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Occurrence and diversity of plasmids in populations of streptomycetes in soil

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Abstract

Studies were made of naturally occurring plasmids hosted in *Streptomyces* strains isolated from two different terrestrial ecosystems: an agricultural field and a protected forest area. Six out of the 147 screened isolates contained plasmids. The strains containing these plasmids were all isolated from the agricultural soil. Plasmids were not found among the strains isolated from the forest area. Cross hybridization of the six newly isolated plasmids revealed very high similarities between four of them. However, no similarities were found between the six newly isolated plasmids and well studied streptomycete plasmids such as pIJ101 and SCP2*. The host strains of the four similar plasmids belonged to three different species *S. anulatus*, *S. rochei* and *S. diastaticus*. This implies a possible conjugative transfer of these plasmids within the streptomycete population in the agricultural area. The reason for the absence of streptomycete plasmids from the populations derived from the forest area is discussed.

Introduction

Members of the genus *Streptomyces* are Gram positive bacteria that belong to the class Actinobacteria. They are ubiquitous soil microorganisms that are found in many different soil types where they play an important role in soil decomposition processes. One of the most important features of this genus is that many members are capable of producing antibiotics and other useful secondary metabolites for medical and agricultural applications (Crandall & Hamill 1986). Bérdy (1995) reported that among the 11900 antibiotics discovered up to 1994, approximately the 55% of them were produced by *Streptomyces* species. The complex streptomycete life cycle which includes a series of morphologically distinct forms involves differentiation of the mycelium into substrate and aerial hyphae followed by sporulation. *Streptomyces* species are also distinguished by the high G+C content (69–78%) of their DNA. In most cases their chromosome has been reported to be linear (Leblond et al. 1993; Lin et al. 1993; Chen et al. 1994).

A large number of *Streptomyces* plasmids has been reported (Chen et al. 1993; Evans et al. 1994; Zotchev & Schrempf 1994). According to Kendall & Cohen (1988) streptomycete plasmids contain genes involved in plasmid spread: this allows their dissemination through the mycelium. The plasmids may be present as covalently closed circular (ccc) or linear DNAs, autonomously replicating or chromosomally integrated, ranging in size from a few to several hundred kilobases (Servin-Gonzalez et al. 1995). In most cases, they are conjugative with the ability to mobilize chromosomal markers (Cma) (Hopwood & Kieser 1993).

Interspecific plasmid transfer within the genus *Streptomyces* was established in 1973 by Hopwood & Wright. Horizontal gene transfer of introduced plasmids among streptomycetes has been studied in sterile and non sterile soil microcosm systems (Herron & Wellington 1990; Wellington et al. 1990; Vionis et al. 1998). However, little is known about the ecological role of self-transmissible *Streptomyces* plasmids.

When a plasmid carries genes which promote survival in a specific environment it is easier to correlate plasmid dynamics with forces that determine its diversity. In contrast with nocardioform actinomycetes, such as *Mycobacterium*, *Nocardia* and *Rhodococcus*, which often contain catabolic plasmids, in *Streptomyces* only a single phenotype is associated with their plasmids: pock formation. Even in the case of large linear plasmids, *Streptomyces* plasmids rarely seem to

code for other properties affecting the host phenotype besides those associated with conjugation (Hopwood & Kieser 1993; Wu & Roy 1993; Netolitzky et al. 1995). As a consequence, the forces that determine their dissemination in various environments are poorly understood. According to Kinashi (1994) the ability of plasmids to transfer and integrate into the chromosome could have played an important role in DNA shuffling resulting in the spreading of antibiotic synthetic gene clusters between these microrganisms.

Knowledge about plasmid distribution, diversity and evolution in relation to natural habitats is needed in order to understand the role of plasmids in the flow of genetic information between bacterial communities in nature. This knowledge could be obtained by the identification and characterisation of new naturally occurring plasmids originating from indigenous bacterial populations.

The aim of this work was to study the occurrence and diversity of plasmids recovered from streptomycete populations isolated from two different Mediterranean soil ecosystems: an agricultural field and a protected forest area. In addition, attempts were made to detect potential gene transfer events.

Material and methods

Bacterial strains and plasmids

One hundred and forty seven *Streptomyces* strains, which had previously been isolated and identified, were studied (Katsifas et al. 1999). The collection of isolates is presented in Table 1. *Streptomyces lividans* TK24 (Str^r) was used in the pock formation and conjugation experiments. Plasmids pIJ673, pQR210 and pQR213 were used as probes in the hybridization experiments. pIJ673 is a derivative of pIJ101, a conjugative, multicopy plasmid with cloned resistance determinants against viomycin (vph), thiostrepton (tsr) and neomyin (Tn5) (Kieser et al. 1982). pQR210 and pQR213, derivatives of pUC18, contain the *tra* and *cma* region of *Streptomyces* plasmid SCP2*, respectively (Rehman 1995). Plasmid pIJ702 (Katz et al. 1983), a pIJ101 derivative lacking the *tra* region with cloned resistance determinant against thiostrepton (tsr) and genes encoding for melanin production was used in the transformation experiments.

Soils

Two soil types were selected, these differed markedly in terms of structure, phosphorus and organic matter content as described by Katsifas et al. (2000). Seventy *Streptomyces* isolates were obtained from a sandy silt/sandy silt loam soil, according to Alexander's triangle (1977), collected from the selected agricultural area and named soil type A. The pH of the soil was 7.9, the phosphorus content 124 mg l^{-1} dry soil and the organic carbon content 1.23%. The remaining 77 *Streptomyces* isolates were isolated from a selected preserved forest area with a sandy loam soil and named soil type B. The pH of this second soil was 8.2, the phosphorus content 4 mg l^{-1} dry soil and the organic carbon content 2.7%.

Media and culture conditions

For plasmid isolation the *Streptomyces* strains were grown in liquid YEME medium (Hopwood et al. 1985) and the *E. coli* strains in LB medium. For the pock formation experiments either solid R2YE medium or R5 medium (Hopwood et al. 1985) were used. The AGS medium (Herron & Wellington 1990) was used for sporulation of the *Streptomyces* strains. The media were supplemented with appropriate antibiotics (streptomycin 50 μg ml^{-1}; thiostrepton 50 μg ml^{-1}; neomycin 10 μg ml^{-1} and ampicillin 50 μg ml^{-1}) when necessary.

DNA manipulation

Plasmid DNA was isolated from streptomycete mycelia and *E. coli* by an alkaline lysis procedure as described by Hopwood et al. (1985) and Kieser (1984) with some modifications as different indigenous strains required different incubation times with the lysozyme and alkaline SDS solution (0.3 NaOH, 2% SDS). Large scale isolation of plasmid DNA was followed by further purification on caesium chloride-ethidium bromide gradients. All of the digestions were carried out according to the supplier's instructions. The software package Kodak 1D Image Analysis was used to estimate band sizes. Genomic DNA from streptomycete strains was isolated by the method of Hopwood et al. (1985). For pulse field gel electrophoresis, DNA was prepared from mycelium immobilized in agarose blocks as described by Kieser et al. (1992); the running conditions were 145V, pulse time 90 sec for 29 hours.

Table 1. Phenotypic identification of isolates to species

Soil Type A (Agricultural area)			Soil Type B (Forest area)		
Species	Number of Strains (%)		Species	Number of Strains (%)	
S. cyaneus	4	(5.7%)	*S. cyaneus*	26	(33.7%)
S. diastaticus	11	(15.7%)	*S. diastaticus*	2	(2.6%)
S. exfoliatus	7	(10%)	*S. exfoliatus*	3	(3.9%)
S. griseoflavus	1	(1.4%)	*S. griseoruber*	7	(9%)
S. griseoruber	1	(1.4%)	*S. griseoviridis*	1	(1.3%)
S. anulatus	18	(25.7%)	*S. anulatus*	25	(32.5%)
S. rochei	13	(18.6%)	*S. violaceusniger*	1	(1.3%)
S. violaceus	3	(4.3%)	*S. phaeochromogenes*	1	(1.3%)
S. chromofuscus	10	(14.3%)	*S. rochei*	8	(10.4%)
			S. violaceus	2	(2.6%)
S. lavendulae	2	(2.9%)	*S. lavendulae*	1	(1.3%)
Totals	70		Totals	77	

DNA labelling, dot blot analysis and southern hybridization

A nonradioactive nucleic acid labelling-detection system, namely Digoxigenin Kits from Boehringer were used to label pIJ673, pQR210, pQR213 and the new isolated plasmids. Dot blots contained 10 ng heat-denaturated genomic DNA per spot. Southern blotting was done according to Sambrook et al. 1989. The DNA was cross-linked to the nylon filter by subjecting the filter to UV irradiation for 3 min. Prehybridization was performed at 68 °C for 3–4 hours and was followed by hybridization overnight under the same conditions. After hybridization, the filter was washed twice with 2xSSC, 0.1% SDS at room temperature for 10 min and twice with 0.1xSSC, 0.1% SDS at 68 °C for 20 min. The development of the hybridized DNA was performed following the Digoxigenin Kit instructions.

Transformation

Plasmid pIJ702 was introduced into the *S. anulatus* (tsr^s) isolate, host of the newly isolated plasmid pMAR6, by polythylene-glycol-mediated transformation of protoplasts which were subsequently regenerated on R2YE medium (Thomson et al. 1982). Melanin-producing transformants were selected and subsequently maintained by growth on media containing 50 μg thiostrepton ml^{-1}.

Pock formation and conjugation experiments

Pocks were detected by plating approximately 10^7 spores of the plasmid-free strain *S. lividans* TK24 onto R5 plates and incubating at 30 °C. The same number of plasmid-carrying-strain spores were added on the plate after 12 h of incubation. The plates were incubated at 30 °C for up to 10 days and examined daily for the appearance of pocks (Hopwood et al. 1985).

Crosses between the *S. anulatus* isolate hosting both pMAR6 and pIJ702 plasmids (str^s, tsr^r) and *S. lividans* TK24 (str^r, tsr^s) were performed as described by Hopwood et al. (1985).

Results

Streptomycete isolates

In total 147 streptomycete isolates were screened for the presence of plasmids. The majority of the strains isolated from both soil types were identified to *Streptomyces* species-groups; two strains from soil type A and one strain from soil type B were assigned to the phenotypically dinstinct species *S. lavendulae* (Table 1). *S. anulatus*, *S. diastaticus* and *S. rochei* were the most dominant species in streptomycete community of the agricultural area. In the forest samples this was also the case for *S. anulatus* and *S. rochei* but not for *S. diastaticus*.

Table 2. Characteristics of the newly isolated plasmids

Group	Plasmid	Host Strain (cluster/subcluster)	Structure	Size	Stability	Pock formation
1	pMAR1	*S. rochei* (12)	*ccc*	80 kb	very high	yes
2	pMAR2	*S. griseoruber* (21)	*ccc*	50 kb	very high	yes
1	pMAR3	*S. griseus* (1B)	*ccc*	80 kb	very high	yes
3	pMAR 4	*S. exfoliatus* (5)	*ccc*	45 kb	very high	yes
1	pMAR5	*S. diastatochromogenes* (19)	*ccc*	80 kb	very high	yes
1	pMAR6	*S. griseus* (1B)	*ccc*	80 kb	very high	yes

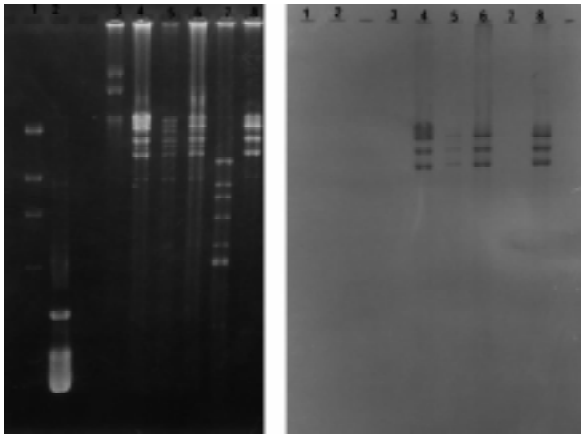

Figure 1. Hybridization among the 6 new plasmids using pMAR1 as probe. Lanes. 1: λ DNA/*Hin*dIII, 2: pBluescript II KS, 3: pMAR2/*Eco*RI, 4: pMAR5/*Eco*RI, 5: pMAR3/*Eco*RI, 6: pMAR6/*Eco*RI, 7: pMAR4/*Eco*RI, 8: pMAR1/*Eco*RI.

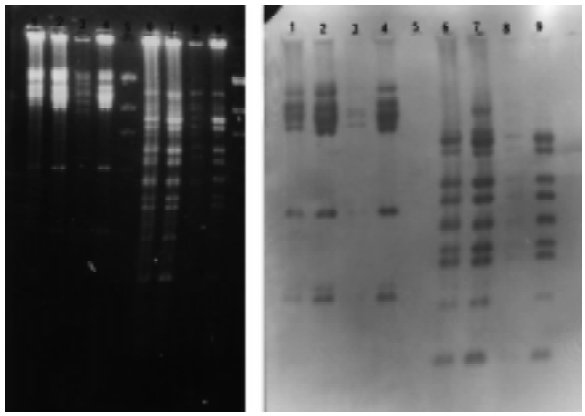

Figure 2. Hybridization among the new plasmids using pMAR1 as probe. Lanes.1: pMAR6/*Eco*RI-*Hin*d III, 2: pMAR5/*Eco*RI-*Hin*d III, 3: pMAR3/*Eco*RI-*Hin*d III, 4: pMAR1/*Eco*RI-*Hin*d III, 5: λ DNA/*Hin*d III, 6: pMAR6/*Cla* I-*Xho* I, 7: pMAR5/*Cla* I-*Xho* I, 8: pMAR3 /*Cla* I-*Xho* I, 9: pMAR1/*Cla* I-*Xho* I.

Plasmids isolated and characterised by restriction analysis

Six out of the 147 strains were found to contain plasmids. The strains containing these plasmids were all derived from the agricultural soil (type A). The sizes of pMAR1, pMAR3, pMAR5 and pMAR6 were estimated by restriction analysis with single and double digestions. According to these results the plasmids were classified into three groups (Table 2).

Cross hybridizations between plasmids

All the new plasmids were first digested with *Hin*d III, *Eco* RI, *Cla* I and *Xho* I. The restriction patterns of plasmids pMAR1, pMAR3, pMAR5 and pMAR6 were very similar and homologies among them were investigated by hybridization experiments using plasmid pMAR1 as the probe. All of the plasmids were digested with *Eco* RI. No similarity was detected between the labelled plasmid pMAR1 and the remaining two plasmids (pMAR2 and pMAR4), whereas the similarity between pMAR 1,3,5,6 was confirmed (Figure 1).

Double digests with *Eco*RI, *Hin*d III and *Cla* I, *Xho* I restriction enzymes of plasmids pMAR1, pMAR3, pMAR5 and pMAR6 and additional hybridization using pMAR1 as probe proved that these 4 plasmids were identical (Figure 2). Plasmid pMAR4 was used as a probe against pMAR2 but no similarity was observed between these two plasmids.

Well characterized plasmids such as pIJ673, pQR210 and pQR213 were used as probes in order to investigate the existence of shared DNA sequences between the new plasmids and the known ones. In all cases no strong hybridizations signals were observed.

Figure 3. Crosses between *S. griseus* pMAR6 and *S. lividans* TK24 reveal pock formation.

Investigation of plasmid function

No obvious phenotypic characteristics were associated with the 6 new plasmids. To investigate potential functions they were used as probes in hybridization experiments against a *Streptomyces coelicolor* A3(2) cosmid library (Redenbach et al. 1996). None of the plasmids showed significant similarity with the chromosomal DNA.

All of the plasmids were also tested for pock formation. Pocks were observed in all cases suggesting that the new plasmids were transferred from the host strains to the plasmid-free strain *S. lividans* TK24 (Figure 3). Crosses using the *S. anulatus* isolate, host of newly isolated plasmid pMAR6, as the donor strain displayed the highest frequency of pock formation among all of the host strains.

Mobilization of nonconjugative plasmids on agar

Plasmid pIJ702 was transformed into the *S. anulatus* strain which was host of the newly isolated plasmid pMAR6. Both plasmids, pMAR6 and pIJ702, seemed to co-exist for more than 100 generations as shown by hybridization experiments. Further crosses between the *S. anulatus* isolate hosting both plasmids and the plasmid-free strain *S. lividans* TK24 were performed in order to investigate mobilization of pIJ702 by pMAR6. Melanin producing transconjugants were obtained and the existence of pIJ702 was confirmed by hybridization experiments.

Detection of linear plasmids

The six host strains were also screened for the presence of linear plasmids by pulse field gel electrophoresis. These experiments revealed the presence of linear plasmids of plasmid group 1 in all four host strains. No linear molecules were present in isolates *S. griseoruber,* host of plasmid pMAR2 or in *S. exfoliatus*, host of plasmid pMAR4.

Figure 4. Dot blot hybridization among pMAR6 and total DNA of strains *S. griseus* host of pMAR6 (A2, positive control), *S. lividans* TK24 (A3, negative control) and isolates *S. venezuelae* (A5), *S. rochei* (A8, 9, 10, 11), *S. viridosporus* (B1, 2, 3, 4), *S. rochei* (B5,6,7,8), *S. rochei* (B9,10, 11, 12), *S. viridosporus* (C1,2,3).

Distribution of plasmid pMAR6

To obtain more information about the distribution of plasmids in the different host backgrounds the 147 isolates from both soil types were screened for the presence of pMAR6 by dot blot hybridization. These experiments revealed similarities between plasmid pMAR6 and 6 more *Streptomyces* strains (Figure 4). Further attempts to isolate ccc plasmids from these strains were not successful. With the exception of an isolate from soil type B (forest area), the new strains showing hybridizing sequences were from soil type A (agricultural area).

Discussion

Previous studies have failed to demonstrate any host-benefiting functions for streptomycete plasmids suggesting that they are cryptic nonmutualistic plasmids (Hopwood & Kieser 1993). This also seems to be the case in this survey. The fact that the plasmids are stably inherited, although not conferring any obvious phenotypic traits, is of considerable interest in the light of their persistence and possible dissemination in the soil ecosystem. It is not known whether these plasmids confer any benefits to their hosts or if they replicate only at the expense of their hosts.

Since plasmids were not isolated from strains originated from the protected and preserved forest areas while all new plasmids were isolated from strains originated from the agricultural area, a heavily disturbed and frequently changing environment, one may assume that these plasmids could provide an adaptation benefit to the indigenous bacterial population. This deduction is also in agreement with the observation that the hosts of plasmid group 1 belong to the most dominant species of the isolated streptomycete population.

The 4 host strains with identical plasmids (group 1) belong to 3 different species-groups: *S. anulatus*, *S. rochei* and *S. diastaticus*. These data strongly imply transfer of the isolated plasmids within the streptomycete communities in the natural environment. This possibility is also supported by their ability to mobilize nonconjugative plasmids such as pIJ702. This very important finding raises the question whether the newly isolated plasmids could also mobilize tra$^-$ plasmids and whether they are actively transferred under natural conditions.

Gene transfer between streptomycetes in soil may not be a rare event, but it has only been clearly demonstrated between closely related strains and is affected by several environmental factors (Herron & Wellington 1990; Vionis et al. 1998; Ravel et al. 2000). Although plasmid transfer between *Streptomyces* spp. in sterile (Rafii & Crawford 1988; Bleakley & Crawford 1989) and non-sterile soil (Wellington et al. 1990) has already been reported, transfer to members of the indigenous soil microflora is difficult to explore due to the lack of knowledge concerning the phenotypic properties of potential recipients. These new plasmids will be used in further experimental work to determine whether they transfer under physicochemical conditions relevant to their natural habitats.

The detection of DNA-DNA homology between plasmid pMAR6 and the *S. rochei* isolate from the forest area poses a number of questions about plasmid occurrence. Since all attempts to isolate the plasmid from *S. rochei* failed, the positive hybridization signal could be attributed to an integrated mobile element which shares similar sequences with pMAR6. However, it is not possible to determine whether these sequences code for plasmid back-bone genes such as replication or transfer determinants or if they are associated with other genes.

The role of cryptic plasmids in the soil environment remains to be elucidated. The dissemination of antibiotic resistance and xenobiotic catabolic genes by mobile genetic elements such as plasmids is well established (de la Cruz & Davies 2000) but studies focused on plasmids isolated from different habitats will provide clues about bacterial responses to other environmental stresses.

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