Survival of a plasmid-bearing strain of *Bacillus subtilis* introduced into compost

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Survival of *Bacillus subtilis* strain 168 containing plasmid pAB224, which carries a gene for tetracycline resistance, was studied in mushroom compost under mesophilic and thermophilic conditions. Stable populations of *B. subtilis* were maintained as spores in both sterile and fresh mushroom compost incubated at 37 °C. At 65 °C, the introduced *B. subtilis* populations declined during incubation but spores were still detectable after 28 d. Survival at the higher temperature was greater in fresh than in sterile compost. There was no apparent loss of plasmid pAB224 or plasmid-determined phenotype from the introduced *B. subtilis* population at either incubation temperature. The frequency of tetracycline resistance in the indigenous *Bacillus* population was very low (10^{-5}), but some tetracycline-resistant isolates contained plasmid DNA. Four plasmid DNA profiles were found associated with five *Bacillus* phenotypes, and some evidence for homology with pAB224 was found. However, pAB224 was found to be a suitable marker for release studies because it was easily recovered, readily distinguished from indigenous plasmids on agarose gels, and was maintained in compost-grown *B. subtilis* 168 in the absence of any selective pressure.

Introduction

Bacillus species are important commercial sources of a variety of industrial enzymes including extracellular proteases and amylases (Priest, 1977). In addition, the insecticidal properties of *Bacillus thuringiensis* are widely employed for the bio-control of insect pests (Payne, 1988). Introduction of cloned genes into *Bacillus* spp. via plasmid vectors can be used to improve the efficacy of some strains (Aiba *et al.*, 1983; Miyagawa *et al.*, 1989; Nakamura & Imanaka, 1989). Consequently concerns have been raised over the deliberate or accidental release of recombinant *Bacillus* strains into the environment, and the potential for gene survival and transfer. Microcosm studies aimed at assessing the risks involved in the release of genetically engineered micro-organisms have been carried out for a number of species under a range of environmental conditions (e.g. Stotzky & Babich, 1986; Trevors *et al.*, 1987). However, such studies have mainly focussed on survival and gene exchange among introduced Gram-negative bacteria (Bentjen *et al.*, 1989; Devanas & Stotzky, 1986; Genthner *et al.*, 1988; Krasovsky & Stotzky, 1987; Morel *et al.*, 1989; Richaume *et al.*, 1989; Trevors & Berg,

1989). Microcosm studies on gene exchange between Gram-positive species have been limited to recent reports of conjugal plasmid transfer between *Streptomyces* spp. (Bleakley & Crawford, 1989; Rafii & Crawford, 1988; Wang *et al.*, 1989; Wellington *et al.*, 1988), transformation of *Bacillus subtilis* in sterile soil (Graham & Istock, 1978) and conjugal gene transfer between *Bacillus cereus* and *B. subtilis* in both sterile and non-sterile soil (Van Elsas *et al.*, 1987). There have also been a few investigations on survival of *Bacillus* strains in rhizosphere and non-rhizosphere soils (Acea *et al.*, 1988; Van Elsas *et al.*, 1987, 1988).

In the work described here, we examined survival of *B. subtilis* in commercially produced mushroom compost, an environment where biological activity is high and where *Bacillus* spp. are present in large numbers (Amner *et al.*, 1988). Survival of both the host and the non-transmissible plasmid pAB224, which encodes tetracycline resistance (Bingham *et al.*, 1980), were monitored under mesophilic (37 °C) and thermophilic (65 °C) conditions. The suitability of tetracycline as a marker for these studies was further assessed by examining plasmid DNA in the indigenous tetracycline-resistant *Bacillus* population of compost.

Methods

Isolation of tetracycline-resistant bacteria from compost. Mushroom compost samples were collected at the end of phase II of commercial preparation, as described previously (Amner *et al.*, 1988). Tetracycline-resistant strains of thermophilic bacteria were isolated from compost samples by dilution plating onto nutrient agar, NA (Lab M), containing 25 µg tetracycline ml⁻¹. The plates were incubated at 50 °C overnight and colonies purified by subculturing on the same medium. Strains were maintained on NA containing tetracycline and as stocks in 15% (v/v) glycerol stored at -70 °C.

Screening for plasmids. Tetracycline-resistant isolates were inoculated into 5 ml nutrient broth (NB) containing 10 µg tetracycline ml⁻¹ and incubated with shaking at 50 °C overnight. Harvested cultures were then subjected to a rapid alkaline extraction procedure (Birnboim & Doly, 1979) for the isolation of plasmid DNA.

Large-scale preparation of plasmid DNA. Cells from overnight cultures in NB (1 litre) containing 10 µg tetracycline ml⁻¹ were harvested at 4 °C, washed in 500 ml TEN buffer (10 mM-Tris/HCl, pH 7.5; 10 mM-EDTA, pH 8.0; 150 mM-NaCl), and resuspended in 40 ml lysozyme solution (2%, w/v, glucose; 10 mM-Tris/HCl, pH 8.0; 10 mM-EDTA, pH 8.0; 2 mg lysozyme ml⁻¹). The mixture was incubated at 37 °C for 15 min then cells were lysed by the addition of 80 ml of a 1% (w/v) SDS solution containing 10 µg heat-treated RNAase A ml⁻¹ (Sigma). The pH of the lysate was adjusted to 12.2–12.8 with 0.2 M-NaOH. After gentle mixing at 4 °C for 12 min, 60 ml sodium acetate (3 M, pH 4.8) was added and the mixture left at 4 °C for at least 1 h. A cleared lysate was obtained by centrifugation at 27 000 g for 30 min and filtering through cheesecloth. Plasmid DNA was concentrated by 2-propanol precipitation at room temperature for 30 min. The precipitate was washed in 70% (v/v) ethanol, dried and dissolved in 9 ml TE buffer (10 mM-Tris/HCl, pH 8.0; 1 mM-EDTA, pH 8.0). Caesium chloride (9 g) and ethidium bromide (1 ml of a 10 mg ml⁻¹ stock solution) were added and CsCl-EtBr equilibrium density gradient centrifugation was performed at 38 000 r.p.m. for 65 h using an LKB ultracentrifuge (model 2330 Ultraspinn 55, with RP55T rotor). Ethidium bromide was removed from plasmid bands by 2-propanol extraction, and caesium chloride removed by dialysis against TES (20 mM-Tris/HCl, pH 8.0; 1 mM-EDTA, pH 8.0; 10 mM-NaCl). The dialysed sample (500 µl) was then incubated at -70 °C for 30 min with 1 ml ice-cold ethanol and 50 µl sodium acetate (3 M, pH 4.8). The precipitated plasmid DNA was dried, redissolved in TE and stored at -20 °C.

Restriction enzyme digestion and agarose gel electrophoresis. Plasmid DNA (20 µl) was digested with restriction endonucleases *Hae*III and *Hind*III (Boehringer Mannheim) using conditions recommended by the manufacturer. The reaction was terminated by heating at 70 °C for 10 min. Electrophoresis was carried out in horizontal gels of 0.7% agarose in TBE buffer (90 mM-Tris/HCl; 90 mM-boric acid; 2.5 mM-EDTA containing 0.2 µg ethidium bromide ml⁻¹). Plasmid sizes were estimated by comparison of band sizes with those of *Hind*III-digested phage λ DNA.

Electroelution of DNA bands from agarose gels. Plasmid DNA (200 µl) from *Bacillus* isolate MC58 (containing 640 µg DNA ml⁻¹) was loaded onto a horizontal agarose gel (as above). After electrophoresis, all DNA bands were excised and electroeluted (Maniatis *et al.*, 1982). Eluted DNA fragments were digested with *Hae*III.

Characterization of tetracycline-resistant isolates. Growth at 37, 50 and 65 °C was tested by inoculating isolates onto NA agar with or without tetracycline (25 µg ml⁻¹) and examining plates after overnight incubation. Antibiotic sensitivity profiles were obtained by inoculating isolates onto NA containing one of the following antibiotics:

chloramphenicol, ampicillin (both at 25 µg ml⁻¹), streptomycin (50 µg ml⁻¹), or kanamycin (100 µg ml⁻¹). Plates were incubated for up to 48 h at 50 °C. For the determination of starch degradation, strains were inoculated onto NA containing 0.2% soluble starch (BDH) and incubated for up to 72 h at 50 °C. Plates were then flooded with a solution of Gram's iodine and examined for zones of clearing indicative of starch hydrolysis.

Survival of *B. subtilis* containing pAB224 in compost samples. *B. subtilis* strain 168 was kindly provided by Richard Sharp (CAMR, Salisbury, UK). Plasmid pAB224 is a 3.0 kb deletion variant of a plasmid originally isolated from *Bacillus stearothermophilus* (Bingham *et al.*, 1980); it carries a gene which confers tetracycline resistance upon the host (MIC 75 µg ml⁻¹).

B. subtilis 168 containing pAB224 was cultured on NA plus 25 µg tetracycline ml⁻¹ for 48 h at 37 °C, and an inoculum was obtained by resuspending colonies in 10 ml sterile distilled water. Compost samples (3 g of fresh or sterile compost in boiling tubes with loose-fitting caps) were inoculated with 0.5 ml of the *B. subtilis* suspension to give an inoculum of 6×10^8 c.f.u. (g compost)⁻¹. Where appropriate, compost was sterilized by autoclaving at 121 °C for 20 min on three successive days. Sterile distilled water (0.5 ml) was added to uninoculated control tubes containing fresh compost. Tubes containing compost were incubated at either 37 °C or 65 °C for 28 d; desiccation was prevented by monitoring weight and adjusting with sterile distilled water. Recoveries were made immediately after inoculation and at regular intervals following extraction and serial dilution in quarter-strength Ringer solution, as described previously (Amner *et al.*, 1988). Dilutions (0.1 ml) were plated onto NA with or without tetracycline (25 µg ml⁻¹) and all plates were incubated at 37 °C overnight. Spore counts were obtained by heating diluted compost samples at 90 °C for 20 min prior to plating out. All experiments were performed in triplicate and data subjected to preliminary analysis by Student's *t*-test.

Maintenance of plasmid pAB224 was monitored throughout the incubation period by screening four to six randomly selected tetracycline-resistant *B. subtilis* isolates per compost sample, from triplicate compost samples. Isolates were inoculated into NB (5 ml) containing 10 µg tetracycline ml⁻¹, shaken overnight at 37 °C, then subjected to the rapid alkaline extraction method (see above). In addition, a similar number of *B. subtilis* colonies recovered on non-selective plates from fresh compost, and all tetracycline-resistant isolates in the controls, were screened for plasmid DNA.

Results

Isolation and classification of tetracycline-resistant bacteria

An incubation temperature of 50 °C was used in isolation experiments to select for thermotolerant and thermophilic groups of bacteria. We have previously analysed the bacterial populations of mushroom compost (Amner *et al.*, 1988) and confirmed that virtually all bacteria isolated at 50 °C and exhibiting a non-hyphal growth form are members of the genus *Bacillus* (see Fermor *et al.*, 1985). Low numbers of indigenous bacteria resistant to tetracycline were isolated from mushroom compost. This population represented a maximum of 10^3 c.f.u. (g fresh compost)⁻¹, compared to a total *Bacillus* population of 10^8 c.f.u. g⁻¹. Differences in colony morphology were used to select six representative tetracycline-sensitive

Fig. 1. Agarose gel electrophoresis of plasmids from tetracycline-resistant *Bacillus* isolates. (a) Uncut plasmid DNA: lanes 1 and 11, *Hind*III-digested phage λ DNA size markers; lane 2, pAB224; lanes 3 to 9, plasmid DNA from isolates CB21, CB30, CB34, CB58, CB9, CB10 and CB18; lane 10, pTB90 (Imanaka *et al.*, 1982). (b) Plasmid DNA restricted with *Hae*III: lane 1, *Hind*III-digested phage λ DNA size markers; lane 2, pAB224; lanes 3 to 6, plasmid DNA from isolates CB21, CB30, CB58 and CB9.

isolates, and these plus 52 tetracycline-resistant isolates were screened for the presence of plasmid DNA. Plasmids were detected in 17 tetracycline-resistant strains and their presence confirmed by large-scale plasmid preparation and purification on caesium chloride gradients. Plasmid DNA was not detected in any of the tetracycline-sensitive isolates tested.

Plasmid DNA preparations from all plasmid-bearing isolates, except strain CB10, gave multiple band patterns on agarose gels (Fig. 1a). This suggested the presence of more than one plasmid, but when the bands from one isolate (CB58) were electroeluted from agarose gels and restricted with *Hae*III, similar digest patterns emerged for the different bands (data not shown). In any event, the banding pattern of plasmid DNA from the indigenous tetracycline-resistant *Bacillus* strains was clearly distinct from that of pAB224 (Fig. 1a) and this could be readily confirmed by restriction with *Hae*III (Fig. 1b).

Plasmid-bearing strains were classified into five groups according to apparent plasmid size and strain phenotype (Table 1). This simple classification scheme includes growth at 37 °C and 65 °C to differentiate thermotolerant and thermophilic species both of which grow at 50 °C, and starch hydrolysis, which is useful for the primary subdivision of thermophilic *Bacillus* strains (R. Sharp, CAMR, Salisbury, UK, personal communication). The majority of strains were unable to grow at 65 °C and were sensitive to all of the antibiotics tested except tetracycline. The plasmid DNA extracted from these strains was approximately 10 kb in size, as determined by restriction analysis with endonucleases *Hae*III (Fig. 1b) and *Hind*III. Two other isolates (CB9

and CB58) appeared to carry smaller, 7 kb plasmids (Fig. 1b). One of these isolates (CB9) was able to grow at 65 °C and was resistant to 50 μ g streptomycin ml⁻¹. The other isolate (CB58) was characterized by a mucoid colony form, sensitivity to streptomycin and resistance to ampicillin (Table 1). The other two tetracycline-resistant isolates (CB10 and CB18) showed similar characteristics to CB9 (Table 1) but appeared to carry larger plasmids, >25 kb in size, which had different restriction maps (data not shown). Isolates CB10 and CB18 differed from one another in their ability to degrade starch (Table 1).

Survival of B. subtilis 168 containing pAB224 in sterile compost

Data on the recovery of *B. subtilis* 168 as spores and total c.f.u. following release into sterile compost are presented in Fig. 2. In compost samples incubated at 37 °C, the population increased within the first 24 h after inoculation, then declined to a constant level of 10⁷ c.f.u. g⁻¹ for the remaining 3 weeks of the incubation period (Fig. 2a). The proportion of the population recovered as spores increased from 0.0001% at inoculation to approximately 100% after 7 d, indicating that *B. subtilis* 168 was persisting as spores throughout most of the incubation period. Some statistically significant differences ($P < 0.01$) were found between viable counts on tetracycline-amended and unamended media, appearing to indicate a possible loss of the plasmid-determined phenotype. However, because these differences were limited to days 1, 3 and 28, this can be discounted. Furthermore, pAB224 DNA was readily recovered at

Table 1. Characteristics of tetracycline-resistant *Bacillus* isolates which contained plasmid DNA

Strain number	Apparent plasmid size (kb)	Growth at 37 °C*		Growth at 65 °C*		Starch hydrolysis	Mucoid colony form	Additional drug resistances† detected (µg ml ⁻¹)			
		NA	NA + Tet	NA	NA + Tet			Kan (100)	Str (50)	Cam (25)	Amp (25)
CB21, 26, 28, 29, 30, 31, 32, 34, 35, 37, 38, 41, 42	10	+	+	—	—	—	—	—	—	—	—
CB9	7	+	+	+	+	—	—	—	+	—	—
CB58	7	+	+	±	±	+	+	—	—	—	+
CB10	> 25	+	+	+	+	+	—	—	+	—	—
CB18	> 25	+	+	+	—	—	—	—	+	—	—

* Growth was tested in the presence and absence of tetracycline (Tet) at 25 µg ml⁻¹.

† Kan, kanamycin; Str, streptomycin; Cam, chloramphenicol; Amp, ampicillin.

every stage of the incubation period from all *B. subtilis* colonies that were screened (60–90 colonies in total).

In sterile compost samples incubated at 65 °C, the population of *B. subtilis* 168 (as recovered at 37 °C) declined steadily from 10⁸ to 10² c.f.u. g⁻¹ over 28 d (Fig. 2b). Throughout this time, the *Bacillus* counts recovered as spores were significantly lower ($P < 0.01$) than the total counts, particularly on the tetracycline-amended medium. However, there was no significant loss of tetracycline resistance within the total population and again plasmid DNA was readily extracted from all *B. subtilis* isolates screened (60–90 colonies).

Survival of *B. subtilis* 168 containing pAB224 in fresh compost

Survival of *B. subtilis* 168 in non-sterile compost was monitored by recovery of the strain from compost microcosms onto NA containing 25 µg tetracycline ml⁻¹ (Fig. 3). The pattern of survival was similar to that described above for sterile compost. In samples incubated at 37 °C, the population declined during the first week then stabilized at a lower level, presumably due to sporulation (Fig. 3a). In samples incubated at 65 °C, the population declined steadily throughout the incubation period and, with the exception of day 14, the total viable counts obtained were significantly greater ($P < 0.01$) than the equivalent spore counts (Fig. 3b). The decrease in population of *B. subtilis* at 65 °C was not as marked in fresh compost as in sterile compost. Viable counts in excess of 10⁴ c.f.u. g⁻¹ were recovered after 28 d in fresh compost, compared to 10² g⁻¹ from sterile compost (Fig. 2b).

There appeared to be no loss of plasmid pAB224 from *B. subtilis* 168 in fresh compost at either incubation

temperature. All of the randomly selected tetracycline-resistant *B. subtilis* isolates yielded plasmid DNA. In addition, plasmid DNA was recovered from *B. subtilis* isolates recovered on non-selective agar from fresh compost samples (120–180 colonies examined). We found that colonies of *B. subtilis* strain 168 were easily recognizable on isolation plates, and furthermore that careful examination of the other colonies enabled a preliminary classification based on colony morphology. The indigenous bacterial population consisted of two colony types: either small (<3 mm in diameter) and fawn, or larger and lighter in colour. The colonies of *B. subtilis* 168 were of similar size to the larger of the two indigenous colony types, but were more translucent and easily distinguished when isolation plates were held to the light. No *B. subtilis* 168 colonies were recovered from uninoculated control compost samples, and this was the only colony type recovered on tetracycline-amended agar from inoculated samples. A small number of tetracycline-resistant colonies (less than three colonies per isolation plate at the detection limit of 10² g⁻¹) were isolated from uninoculated control samples incubated at 37 °C, on days 0, 14 and 28. These were found to contain plasmid DNA which did not co-migrate with pAB224 on agarose gels and which showed the typical multi-band pattern (see Fig. 1a). These plasmids were not characterized further.

Changes in the indigenous bacterial population in fresh compost were observed following recovery of bacteria on NA at 37 °C (Fig. 3). The initial population of 10⁸ viable counts (g compost)⁻¹ decreased to a steady level of 10⁷ g⁻¹ in compost incubated at 37 °C, compared to a decrease of more than two orders of magnitude in compost incubated at 65 °C. Spore counts generally equated with total counts in compost incubated at 37 °C,

Fig. 2. Recovery of *B. subtilis* strain 168 containing pAB224 from sterile compost incubated at (a) 37 °C and (b) 65 °C. Recovery at 37 °C on NA: ●, total counts; ○, spore counts. Recovery at 37 °C on NA containing tetracycline: ■, total counts; □, spore counts. All values are the means of triplicate determinations.

Fig. 3. Recovery of *B. subtilis* strain 168 containing pAB224 from, and population dynamics of indigenous bacteria in, fresh compost incubated at (a) 37 °C and (b) 65 °C. Recovery of total population from uninoculated control composts at 37 °C on NA: ♦, total counts; ◇, spore counts. Recovery of total population from *B. subtilis*-inoculated composts at 37 °C on NA: ●, total counts; ○, spore counts. Recovery of tetracycline-resistant population from composts inoculated with *B. subtilis* at 37 °C on NA containing tetracycline: ■, total counts; □, spore counts. All values are the means of triplicate determinations.

but were lower in compost incubated at 65 °C. In both sets of fresh compost samples, inoculation with *B. subtilis* resulted in no significant increase ($P < 0.01$) in total bacterial counts over control tubes, except on day 0.

Discussion

Mushroom compost was selected as a model environment for studying survival of an introduced plasmid-bearing *B. subtilis* strain because it is a relatively

reproducible substrate with a highly active indigenous microflora in which Gram-positive bacteria, notably *Bacillus* spp. and actinomycetes, are abundant (Amner *et al.*, 1988). Compost also provides a potential system for studying gene exchange between bacterial groups with different temperature optima since the indigenous population comprises both mesophilic and thermophilic species (Fermor *et al.*, 1985). The ability of *B. subtilis* to grow at temperatures up to 50 °C makes it useful for detecting gene transfer into both populations. This study

was aimed at examining survival of *B. subtilis* host and vector in compost at both 37 °C and 65 °C. Tetracycline resistance was chosen as the selectable marker for monitoring *B. subtilis* survival, since our previous studies had indicated an extremely low background resistance to tetracycline within the compost population (Amner *et al.*, 1988). Characterization of this small tetracycline-resistant population, isolated from compost at 50 °C, revealed some plasmid-bearing strains. We distinguished four apparent plasmid types, one of 7, one of 10 and two of > 25 kb in size; these are within the size range of other plasmids detected in thermophilic *Bacillus* spp. (Bingham *et al.*, 1979; De Rossi *et al.*, 1989; Hoshino *et al.*, 1985b; Imanaka *et al.*, 1981). Preliminary restriction analysis including, for one isolate, that of electroeluted bands, suggested that the multiple bands of plasmid DNA (Fig. 1) were of a common origin within each strain. Examination of the electrophoretic separation of uncut plasmid DNA and the band intensities of restricted DNA do however reveal anomalies which limit the conclusions of these experiments. Multiple banding has previously been observed during the screening of thermophilic *Bacillus* strains for plasmids (De Rossi *et al.*, 1989), and we have found this to be a very useful feature for distinguishing the introduced plasmid pAB224 from plasmid DNA in the indigenous microflora screened in this study. Southern hybridization experiments (results not shown) have provided evidence of homology between the plasmid DNA of tetracycline-resistant isolates and pAB224. Both cryptic and tetracycline-resistance plasmids have been shown to be closely related in *Bacillus* spp. (De Rossi *et al.*, 1989; Hoshino *et al.*, 1985a; Polack & Novick, 1982), so it may not follow that the tetracycline resistance shown by such compost isolates is encoded by their plasmids. However, we have now transformed *B. subtilis* to tetracycline resistance with the 7 kb and 10 kb plasmid DNA listed in Table 1 (unpublished data). In view of these relationships, the application of colony hybridization methodology for the detection of pAB224 transfer events to this indigenous microflora would be limited unless stringency conditions could be satisfactorily increased or additional marker DNA inserted.

Inoculation of *B. subtilis* into a range of soil types has typically been accompanied by a drop in numbers, particularly in non-sterile soils (Acea *et al.*, 1988; Van Elsas *et al.*, 1987; 1988). Survival of *B. subtilis* strain 168 containing pAB224 in fresh compost incubated at 37 °C was comparatively high, presumably due to sporulation and maintenance of populations in a dormant metabolic state. Survival at 65 °C was reduced, but viable populations were maintained over a considerable period, especially in fresh compost. This degree of survival was surprising, since although thermophilic strains of *B.*

subtilis have been produced by mutation *in vitro* (Droffner & Yamamoto, 1985), the upper growth temperature of the *B. subtilis* strain used here was found to be 50 °C. Sporulation within the *B. subtilis* population did not appear to be as complete in compost at 65 °C as at 37 °C. However, these results could simply reflect the possibility that prolonged incubation of spores at 65 °C followed by heating at 90 °C for 20 min prior to plating out may cause a reduction in the viability of the spores sampled. The steady decline in numbers of *B. subtilis* at 65 °C may be attributable to high-temperature induction of spore germination and subsequent lethal injury of vegetative forms at this temperature. Predation is unlikely to be a factor since protozoan populations have not been shown to have pronounced effects on *Bacillus* populations in soil (Casida, 1989), and predator numbers are expected to be low in compost due to the pasteurization phase of commercial mushroom preparation. Nutrient limitation is similarly expected to be unimportant since compost supports high populations of *Bacillus* spp. (Amner *et al.*, 1988; Fermor *et al.*, 1985) and because survival was found to be reduced in sterile compost where the autoclaving process would release bound nutrients, and competition with the indigenous population would be absent. The greater survival of *B. subtilis* in fresh rather than sterile compost at 65 °C may be due to the release of excess or toxic nutrients by the combined effects of compost sterilization and high-temperature incubation, or the provision of growth-promoting substances by the indigenous microflora.

There was no apparent loss of plasmid pAB224 in compost. This study therefore demonstrates how an introduced plasmid can survive in a natural population even in the absence of selective pressure. This, together with the maintenance of viable *Bacillus* populations at both 37 °C and 65 °C, provides an opportunity for studying gene exchange between mesophilic and thermophilic populations. Gene exchange between *Bacillus* strains has previously been demonstrated in soil (Graham & Istock, 1978; Van Elsas *et al.*, 1987), and gene exchange between mesophilic and thermophilic groups has been demonstrated *in vitro* (Imanaka & Aiba, 1986). Gene transfer may be limited due to the production of metabolically inactive spores by the introduced and the indigenous populations. Future experiments will be aimed at increasing the possibilities for gene transfer by, for example, nutrient amendments to compost to maintain vegetative cell rather than spore populations. Furthermore, recombinant plasmids containing appropriate combinations of selectable markers are being developed from the work described here, in order to enable more rigorous evaluation of plasmid survival and transfer potential. The increased longevity of endospore-forming bacteria has important implications for the

long-term survival of genetically-engineered micro-organisms in the environment.

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