analysis of RAPD patterns of transformants

To investigate the genetic distances among the *Bacillus* species and the intergenotes obtained in the heterologous exchanges, DNA of all the strains (Table 1) was subjected to amplification by the RAPD technique using three 10-mer random primers of different G-C content (60 to 80%) (Table 2). The amplification patterns (Fig. 1) showed that all the primers succeeded in differentiating the *Bacillus* species and the intergenotes, and that different degrees of polymorphism of the profiles were obtained according to the primer used. 19, 25, and 33 bands of different size (200 to 3000 bp) were produced with primers RF2, 1253, and 1247, respectively. The highest level of polymorphism, obtained with the primer 1247, could be due to its G-C content which is the closest to that of the *Bacillus* species considered (30 to 45 %) (Priest, 1993). The reproducibility of the results was verified in two independent experiments.

The relationships among the 14 strains studied were represented by a dendrogram (Fig. 2) constructed as described in Materials and Methods. The unrooted dendrogram shows that the intergenotes and *B. amyloliquefaciens* are

highly similar to *B. subtilis*, and that there is a high degree of divergence between *B. subtilis* and the other *Bacillus* species. These data are in agreement with the results of the transformation experiments (Table 3). In fact, the highest transformation frequencies were obtained with DNA isolated from *B. amyloliquefaciens* and intergenotes.

These results, obtained in a system simulating a natural habitat (clay-DNA complex and presence of non-sterile soil), reinforce the hypothesis that transformation can play a key role in the transfer of genetic information among bacteria of different species in soil. The presence of intergenotes, reducing the genetic distance between the donor and recipient strain, can contribute to the reshuffling of genes in the bacterial populations and hence determine their panmictic structure.

Acknowledgements

We are grateful to D. Paffetti for assistance with computer work.

REFERENCES

Chamier, B., Lorenz, M.G., and Wackernagel, W. 1993. Natural transformation of *Acinetobacter calcoaceticus* by plasmid DNA adsorbed on sand and groundwater aquifer material. *Applied and Environmental Microbiology* **59**: 1662–1667.

Cohan, F.M., Roberts, M.S., and King, E.C. 1991. The potential for genetic exchange by transformation within a natural population of *Bacillus subtilis*. *Evolution* **45**: 1383–1421.

Davis, B.D. and Mingioli, E.S. 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B12. *Journal of Bacteriology* **60**: 17–28.

Duncan, K.E., Istock, C.A., Graham, J.B., and Ferguson, N. 1989. Genetic exchange between *Bacillus subtilis* and *Bacillus licheniformis*: variable hybrid stability and the nature of bacterial species. *Evolution* **43**: 1585–1609.

Duncan, K.E., Ferguson, N., Kimura, K., Zhou, X., and Istock, C.A. 1994. Fine-scale genetic and phenotypic structure in natural populations of *Bacillus subtilis* and *Bacillus licheniformis*: implications for bacterial evolution and speciation. *Evolution* **48**: 2002–2025.

Excoffier, L., Smouse, P.E., and Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**: 479–491.

Fusi, P., Ristori, G., Calamai, L., and Stotzky, G. 1989. Adsorption and binding of proteins on "clean" (homoionic) and "dirty" (coated with Fe oxyhydroxides) montmorillonite, illite, and kaolinite. *Soil Biology & Biochemistry* **21**: 911–920.

Gallori, E., Bazzicalupo, M., Dal Canto, L., Fani, R., Nannipieri, P., Vettori, C., and Stotzky, G. 1994. Transformation of *Bacillus subtilis* by DNA bound on clay in non-sterile soil. *FEMS Microbiology Ecology* **15**: 119–126.

Harford, N. and Mergeay, M. 1973. Interspecific transformation of rifampicin resistance in the genus *Bacillus*. *Molecular and General Genetics* **120**: 151–155.

Harris-Warrick, R.M. and Lederberg, J. 1978. Interspecies transformation in *Bacillus*: sequence heterology as the major barrier. *Journal of Bacteriology* **133**: 1237–1245.

Harter, R.D. and Stotzky, G. 1971. Formation of clay-protein complexes. *Soil Science Society of America Proceedings* **35**: 383–389.

Huff, D.R., Peakall, R., and Smouse, P.E. 1993. RAPD variation among natural populations of outcrossing buffalograss [*Buchloë dactyloides* (Nutt.) Engelm.]. *Theoretical and Applied Genetics* **86**: 927–934.

Istock, C.A., Duncan, K.E., Ferguson, N., and Zhou, X. 1992. Sexuality in a natural population of bacteria-*Bacillus subtilis* challenges the clonal paradigma. *Molecular Ecology* **1**: 95–103.

Khanna, M. and Stotzky, G. 1992. Transformation of *Bacillus subtilis* by DNA bound on montmorillonite and effect of DNase on the transforming ability of bound DNA. *Applied and Environmental Microbiology* **58**: 1930–1939.

Lorenz, M.G. and Wackernagel, W. 1987. Adsorption of DNA to sand and variable degradation rates of adsorbed DNA. *Applied and Environmental Microbiology* **53**: 2948–2952.

Lorenz, M.G. and Wackernagel, W. 1994. Bacterial gene transfer by natural genetic transformation in the environment. *Microbiological Reviews* **58**: 563–602.

Maynard Smith, J., Dowson, C.G., and Spratt, B.G. 1991. Localized sex in bacteria. *Nature* **349**: 29–31.

Maynard Smith, J., Smith, N.H., O'Rourke, M., and Spratt, B.G. 1993. How clonal are bacterial? *Proceeding of the National Academic of Science USA* **90**: 4384–4388.

Paffetti, D., Scotti, C., Gnocchi, S., Fancelli, S., and Bazzicalupo, M. 1996. Genetic diversity of an Italian *Rhizobium meliloti* population from different *Medicago sativa* varieties. *Applied and Environmental Microbiology* **62**: 2279–2285.

Paget, E., Jocteur Monrozier, L., and Simonet, P. 1992. Adsorption of DNA on clay minerals: protection against DNase I and influence on gene transfer. *FEMS Microbiology Letters* **97**: 31–40.

Priest, F.G. 1993. Systematic and ecology of *Bacillus*. In: *Bacillus subtilis and other Gram-Positive Bacteria*. J.A. Hoch and R. Losick, eds. American Society for Microbiology, Washington, DC.

Roberts, M.S. and Cohan, F.M. 1993. The effect of DNA sequence divergence on sexual isolation in *Bacillus*. *Genetics* **134**: 401–408.

Romanowski, G., Lorenz, M.G., and Wackernagel, W. 1991. Adsorption of plasmid DNA to mineral surfaces and protection against DNase I. *Applied and Environmental Microbiology* **57**: 1057–1061.

Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4**: 406–425.

Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, New York.

Smith, M.W., Feng, D.F., and Doolittle, R.F. 1992. Evolution by acquisition: the case for horizontal gene transfer. *Trends in Biochemistry Science* **17**: 489–493.

Stewart, G.J. 1992. Natural transformation and its potential for gene transfer in the environment. In: *Microbial Ecology*. M.A. Levin, R.J. Seidler and M. Rogul, eds. McGraw-Hill, Inc., pp. 253–281.

te Riele, H.P.J. and Venema, G. 1982. Molecular fate of heterologous bacterial DNA in competent *Bacillus subtilis*. I. Processing of *B. pumilus* and *B. licheniformis* DNA in *B. subtilis*. *Genetics* **101**: 179–188.

Vettori, C., Paffetti, D., Pietramellara, G., Stotzky, G., and Gallori, E. 1996. Amplification of bacterial DNA bound on clay minerals by the random amplified polymorphic DNA (RAPD) technique. *FEMS Microbiology Ecology* **20**: 251–260.

Wilson, G.A. and Young, F.E. 1972. Intergenotic transformation of the *Bacillus subtilis* genospecies. *Journal of Bacteriology* **111**: 705–716.