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Survival, metabolic activity and conjugative interactions of indigenous and introduced streptomycete strains in soil microcosms

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Abstract

The growth and activity of introduced (*S. lividans* TK24 pIJ673 and *S.lividans* TK23) and indigenous (*S.griseus* CAG17) streptomycete strains in soil was studied, under controlled conditions. The effect of environmental parameters such as temperature, soil water content and nutrient availability on the growth and activity of these strains, was studied using a highly dynamic fed-batch soil microcosm system. Using this new system, repeated cycles of active streptomycete growth were achieved, allowing long-term investigation of metabolic activity, plasmid stability and conjugative plasmid transfer. In long-term experiments, respiration rates and enzyme activity patterns matched the pattern of germination/sporulation cycles of the inoculants. *In situ* hybridisation, using fluorescently labelled oligonucleotides, also proved the presence of metabolically active streptomycete mycelia in sterile soil. Plasmid stability under varying temperatures and selective pressure was studied using the above system. In both sterile and non sterile amended antibiotic containing soil, no intraspecific transfer of plasmid pIJ673 from *S.lividans* TK24 to *S.griseus* CAG17 was detected. The soil microcosm system used, though, permitted detection of intraspecific conjugative transfer of this plasmid from *S.lividans* TK24 to *S.lividans* TK23 in soil.

Introduction

Over the last few years streptomycetes have provided a useful model for studying microbial interactions and activities in soil, because of their importance in soil decomposition processes and their ubiquitous presence in many different soil types (Locci, 1989; McCarthy, 1987; Crawford, 1988). *Streptomyces* strains are well adapted to growth in the terrestrial environment (Mayfield et al., 1972). They survive stress conditions by sporulation and undergo short periods of comparatively rapid growth, when nutrients are available (Williams, 1978; Mayfield et al., 1972). The supply of nutrients in soil is discontinuous in space and time (Williams, 1978 & 1985). Therefore streptomycetes follow a cyclic pattern of germination and sporulation depending on nutrient supply. The difference of total viable plate counts of soil extracts and the spore-specific extraction method (Herron & Wellington, 1990) allow estimation of the active face of streptomycete propagules but additional methodology for monitoring *in situ* is needed, such as microbial activity and molecular markers. Sterile and non-sterile soil model systems have been employed to detect metabolic activity and gene transfer in soil (Lorenz et al., 1988; Bleakly & Crawford, 1989; Wellington et al, 1990). Factors such as soil type, volume and moisture, donor/recipient ratios, temperature, pH and nutrient status have been investigated and the complexity of microcosms used varied depending on the aims of each experiment (reviewed by Cresswell & Wellington, 1992). Most systems used so far were run as batch, with the exception of the report by Wellington et al. (1992), where a fed-batch system was used, allowing sporadic inputs of nutrients with consequent population oscillations. This system, though, did not turn out to be suitable for long-term experiments.

Gene transfer between streptomycetes in soil is a relatively rare event (Cresswell & Wellington, 1992). It has only been clearly demonstrated between closely related strains and it is affected by several envi-

ronmental factors (Wellington et al., 1988; Bleakley & Crawford, 1989; Herron & Wellington, 1990). Although genetic interactions between marked donors and indigenous populations have been demonstrated, horizontal gene transfer to members of the indigenous soil microflora is rather tedious to prove, due to the lack of knowledge concerning phenotype and properties of potential recipients. Henschke and Schmidt (1990) reported that indigenous soil bacteria can receive a gene from an introduced donor in non-sterile unamended soil. Genetic interactions have proved useful indicators of the streptomycete life cycle in soil. Plasmid transfer in batch microcosms occured only in the first two days, when the inoculants were mostly in the mycelial state (Clewlow et al., 1990). Since gene transfer requires an active growth phase, such events could only take place between streptomycetes after germination, during the mycelial growth phase. Model ecosystems which allow active growth of microorganisms have the potential to facilitate detection of gene transfer, which requires metabolically active states.

In the present study, a dynamic fed-batch soil microcosm system was developed, for use in the investigation of long-term growth and activity of indigenous and introduced laboratory streptomycete strains under controlled conditions. The new model microcosm system was designed to allow sampling, stirring, addition and withdrawal of soil in a constant working volume and to provide a more uniform soil particle size and texture. Enumeration of the seeded strains was made by both total viable counts and differential extraction of spores from soil samples. Microbial activity was estimated by measuring several enzyme activities and respiration rates. Population trends were studied and the effect of handling of soil microcosms on the outcome of studies on genetic interactions between filamentous spore-forming bacteria were assessed. The study aimed to provide additional information concerning the survival, activity and interactions of streptomycetes in soil.

Materials and methods

Bacterial strains and plasmids

Streptomyces griseus CAG17 mutant resistant to rifampicin (100 μg ml^{-1}) and sensitive to neomycin (10 μg ml^{-1}) was isolated from soil in our laboratory (Vionis et al., 1996). *Streptomyces lividans* TK24 and TK23 are mutants of the natural isolate *S.lividans* 66, bearing chromosomal resistances to streptomycin (10 μg ml^{-1}) and spectinomycin (10 μg ml^{-1}) respectively (Hopwood et al., 1985). pIJ673 (Wellington et al., 1992) is a derivative of the multicopy streptomycete plasmid pIJ101 (Hopwood et al., 1985) conferring resistance to neomycin (10 μg ml^{-1}), thiostrepton (50 μg ml^{-1}) and viomycin (10 μg ml^{-1}).

All strains were grown and maintained on AGS medium (Herron and Wellington, 1990) amended with the appropriate selective agents. Filtered spore suspensions were used for inoculation of soil microcosms (Hopwood et al., 1985). Inoculum size, estimated directly using a haemocytometer and by viable counts on AGS plates, was approximately 10^5 cfu of each strain g^{-1} soil. All plates were incubated at 28 ˚C for 5 to 6 days.

Soil

A sandy silt/sandy silt loam soil was used in this study. It was taken from an agricultural field at Marathon area (42 km from the centre of Athens). Soil composition and particle size analysis were as previously described (Karagouni et al., 1992) The pH of the soil was 7.6, soil handling after collection from the field and sterilisation was carried out as described by Karagouni et al. (1992).

Microcosms

450 g of sterile soil were placed in sterile polyethylene pots (growth chambers) composing the microcosm. Where appropriate, nutrients for amendment (chitin, or chitin and starch), were added to soil prior to sterilisation. In experiments where the effects of neomycin were being investigated, neomycin was added to the water used for rewetting the air-dried soil. When investigating the effects of thiostrepton on plasmid stability, thiostrepton was mixed with the sterile air-dried soil, prior to inoculation. About 10^5 cfu of each streptomycete strain per gram of dry soil were added in the sterile distilled water used for rewetting the soil to the desired water content. Incubation temperatures varied (22 ˚C, 25 ˚C, 28 ˚C or 37 ˚C) depending on the requirements of each experiment. Moisture contents of -480kPa, -210kPa, -69kPa and -38kPa (8.70%, 10.97%, 15.05% and 18.20% [w/w] respectively) were used in order to study the effect of soil water content on the physiology of the strain studied. For each microcosm system water content was kept constant throughout the experiment. Stirring of microcosms occurred only on sampling days (prior to sampling) or upon cor-

rection of the moisture content. Microcosms were sampled at days 0, 1, 2, 5, 10 and 15 after inoculation, each sample not exceeding 10% of the total soil volume. For some of the microcosms, samples taken were replaced immediately with equal amount of uninoculated sterile (or non-sterile) soil, keeping the total amount of soil in the system constant. The replacement soil was either added without mixing (microcosm C, Table 1) or it was thoroughly mixed with the remaining volume of the microcosm for homogeneity (microcosm D, Table 1). Microcosms devised were as shown in Table 1.

In order to achieve repeated cycles of streptomycete growth in soil, at days 15, 30, 45 etc., larger portions of the microcosm volume (10%, 20%, 30%, 40%, 60% and 75% [w/w]) were removed and replaced in the same way as samples, to avoid fluctuations of the system mass (soil turnover). Samples were taken at days 0, 1, 2, 5, 10 and 15 after each turnover. Before removal and after replacement of the soil subtracted during every turnover, the system was thoroughly stirred, to ensure homogeneity.

Extraction and enumeration of spores and mycelium

Soil samples were treated as described by Wellington et al. (1992): Spores and mycelia were extracted from three 1g aliquots with 1/4 strength Ringer's solution and spores were extracted from 10g of soil using the method described by Herron and Wellington (1990) as modified by Marsh (personal communication).

S.griseus CAG17 was recovered on AGS plates containing rifampicin. To demonstrate the presence of transconjugant CAG17 cfu (containing pIJ673), samples were plated on AGS containing rifampicin, neomycin and thiostrepton. *S.lividans* TK24 pIJ673 was recovered on AGS containing streptomycin, neomycin and thiostrepton. Total *S.lividans* TK24 population (irrespective of the presence of pIJ673) was enumerated on AGS containing streptomycin. *S.lividans* TK23 was recovered from soil by plating on AGS containing spectinomycin. *S.lividans* TK23 transconjugants, harbouring pIJ673, were enumerated on AGS supplemented with spectinomycin, neomycin and thiostrepton. For the recovery of streptomycete inoculants from non-sterile soil, AGS was additionally supplemented with cycloheximide and nystatin, to suppress growth of indigenous fungi.

Determination of metabolic activity

Respiration rates: Determination of respiration rates of microorganisms in soil was based on the method described by Kibble (1966) and modified as follows: small sterile pots, containing 20 ml each of 1N NaOH solution were placed in the growth chambers of inoculated microcosms. The NaOH solution was replaced daily. To estimate the amount of CO_2 produced by microorganisms, the samples were titrated with 1N HCL, using phenolphthalein as indicator. Microcosms containing non-inoculated sterile soil were incubated under the same conditions, for estimation of 'background respiration' (CO_2 contamination from air trapped in microcosm growth chambers or contaminating the NaOH solution during sampling and replacement procedures).

Urease enzyme activity was determined using the method of Tabatabai (1982).

Phosphatase enzyme activity was determined by a colorimetric method, based on the estimation of p-nitrophenol according to Tabatabai (1982).

Activity of dehydrogenases was determined using a colorimetric method, by estimation of triphenyl formazan (TPF) produced (Tabatabai, 1982).

Chitinase activity: Extraction and precipitation of extracellular enzymes from soil samples and determination of chitinase activity in soil extracts was carried out as previously described by Vionis et al. (1996).

In situ hybridisation

In situ hybridisation of streptomycete inoculants with a fluorescently labelled oligonucleotide (Roller et al., 1994) in soil, to demonstrate the presence of mycelia in microcosms after turnovers, was carried out as described by Vionis et al. (1996). The oligonucleotide used can only detect actively growing mycelia but not spores of streptomycetes (Roller et al., 1994).

Colony hybridisation and confirmation of transconjugants

Colonies of *S.lividans* TK23 displaying a transconjugant phenotype, isolated from soil samples, were transferred onto nylon membranes and screened for the presence of pIJ673 as described by Kuczek and Modarski (1989). A fragment of pIJ673 containing the tra and spd genes was labelled with digoxigenin non-radioactive labelling kit (Boehringer Mannheim) and used as probe for the screening. Hybridisation of filters

Table 1. Microcosm systems used in this study.

Micro-cosm type	Soil	Amendment	Antibiotic	Stirring	Replacement	Turnover (%)	Moisture (kPa)	Temperature (˚C)	Inoculum
A	sterile	-	-	-	-	10	-210	22	*S.griseus* CAG17
B	sterile	-	-	+	-	10	-210	22	*S.griseus* CAG17
C	sterile	-	-	-	+	10	-210	22	*S.griseus* CAG17
D	sterile	-	-	+	+	10	-210	22	*S.griseus* CAG17
E	sterile	-	-	+	+	10 (control)	-210	22	*S.griseus* CAG17
F	sterile	-	-	+	+	20	-210	22	*S.griseus* CAG17
G	sterile	-	-	+	+	30	-210	22	*S.griseus* CAG17
H	sterile	-	-	+	+	40	-210	22	*S.griseus* CAG17
I	sterile	-	-	+	+	60	-210	22	*S.griseus* CAG17
J	sterile	-	-	+	+	75	-210	22	*S.griseus* CAG17
K	sterile	crab chitin (4% w/w)	-	+	+	40	-210	22	*S.griseus* CAG17
L	sterile	-	-	+	+	40	-480	22	*S.griseus* CAG17
M	sterile	-	-	+	+	40	-69	22	*S.griseus* CAG17
N	sterile	-	-	+	+	40	-38	22	*S.griseus* CAG17
O	sterile	-	-	+	+	40	-210	25	*S.griseus* CAG17
P	sterile	-	-	+	+	40	-210	28	*S.griseus* CAG17
Q	sterile	-	-	+	+	40	-210	37	*S.griseus* CAG17
R	sterile	-	-	+	+	40	-210	22	*S.lividans* TK24 pIJ673
S	sterile	-	-	+	+	40	-210	28	*S.lividans* TK24 pIJ673
T	sterile	-	-	+	+	40	-210	37	*S.lividans* TK24 pIJ673
U	sterile	-	thio (100 μgg^{-1})	+	+	40	-210	22	*S.lividans* TK24 pIJ673
V	non sterile	chitin & starch (1% w/w each)	-	+	+	10	-210	22	*S.lividans* TK24 pIJ673 + *S.griseus* CAG17
W	non sterile	chitin & starch (1% w/w each)	neo (10 μgg^{-1})	+	+	10	-210	22	*S.lividans* TK24 pIJ673 + *S.griseus* CAG17
X	sterile	-	-	+	+	40	-210	22	*S.lividans* TK24 pIJ673 + *S.griseus* CAG17
Y	sterile	-	-	+	+	40	-210	22	*S.lividans* TK23
Z	sterile	-	-	+	+	40	-210	22	*S.lividans* TK24 pIJ673 + *S.lividans* TK23

Key: thio: thiostrepton, neo: neomycin

and detection of signals was carried out according to the manufacturer's instructions.

Soil experiments

The effect of stirring and replacement of samples on metabolic activity of *S.griseus* CAG17 (microcosms A–D): Total and spore viable counts were determined. Respiration rates and activities of phosphatase, urease and dehydrogenases were determined, to estimate metabolic activity of inoculants in soil.

The effect of soil 'dilution' on survival and metabolic activity of *S.griseus* CAG17 (microcosms E–J): Total and spore viable counts were determined. *In situ* hybridisation of day 1, 16, 31 etc. samples was carried out to confirm the presence of mycelia in soil. Data from different microcosms were compared.

Confirmation of cycles of metabolic activity of *S.griseus* CAG17 in soil amended with crab chitin

(microcosm K): Total and spore viable counts were determined. Extractable chitinase activity was measured at each sampling day and patterns of chitinase production and spore cfu were compared.

The effect of soil water content on survival and metabolic activity of *S.griseus* CAG17 in sterile soil (microcosms H and L–N): Total and spore viable counts were determined. Metabolic activity of the inoculant in each microcosm system was estimated from respiration rates and enzymatic activities.

The effect of temperature on streptomycete survival and activity and on the stability of pIJ673 in sterile soil (microcosms H and O–T): Total and spore cfu were determined. Measurements of respiration rates and enzyme activities were used to assay the metabolic state of the inoculant.

The effect of selective pressure on strain survival, pIJ673 stability and horizontal gene transfer in sterile and non-sterile soil (microcosms U–X): Total and spore viable counts were determined. Interspecific crosses were set up by inoculating donor (*S.lividans* TK24 pIJ673) and potential recipient (*S.griseus* CAG17) in the same microcosm systems. The effect of neomycin on survival, competition and conjugal transfer of pIJ673 between the two streptomycete strains, and the effect of thiostrepton on stability of pIJ673 in sterile soil was investigated.

Conjugal intraspecific plasmid transfer between *S.lividans* strains in sterile soil (Microcosms R, Y and Z) Total and spore viable counts of donor (*S.lividans* TK24 pIJ673), recipient (*S.lividans* TK23) and transconjugants (*S.lividans* TK23 pIJ673) were determined on selective agar media. Transconjugants were confirmed by colony hybridisation.

Statistical analysis

All points on graphs are: a) the means of three replicate samples counted in triplicate for total viable counts, b) the means of triplicate platings of spores extracted from a 10 g sample, c) the means of three replicate samples for urease, acid and alkaline phosphatase, dehydrogenase and chitinase activity and d) the result of a single sample titration for respiration rates. Statistical analyses were accomplished using the MINITAB statistical package (Minitab Statistical Software. State College.Pa.). Minimum significant differences (MSD) were calculated from analysis of variance using the Tukey-Kramer method (Petersen, 1985; Fry, 1989).

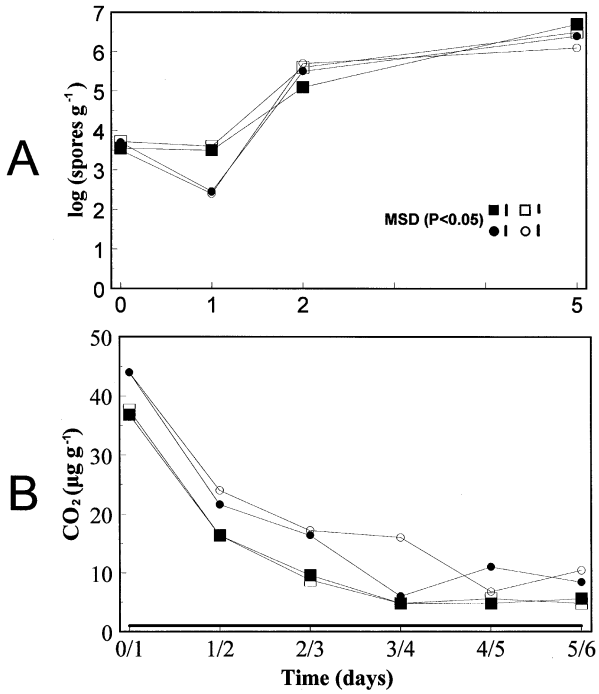

Figure 1. Effect of microcosm handling on metabolic activity of *S.griseus* CAG17. Spore counts (A) and respiration rates (B) in microcosms A (■), B (□), C (●) and D (○), at 28 ˚C and -210kPa.

Results and discussion

Survival of Streptomyces strains in a new, highly dynamic, fed-batch microcosm system (microcosms A–K)

Four soil microcosms (A–D, Table 1) were set up, to determine whether the disturbance of soil brought about through sampling, stirring, mixing, withdrawal and addition of soil affects microbial numbers and activity. In each of these microcosms, moisture content and temperature were maintained at -210kPa and 22 ˚C respectively. Moreover, as shown in Table 1, these microcosms differed in handling of the soil.

S. griseus population levels recorded in microcosms A, B, C and D are presented in Figure 1(A). In microcosm A, where no stirring and mixing of the soil occurred and samples were not replaced, germination of the spore inoculum was expected to be higher than in cases C and D, where soil was thoroughly stirred, but that was not the case. In microcosm A, where microsites were not disturbed, an extended period of mycelial development was expected. This was also not the case. In microcosms C and D, on the other hand, where soil was thoroughly stirred, higher

percentages of spore germination were observed. Total and spore viable counts, indicated no significant differences between the four treatments. Stirring, therefore, and mixing, which resulted in more uniform soil particle sizes and texture, obviously enhanced spore germination compared to sample replacement (10% of the total soil volume). During growth of the natural isolate in our soil, germination efficiency was lower than that observed by Herron and Wellington (1990). In general, our experimental data suggest that the germination of streptomycete spores in soil mainly depended on the soil type (Katsifas et al., 1997) and to a lesser extent on the environmental factors.

In all four microcosms, a constant cumulative increase of urease activity was observed after day 2 (data not shown). This was expected, since it is well known that urease is degraded very slowly and therefore accumulates in soil. The drop in spore counts which was observed at day 1 corresponded to the increase of urease activity. These results suggest that disturbance imposed upon the soil of these microcosms (stirring, mixing, replacement) brought about an increase in urease activity measured in soil. Figure 1(B) shows respiration rates monitored in microcosms A–D. Respiration is often a good index of microbial activity, although many investigations failed to show this correlation (Edwards et al., 1981). At day 5, levels of CO_2 evolved from microcosms were four times lower than those of day 1. The pattern of respiration observed during the five first days was repeated after each turnover. Microcosms C and D, in which samples were replaced, yielded one fold higher respiration rates than microcosms A and B. Since the soil used for this study was autoclaved, addition of uninoculated soil in the microcosm could be considered as addition of nutrients (organic material from inactivated indigenous microflora) as well as a means of providing new microsites for colonisation by the inoculum.

From these data, it was concluded that stirring and replacement of samples were the practices of choice for the purposes of this study. Based on this result, a highly dynamic soil microcosm system was devised, for use in studies of survival, metabolic activity and interactions of streptomycetes in soil. To allow repeated growth cycles in soil to occur, turnover was introduced. Sterile soil added as replacement for the amount of soil subtracted at turnover, provided *S.griseus* CAG17 with nutrients and fresh microniches for colonisation.

Long-term growth patterns of *S.griseus* CAG17 in microcosms E and H are shown in Figure 2(A). In all cases examined, at days 1 and 2 after inoculation,

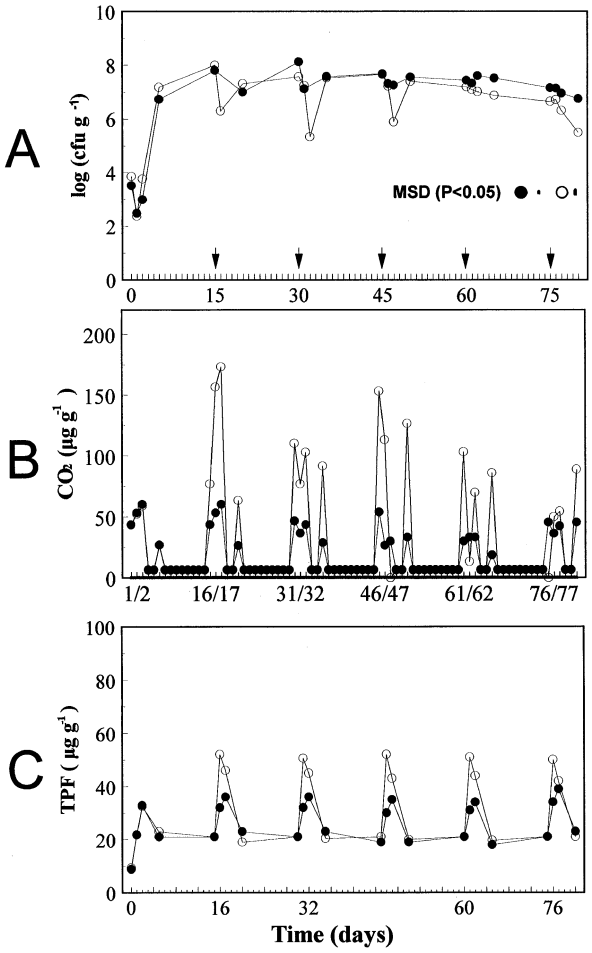

Figure 2. Survival and metabolic activity of *S.griseus* CAG17 in microcosms E (10% turnover - ●) and H (40% turnover - ○). (A) Spores. (B) Respiration rates. (C) Dehydrogenase activity. ↓: Soil turnover.

germination of spores resulted in reduction of extracted spore viable counts, by three log units. After 5 days, the natural strain population had completed one life/differentiation cycle in soil and was predominantly in the form of (new) spores. At day 15, population levels reached 10^8 cfu g^{-1} soil, exhibiting an increase of 3 log units, compared to inoculum levels. Following turnover of 40% (Figure 2[A]) at days 15, 30, 45, 60 and 75, reduction of spore numbers by 2 log units was observed. This germination effect of turnover on streptomycete populations in microcosm soil was observed after each turnover. However, the percentage of germinating spores tended to diminish with time. (from 97% immediately after inoculation to 85% after turnover at day 75). Germination and mycelium development was

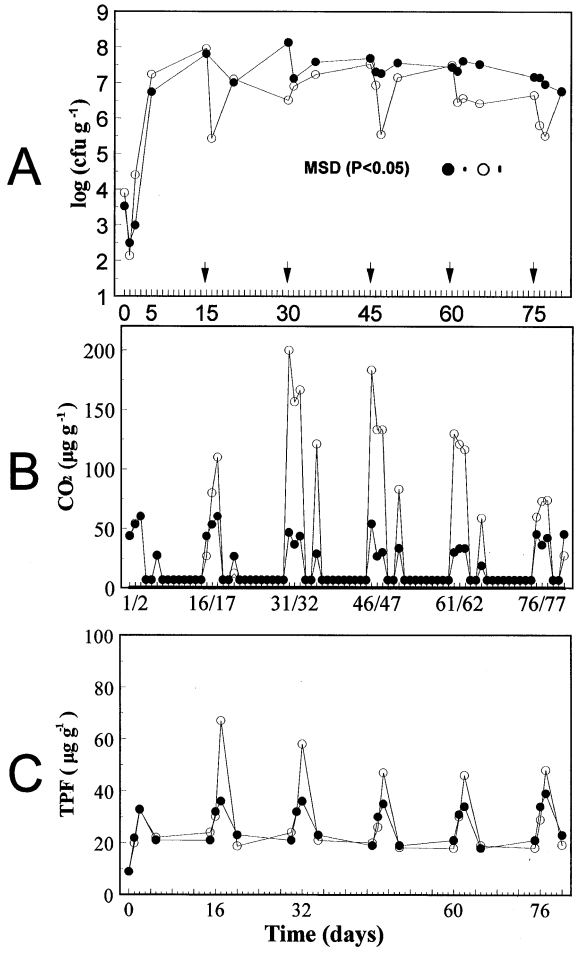

Figure 3. Survival and metabolic activity of *S.griseus* CAG17 in microcosms E (10% turnover - ●) and J (75% turnover - ○). (A) Spores. (B) Respiration rates. (C) Dehydrogenase activity. ↓: Soil turnover.

also confirmed after in situ hybridisation using the fluorescently labelled oligonucleotide described in Roller et al. (1994) (data not shown).

The effect of the amount of soil subtracted and replaced (during turnovers) on growth and activity patterns of *S.griseus* CAG17 in sterile soil was investigated in microcosms E–J. Turnovers of 10% (sample size, negative control), 20%, 30%, 40%, 60% and 75% (w/w) took place every 15 days after initial inoculation. Examples of growth and metabolic activity patterns of the inoculum are shown in Figures 2 and 3. In all turnover treatments, *S.griseus* population remained well above detection limits. On the first day after inoculation and turnovers, reduction of spore levels due to germination was observed. The ratio of spores germinating after subsequent turnovers however, decreased with time. In all 6 microcosms, repeated germination cycles of the inoculum were observed, over a period of 80 days. In all cases, germination of spores was completed during the first two days after each turnover. The percentage of germinating spores was about 90–98% at day 1 after inoculation and after the first turnover (day 16). After 30 days of growth in the microcosms, germination period was extended to the second day after turnover. Highest final population levels were attained in microcosm H. The percentage of spores germinating after the first three turnovers in microcosm I were similar to those observed in microcosm H. After day 47 however, the percentage declined, and by day 77, only 50% of the spores present in the system germinated. The reduction of spore numbers in microcosms H, I and J was mainly due to germination and to a limited extent (below 10%) to removal of part of the population from the systems. Growth pattern of *S.griseus* CAG17 in microcosm J was similar to the one observed in microcosm I.

Respiration and enzyme activities monitored (Figure 2 [B] & [C] and Figure 3[B] & [C]), confirmed viable count data. After turnover, the decrease of spore counts in the systems coincided with peaks in respiration rates and metabolic activity. We observed in all cases that, after a period of approximately 60 days (after the third turnover) the percentage of spores germinated tended to decrease. This was attributed to decreased fitness of the population in the system. This phenomenon could be due to limitations occurring after several rounds of replication within soil. Nonetheless, population levels attained in this system were considerably higher than those reported by Wellington et al. (1992).

To prove that after each turnover *S.griseus* CAG17, which was introduced in the form of spores in sterile soil, was also capable to perform biogeochemical cycling processes, microcosm K was set up. From our previous data (Vionis and Karagouni, unpublished) it was known that *S.griseus* CAG17 possesses chitinolytic properties, like most of *Streptomyces* strains examined (Korn-Wendisch & Kutzner, 1992). Vionis et al. (1996) had proved that the presence of chitinous substrates in soil induces *in situ* production of extracellular chitinolytic enzymes from streptomycete inoculants. It followed that development of metabolically active mycelium in soil should coincide with production of extracellular chitinolytic activity, provided that chitin was available in the growth environment. Figure 4(A) shows survival and growth pattern of the inoculant in

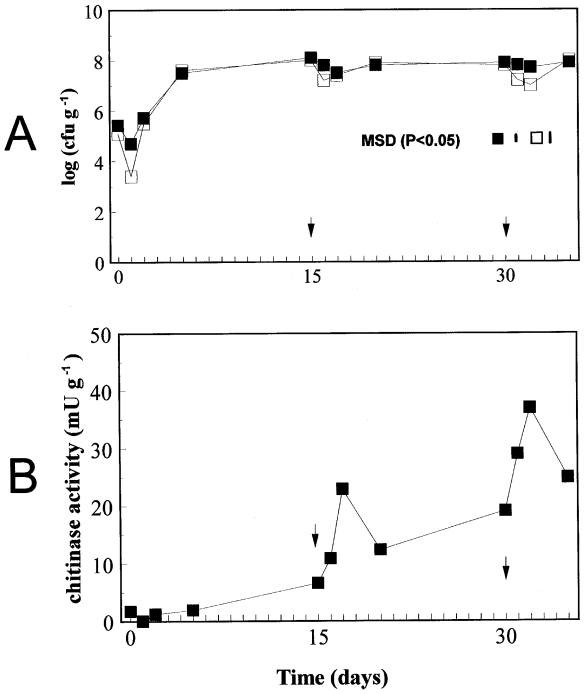

Figure 4. (A) Total viable counts (■) and spores (□) of *S.griseus* CAG17 inoculated in the microcosm K., in the presence of 4% (w/w) crab chitin (B) Confirmation of metabolic activity signalising mycelium development in Microcosm K. Extracellular chitinase activity produced by *S.griseus* CAG17 in chitin-amended soil. ↓: Soil turnover (40%).

sterile soil amended with 4% (w/w) chitin. The corresponding pattern of chitinolytic activity detected in soil samples is shown in Figure 4(B). Spore numbers of CAG17 decreased at days 16 and 31, immediately after the respective turnovers. At the same times, a corresponding increase of extracellular chitinase activity in soil samples was observed. Extracellular chitinolytic enzymes accumulated in soil.

The effect of soil moisture content on survival and activity of streptomycetes in soil

To determine the effect of soil water content, on the survival and metabolic activity of *S.griseus* CAG17 in sterile soil, microcosms H and L–N were set up. As can be seen in Table 1, soil water content varied from -480kPa (microcosm L), to -210kPa (microcosm H), -69kPa (microcosm M) and -38kPa (microcosm N). Moisture content of each microcosm was kept constant throughout the duration of the experiment. Inoculant population levels declined in treatments where moisture content was above -69kPa (microcosms H and L – data not shown). In many previous studies (Stevenson, 1982; Trevors, 1984; Cochran et al., 1989), soil used for the preparation of microcosms was wetted to -67kPa, and under these conditions germination and extensive mycelial development of streptomycete inoculants was observed. This water potential was considered optimal to dry for streptomycete inocula in the type of soil used in those studies. In our experiments, however, soil wetted to this water content was considered waterlogged. Bleakley & Crawford (1989) studied microbial activity in sterile soils at 20%, 40% and 60% MHC and found that 20% MHC was optimal for plasmid transfer between strains of *S.lividans*, but 60% MHC gave the highest soil enzyme activity levels. In many soil types, microbial activity recorded was optimal at approximately -30kPa (60% MHC) (Trevors, 1984) and streptomycetes have been reported to be able to grow and be metabolically active in soils with matric potentials between -1000 to -2000kPa, which inhibit the activity of non-filamentous, unicellular bacteria (Harrison, 1979; Van Loosdrecht et al., 1990). In our experiments, spore counts showed that in all cases examined germination occurred mainly at day 1 and spore formation was rapid and enhanced at elevated moisture levels (-69kPa and -33kPa). At -210kPa, streptomycete inocula germinated well, sporulation rate was slower than for other moisture treatments and spore numbers were just above inoculum level. The comparison of data obtained in different studies concerning the effects of water availability in the soil environment, is complicated and in some cases almost impossible, since the terms used to express water content vary widely. Expression of soil water content as % MHC is a term which depends greatly on the type of soil used in each study. Values of matric potential (in kPa) should overcome this obstacle, and facilitate comparisons (Cresswell & Wellington, 1992). From our data, however, we concluded that a matric potential of -210 kPa (microcosm H) was optimal for the survival of streptomycete inoculants in the type of soil used in these studies.

The effect of incubation temperature on survival and activity of streptomycetes and the stability of plasmid pIJ673 in soil

Temperature is a major environmental factor, with great influence on the physiology and activity of soil microorganisms. Four soil microcosms (microcosms D, and O–Q) were incubated at 22 ˚C, 25 ˚C, 28 ˚C and 37 ˚C, to determine the effect of this important environmental parameter on the life cycle of *S. griseus*

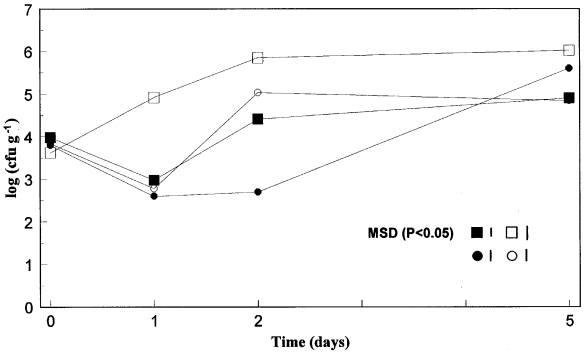

Figure 5. The effect of incubation temperature on spore numbers of *S. griseus* CAG17 at 22 ˚C (●), 25 ˚C (○), 28 ˚C (■) and 37 ˚C (□).

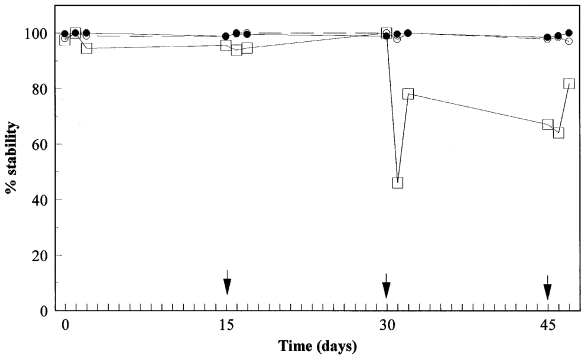

Figure 6. The effect of incubation temperature on stability of pIJ673. % stability of pIJ673 within *S.lividans* TK24, in microcosms incubated at 22 ˚C (microcosm R - ●), 28 ˚C (microcosm S - ○) and 37 ˚C (microcosm T - ■). ↓: Soil turnover.

CAG17 in sterile soil. Total viable counts in soil were higher with increasing temperature (data not shown) but spore counts (Figure 5) suggested that the number of spores produced increased with increasing temperature. It was obvious that, after germination at day 1, streptomycete populations persisted mainly in the form of spores, in microcosms O–Q which were incubated at temperatures higher than 25 ˚C. Only at 22 ˚C (microcosm D) was observed that germination period of *S. griseus* spores in soil is extended up to day 2 (after day 1, 93,5% of spores germinated and a further germination of 90,34% was observed at day 2). In this microcosm, a lower ratio of sporulation was observed in comparison with other incubation temperatures. Therefore, for the purposes of our investigation 22 ˚C was the temperature of choice for the study of activity and interactions between streptomycetes.

Microcosms R, S and T were set up, to investigate the stability of plasmid pIJ673 and the overall survival

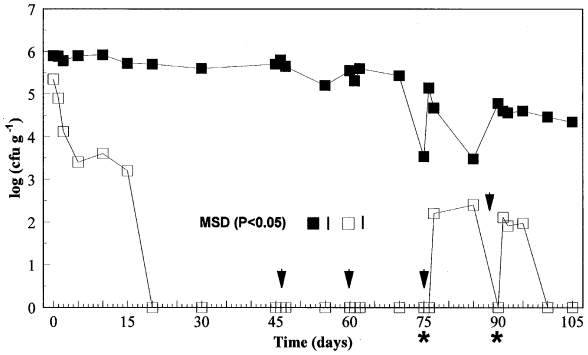

Figure 7. The effect of neomycin on survival and competition between *S.griseus* CAG17 (■) and *S.lividans* TK24 pIJ673 (□) in a highly dynamic microcosm system, containing non sterile soil. ↓: Soil turnover. *: neomycin addition.

of its host *S.lividans* TK24 in sterile soil. Figure 6 shows the stability of pIJ673, in these microcosms. Survival of the host strain was similar at 22 ˚C and 28 ˚C, with final population levels approximately 2 log units higher than the inoculum. At 37 ˚C, the host population declined, and by day 45 it was 1.5 log units lower than inoculum levels (data not shown). In all three microcosms, the plasmid was stably maintained. pIJ673 is a derivative of the multicopy broad-host range conjugative streptomycete palsmid pIJ101 (Hopwood et al., 1985), which has been used for the construction of several cloning vectors. The fact that this plasmid is stably inherited in its host in sterile soil, although not conferring any obvious survival advantage, is of considerable interest in the light of its persistence and possible dissemination in natural soil. A similar observation was made by Karagouni et al. (1992), when pIJ673 was hosted by *S.violaceolatus* ISP5438.

The effect of selective pressure on survival and competition of streptomycetes and on plasmid stability and conjugal horizontal transfer in sterile and non-sterile soil

To test the effect that the presence of antibiotics may have on streptomycetes and plasmid stability in soil, microcosms R and U–W were set up. *S.griseus* CAG17 and *S.lividans* TK24 pIJ673 were simultaneously inoculated into sterile or non-sterile soil, amended with starch and chitin (1% [w/w] of each) (microcosms V and W). The highly dynamic microcosm systems were incubated at 22 ˚C for 105 days. During the first 45 days, the systems were allowed to establish, sampling and water content adjustments being the only disturbances. The establishment period aimed to allow the

inoculant strains to colonise microniches while competing with the indigenous microflora. 10% (w/w) turnovers were carried out at days 45, 75 and 90. Streptomycetes are major antibiotic producers. Previous work has shown that antibiotics are probably produced in soil (Gottlieb, 1976). Moreover, common applications of antibiotics increase the possibility of artificial antibiotic enrichment of soil, through manure, or industrial waste disposal. Therefore, at day 75 and 90, neomycin was added in the soil used for replacement of subtracted microcosm mass, to a final concentration of 10 μg g^{-1} throughout the microcosm system (microcosm W). Neomycin addition was intended to impose selective pressure favourable for *S.lividans* TK24 pIJ673, whose population levels had diminished below detection limits. Figure 7 shows the results of these experiments.

Survival of both strains in microcosm X, corresponded to the patterns obtained from microcosms E and R (data not shown). In microcosms V and W, the absence of disturbance during the first 45 days was intended to enhance the chances of establishment of the introduced strains. Stirring and sample replacement did not affect population levels. *S.griseus* CAG17 survived, remaining at inoculum levels (10^6 cfu g^{-1}soil) and population levels observed in non-sterile soil were two logs lower than those obtained in sterile soil microcosm systems (Figure 7). *S.lividans* TK24 pIJ673 population rapidly declined and was below detection limits after day 20. After the first and second turnover, at days 45 and 60 respectively, a small decline of CAG17 spore numbers was observed, due to the repetition of its life cycle in soil. TK24 plasmid bearing population remained at levels below detection limits. At day 75, before neomycin addition, total viable counts of CAG17 were two log units lower than inoculum size. Following antibiotic addition (day 76), the levels of the indigenous isolate population increased, reaching initial inoculum level. *S.lividans* TK24 pIJ673 population levels shortly rose above detection limits (day 77), but by day 90 they had fallen again below 2 log units. By day 90, CAG17 population had also decreased, reaching values similar to those of day 75. Turnover and neomycin amendment at day 90 resulted in increased population levels, but not above inoculum levels (for *S.griseus* CAG17) or above 3 log units (for *S.lividans* TK24 pIJ673). 105 days after inoculation, *S.griseus* CAG17 population was 1.5 log units below inoculum level, and 4 log units below population sizes observed in sterile non amended soil. 15 days after the last turnover, *S.lividans* TK24 pIJ673 was no longer detectable. In none of these three microcosms was conjugal transfer of pIJ673 to *S.griseus* CAG17 detected. This could be due to extremely low transfer frequencies. Performing *in vitro* conjugation, transformation and phage infection control studies (Vionis and Karagouni, unpublished data) we ascertained that the natural soil isolate could not acquire this plasmid under *in vitro* conditions either, and that it possesses restrictive properties. For this reason we are inclined to believe that restriction barriers, and an indigenous natural plasmid of the new strain (Vionis and Karagouni, unpublished) are more likely candidates for explaining the lack of conjugal plasmid transfer in soil, despite positive selective pressure by the presence of neomycin. Based on our results, we tend to believe that conjugal spread of plasmids among different bacterial species or genera in soil might not be as easy, as originally postulated. Indigenous soil bacterial strains might not be such good recipients after all, giving the risk assessment question a whole new perspective (Mazodier et al., 1989). Interspecific gene transfer in soil might be limited to strains without restrictive properties. Intergeneric conjugal plasmid transfer has so far been reported as transfer of shuttle plasmid constructs, in *in vitro* experiments (Tabakov et al., 1994; Gormley & Davies, 1991), but actual transfer of this kind in soil has yet to be demonstrated.

To determine the effect of selective pressure on plasmid stability in soil, microcosms R and U were devised. The presence of thiostrepton did not affect the stability of the plasmid within its host in sterile soil (data not shown). Despite the fact that pIJ673 bears the Tn5 *tsr* gene, survival of its host strain in sterile soil was not enhanced compared to non-selective soil. However, this plasmid was also maintained stably in non-selective sterile soil, under different temperatures.

Intraspecific conjugal transfer of pIJ673 among S.lividans strains in the new highly dynamic microcosm system

Microcosms R and Y, Z were devised to study conjugal plasmid tranfer among streptomycetes in soil. Figure 8 shows viable counts of *S.lividans* TK24 pIJ673 (plasmid donor- Figure 8[A]), *S.lividans* TK23 (plasmid recipient- Figure 8[A]) and *S.lividans* TK23 pIJ673 (transonjugant population- Figure 8[B]).

As can be seen in Figure 8, system turnovers occurred at days 15, 30 and 45. Total viable counts of the donor (data not shown) increased by one log unit by day 15, while recipient population at the same

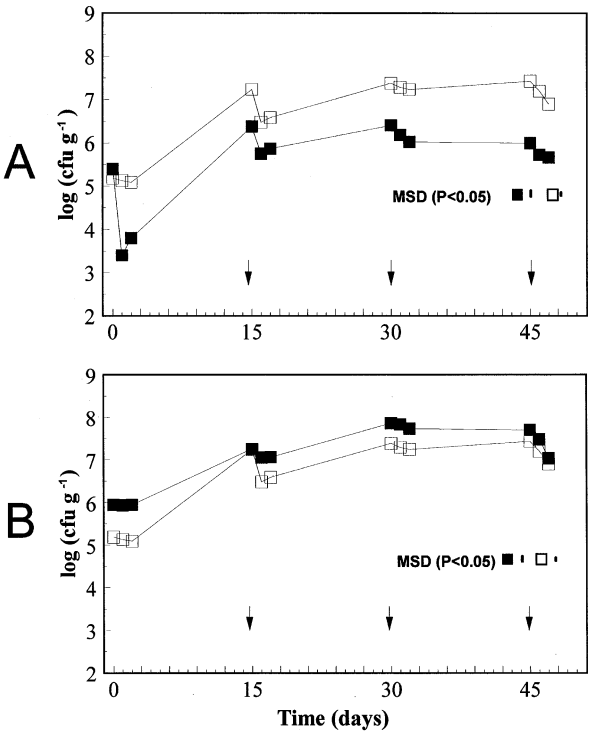

Figure 8. Demonstration of intraspecific conjugal transfer of pIJ673 between *S.lividans* strains in soil, using the new highly dynamic microcosm system. (A) Spore counts of donor *S.lividans* TK24 pIJ673 (■) and recipient *S.lividans* TK23 (□). (B) Total (■) and spore (□) counts of transconjugant *S.lividans* TK23 pIJ673. ↓: Soil turnover.

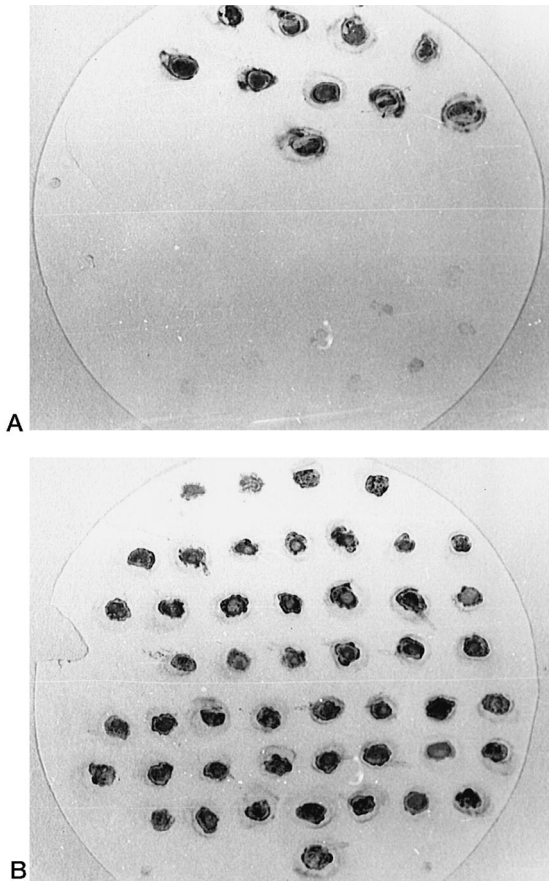

Figure 9. Confirmation of transconjugants from microcosm Z samples by colony hybridisation. (A) Upper half: positive control (*S.lividans* TK24 pIJ673 colonies) Lower half: negative control (*S.lividans* TK23 colonies). (B) Transconjugant colonies recovered at day 1.

period increased by 1.5 log units (data not shown). Spore numbers of both parental strains (Figure 8[A]) were reduced after the first turnover (days sixteen and seventeen), while total viable counts were at levels slightly lower than those recorded at day 15. Germination of donor spores was observed (although to a lesser extent) after both subsequent turnovers (days 31 and 32 and 46 and 47 respectively), while the recipient strain survived predominantly in the form of spores, with low germination efficiencies. Transconjugants were detected already after the first day and their population (Figure 8[B]) amounted to $3.1x10^5$ cfu g^{-1} soil by day 30. Colonies of TK23 with transconjugant phenotype were screen by colony hybridisation to confirm acquisition of pIJ673 (Figure 9).

General conclusions

Experimental data derived from soil microcosms run for the study of fate, growth and survival of streptomycete inoculants in soil, can vary considerably when different soil types are used. Reproducibility of experimental data, on the other hand, is not only dependent on physicochemical parameters but also mostly influenced by the design and handling of microcosm systems used. Small-size microcosm systems present the disadvantage of wall growth, while systems of larger size present low reproducibility problems (Cresswell, 1992). Our data suggest that the system we devised allows monitoring of suvival, metabolic activity and genetic interactions among streptomycetes in soil with high reproducibility. Using the system presented in this study, we achieved repeated rounds of germination/sporulation of the strains in sterile soil. These facts make this system particularly valuable as a tool for long-term studies of survival, metabolic activity and interactions of streptomycetes in soil. We estimated

that even the highest of the turnover treatments studied was not sufficient to cover nutritional demands of the populations growing in this system. Hence, the reduction of the extent of spore germination observed after repeated turnovers of the systems. As expected, dryer, rather than more humid, states of the particular soil used proved more favourable for streptomycetes. Temperature did not seem to greatly affect the overall survival of the inoculants in soil. Duration of germination period, though, was longer at 22 °C, than at any other temperature examined. The indigenous *S.griseus* soil isolate proved to be a good colonizer of soil, whereby the introduced *S.lividans* strains survived equally well. Only in non-sterile soil was the natural isolate at an obvious advantage, since it survived and remained above detection limits, while population levels of *S.lividans* TK24 pIJ673 rapidly declined and were no longer detectable. Plasmid pIJ673, hosted by *S.lividans* TK24, was stable under all environmental conditions examined. This is a very important finding, since any such plasmids that may be introduced into the natural environment would be likely to persist for long periods within the soil gene pool. The addition of antibiotics into soil did not greatly affect the survival of sensitive and resistant strains, or the stability of the plasmid. For neomycin, this may be attributed to adsorption of the water soluble antibiotic to clay particles (Recorbet et al., 1990). The fact that the indigenous *S.griseus* strain did not acquire pIJ673 in soil, or *in vitro*, is very significant for risk assessment. It has so far been postulated that genetic elements introduced into soil microbial communities might readily spread among the natural microorganisms. Since most *Streptomyces* strains possess restriction systems, and plasmids, conjugal horizontal plasmid transfer to natural populations might not be as easy, as it is among well studied introduced strains. Intraspecific horizontal plasmid transfer has also been reported previously, and we proved that the system we devised is adequate for the detection of such events in sterile soil.

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