A gene cloning system for '*Streptomyces toyocaensis*'

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We explored different methods of introducing DNA into '*Streptomyces toyocaensis*' and *Streptomyces virginiae* to construct stable recombinant strains. Plasmid pIJ702 isolated from *Streptomyces lividans* transformed protoplasts of '*S. toyocaensis*' at a frequency of 7×10^3 transformants ($\mu\text{g DNA}$)⁻¹. pIJ702 prepared from '*S. toyocaensis*' transformed '*S. toyocaensis*' protoplasts at a frequency of 1.5×10^5 ($\mu\text{g DNA}$)⁻¹, suggesting that '*S. toyocaensis*' expresses restriction and modification. Plasmid pRHB126 was transduced by bacteriophage FP43 into '*S. toyocaensis*' at a frequency of 1.2×10^{-6} (p.f.u.)⁻¹. Plasmids pOJ436 and pRHB304 were introduced into '*S. toyocaensis*' by conjugation from *Escherichia coli* S17-1 at frequencies of about 2×10^{-4} and 1×10^{-4} per recipient, respectively. Analysis of several exconjugants indicated that pOJ436 and pRHB304 inserted into a unique $\phi\text{C31 attB}$ site and that some of the insertions had minimal deleterious effects on glycopeptide A47934 production. The results indicate that '*S. toyocaensis*' is a suitable host for gene cloning, whereas *S. virginiae* does not appear to be.

Keywords: '*Streptomyces toyocaensis*', transformation, transduction, conjugation, glycopeptide

INTRODUCTION

'*Streptomyces toyocaensis*' produces antibiotic A47934 (Boeck & Mertz, 1986; Zmijewski *et al.*, 1987) and *Streptomyces virginiae* produces antibiotic A41030A (Boeck *et al.*, 1985). A47934 and A41030A contain the crosslinked aromatic heptapeptide structure common to actaplanin, teichoplanin, ristocetin and other glycopeptides (Lancini & Cavalleri, 1990). Although both A47934 and A41030 lack sugars, they are included in the glycopeptide class since they maintain the characteristic heptapeptide structure. A41030A differs from A47934 by the absence of sulfate residues. A47934 and A41030A differ from the heptapeptide of the vancomycin-type glycopeptides at the N-terminal amino acid positions one and three (Lancini & Cavalleri, 1990). Although A47934 and A41030A lack sugar residues, they have potent *in vitro* antibiotic activity against strains of *Staphylococcus*, *Streptococcus* and *Enterococcus* (Lancini & Cavalleri, 1990).

We are interested in the organization and regulation of glycopeptide biosynthetic genes. We chose '*S. toyocaensis*' and *S. virginiae* as potential hosts because of the diverse molecular genetic tools available in streptomycetes (Baltz, 1994). Also, '*S. toyocaensis*' protoplasts are transformable by plasmid pFJ342 (Fayerman *et al.*, 1985).

Many other actinomycete strains produce glycopeptide

antibiotics (Lancini & Cavalleri, 1990), and a protoplast transformation system has been reported for *Amycolatopsis orientalis*, a vancomycin producer, using streptomycete self-replicating plasmids (Matsushima *et al.*, 1987b). However, the effects of self-replicating plasmids on vancomycin production have not been studied, and it is not known if DNA can be readily inserted into the *A. orientalis* chromosome. The availability of chromosomal insertion vectors that integrate site-specifically using the bacteriophage $\phi\text{C31 att/int}$ system (Bierman *et al.*, 1992) makes the availability of a streptomycete cloning system potentially very attractive. To determine if '*S. toyocaensis*' or *S. virginiae* is a suitable host for gene cloning and molecular genetic manipulation, we explored different methods to introduce DNA into them. We show that plasmid DNA can be introduced into '*S. toyocaensis*' by protoplast transformation, by bacteriophage-FP43-mediated transduction, and by conjugation from *Escherichia coli* S17-1. Cosmid DNA containing a large insert of heterologous DNA inserted into the bacteriophage $\phi\text{C31 attB}$ site in '*S. toyocaensis*', and stable recombinants were obtained that produced nearly normal levels of A47934.

METHODS

Strains, plasmids and bacteriophages. The strains, plasmids and bacteriophages used in this study are listed in Table 1.

Media and culture conditions. *E. coli* DH5 α was grown in TY broth (Rao *et al.*, 1987), with addition of 100 μ g apramycin (Am) ml⁻¹ when necessary to maintain plasmid DNA. The streptomycete strains were grown in TS broth and fragmented by ultrasonic vibration (Baltz, 1978). Protoplast regeneration was carried out on modified R2 agar (Baltz, 1978), R2M medium (Matsushima & Baltz, 1988), R2LE medium (Matsushima & Baltz, 1989), R2T20 medium (Yamamoto *et al.*, 1986) and R6 medium (Illing *et al.*, 1989). AS-1 agar (Baltz, 1980), Bennett's (Bnt) agar (Waksman, 1961) and modified R2 agar were used for conjugation analysis. Modified R2 soft agar was as described by Baltz (1978). Bacteriophage FP43 was grown on *Streptomyces griseofuscus* MM30 mycelial fragments on NC agar (Cox & Baltz, 1984) to prepare a transducing lysate as described by McHenney & Baltz (1988). P medium was as described by Hopwood *et al.* (1985).

Protoplast formation and transformation. Protoplasts were prepared and transformations were carried out by procedures optimized for the following micro-organisms: *Streptomyces fradiae* (Matsushima & Baltz, 1985); *A. orientalis* (Matsushima *et al.*, 1987b); *Micromonospora rosaria* (Matsushima & Baltz, 1988); *Saccharopolyspora erythraea* (Yamamoto *et al.*, 1986); *Saccharopolyspora spinosa* (Matsushima & Baltz, 1994). The transformation procedure for '*S. toyocaensis*' was modified from that described by Fayerman *et al.* (1985) as follows: cells were grown in TS broth, then passaged in TS broth plus 0.4% glycine at 37 °C with shaking as described by Baltz (1978). Cells were washed twice by centrifugation and resuspended in P medium (Hopwood *et al.*, 1985), then incubated in P medium plus 1–2 mg lysozyme ml⁻¹ for 1 h at 37 °C. The resulting protoplasts were washed twice by centrifugation and resuspended in P medium, and were finally resuspended in one tenth the original volume. Plasmid DNA (1–5 μ l) was added to 100 μ l protoplast suspension and mixed. PEG 1000 (500 μ l of 20%, v/v, in P medium) was added and mixed in. An additional 500 μ l P medium was added and mixed quickly; dilutions were made in P medium and immediately added to modified soft agar overlays and spread on dehydrated modified R2 agar plates (Matsushima & Baltz, 1985). The plates were incubated at 29 °C for 16–24 h, then modified R2 soft agar overlays containing the appropriate antibiotic were added to each plate. Thiostrepton (Ts) or Am was added to give final bottom agar concentrations of 25 μ g ml⁻¹. Percentage regeneration was determined on control plates lacking antibiotic as described by Baltz & Matsushima (1981).

Bacteriophage host range. The bacteriophage host range analysis was carried out as described by Cox & Baltz (1984) using bacteriophage lysates prepared on *S. griseofuscus*.

Transduction of plasmid DNA by bacteriophage FP43. Transduction of plasmid pRHB126 was carried out generally as described by McHenney & Baltz (1988). '*S. toyocaensis*' A80934 was grown at 37 °C to exponential phase. Cells (100 μ l; $\sim 2 \times 10^7$ c.f.u.) were mixed in an R2 soft agar overlay with 8×10^6 p.f.u. of an FP43 lysate prepared on *S. griseofuscus* MM30. The contents were spread on a modified R2 agar plate and incubated at 34 °C. After 4 h, anti-FP43 antiserum was added and Ts or Am was added at 6 h as described by McHenney & Baltz (1988).

Conjugation from *E. coli* S17-1 to '*S. toyocaensis*'

Spores. Spore stock (100 μ l) of '*S. toyocaensis*' A80934 ($\sim 4 \times 10^8$ ml⁻¹) was mixed with 50 μ l stationary phase *E. coli* S17-1 containing plasmid DNA and spread on the surface of modified R2, AS-1 or Bnt agar with a glass spreader. The agar plates were incubated for 16–24 h at 29 °C, then Am plus naladixic acid (Nal) in 3 ml soft nutrient agar was added to the

surface of the plates to give final bottom agar concentrations of 25 μ g ml⁻¹ for each antibiotic. The agar plates were incubated again for 5–10 d, and Am^R or Ts^R exconjugants were counted.

Mycelia. '*S. toyocaensis*' A80934 was grown for 16 h at 37 °C to stationary phase in TS broth, then fragmented by ultrasound. The culture was diluted 1:10 in fresh TS broth and incubated for an additional 5 or 7 h. Mycelia from the stationary phase culture and from the 5 and 7 h cultures were mixed with *E. coli* S17-1 containing plasmid DNA and spread on the surface of modified R2, AS-1 or Bnt agar. The agar plates were incubated and overlaid with appropriate antibiotics as described in the spore protocol above.

Molecular techniques. Methods for the manipulation of DNA were as described by Sambrook *et al.* (1989) and Hopwood *et al.* (1985). Southern hybridizations were carried out as described by Solenberg & Burgett (1989) using digoxigenin-labelled pOJ436 as a probe (Boehringer Mannheim Genius protocol). Restriction endonucleases were obtained from New England Biolabs, Bethesda Research Labs, Boehringer Mannheim Biochemicals, Amersham, United States Biochemical or Promega, and were used according to the specifications of the manufacturers.

Analysis of antibiotic production. Antibiotic production was determined by fermentation and subsequent HPLC analysis of culture broths as described by Zmijewski *et al.* (1987).

RESULTS AND DISCUSSION

Bacteriophage host range

Many streptomycete bacteriophages display a broad host specificity that is limited primarily by the expression of restriction barriers in particular hosts (Cox & Baltz, 1984; Matsushima *et al.*, 1987a; McHenney & Baltz, 1988; Matsushima & Baltz, 1989; Hahn *et al.*, 1990). Streptomycetes that propagate a high percentage of streptomycete bacteriophages tend to be much less restricting for plasmid transformation than those that propagate a smaller percentage of bacteriophages (Cox & Baltz, 1984; Larson & Hershberger, 1984; Matsushima & Baltz, 1985, 1989; Matsushima *et al.*, 1987a). Therefore, as a first test to characterize the two streptomycete glycopeptide producers as potential hosts for genetic manipulation, we carried out a bacteriophage host range analysis. '*S. toyocaensis*' A80934 propagated all seven bacteriophages (FP4, FP22, FP43, FP46, FP54, FP55 and FP60). *S. virginiae* A41030.4 propagated only three (FP4, FP43 and FP55) of the seven. These data suggest that *S. virginiae* may express a significant restriction barrier, and that the '*S. toyocaensis*' strains may be better suited for transformation studies.

Protoplast regeneration and transformation

Protoplasts of *S. virginiae* were prepared by five different methods developed for *S. fradiae*, *A. orientalis*, *M. rosaria*, *Sacch. erythraea* and *Sacch. spinosa*, but only those prepared by the method optimized for *S. fradiae* were able to regenerate viable cells. None of the protoplasts were transformed by the broad host range streptomycete plasmid pIJ702. '*S. toyocaensis*' protoplasts prepared by the *S. fradiae* method also regenerated viable cells at a 6%

Table 1. Bacterial strains, bacteriophages and plasmids

Strain, bacteriophage or plasmid	Relevant characteristics	Reference/source
Strain		
<i>Escherichia coli</i>		
S17-1	RP4 derivative integrated in chromosome	Simon <i>et al.</i> (1983)
DH5 α	F ⁻ <i>endA1 hsdR17</i> (τ_K^-) <i>supE44 thi-1 λ^- recA1 deoR gyrA96 relA1 Δ(argF-lacZYA) U169 ϕ80d lacZΔ M15 Nal^R</i>	Bethesda Research Labs
<i>Streptomyces griseofuscus</i>		
ATCC 23916	Host for preparing bacteriophage lysates	Cox & Baltz (1984)
MM30	ATCC 23916 containing pRHB126	McHenney & Baltz (1991)
<i>Streptomyces lividans</i>		
TK23	Host for preparing plasmid DNA	Hopwood <i>et al.</i> (1985)
<i>'Streptomyces toyocaensis'</i>		
A80934	Produces glycopeptide A47934	Fayerman <i>et al.</i> (1985)
PM317	A80934::pOJ436	This work
PM318	A80934::pOJ436	This work
PM319	A80934::pOJ436 Δ 1	This work
PM320	A80934::pOJ436 Δ 2	This work
PM321	A80934::pOJ436 Δ 3	This work
PM322	A80934::pOJ304	This work
PM323	A80934::pOJ304	This work
PM324	A80934::pOJ304	This work
PM325	A80934::pOJ304	This work
PM326	A80934::pOJ304	This work
<i>Streptomyces virginiae</i>		
A41030.4	Produces glycopeptide A41030A	Boeck <i>et al.</i> (1985)
Bacteriophage		
FP43	Transduces plasmid pRHB126; broad host range	McHenney & Baltz (1991); Cox & Baltz (1984); Hahn <i>et al.</i> (1991)
FP22	Broad host range	Cox & Baltz (1984); Hahn <i>et al.</i> (1990)
FP4, FP46, FP54, FP55, FP60	Broad host range	Cox & Baltz (1984)
Plasmid		
pIJ702	<i>tsr mel</i> , 5.65 kb, broad host range	Katz <i>et al.</i> (1983); Baltz (1994)
pOJ436	Am ^R (cos)3' <i>rep</i> ^{puC} <i>oriT</i> <i>int</i> ^{ϕC31} , 11.2 kb	Bierman <i>et al.</i> (1992)
pRHB126	<i>rep</i> ^{ts} <i>pac</i> ^{FP43} <i>tsr</i> , 17.2 kb, broad host range	McHenney & Baltz (1991)
pRHB304	pOJ436 containing 37 kb insert of <i>Sacch. spinosa</i> DNA	Matsushima <i>et al.</i> (1994)

efficiency, but were not transformed under those conditions. However, protoplasts prepared by the method optimized for '*S. toyocaensis*' regenerated viable cells at 16% efficiency and were transformed at a frequency of 7.1×10^3 ($\mu\text{g pIJ702 DNA}$)⁻¹ prepared from *Streptomyces lividans*. pIJ702 prepared from a '*S. toyocaensis*' transformant transformed '*S. toyocaensis*' at a 20-fold higher frequency [1.5×10^5 transformants ($\mu\text{g DNA}$)⁻¹]. The transformation frequency obtained with modified pIJ702 is similar to the transformation frequencies in '*S. toyocaensis*' reported by Fayerman *et al.* (1985). '*S. toyocaensis*' was not transformed by pRHB126 prepared from *S. griseofuscus*. pRHB126 is derived from, but is larger than, pIJ702 (Table 1). These data suggest that '*S. toyocaensis*'

expresses some restriction and modification. *S. virginiae* did not appear to be readily transformed.

FP43-mediated transduction of '*S. toyocaensis*' by pRHB126

Since the transformation study suggested that pRHB126 was effectively restricted during protoplast transformation, we were interested in determining if this plasmid could be introduced by transduction. pRHB126 contains the DNA packaging origin (*pac*) from bacteriophage FP43, and it and its parent plasmid pRHB106 are readily transduced into many streptomycetes, including some that are highly restricting for bacteriophage plaque

Table 2. Conjugal transfer of plasmids from *E. coli* S17-1 to glycopeptide-producing streptomycetes

Recipient strain	Plasmid	Protocol	Mating ratio (streptomycete: <i>E. coli</i>)	Conjugation medium	Exconjugants per recipient
' <i>S. toyocaensis</i> ' A80934	pOJ436	Mycelia	10:90	R2, AS-1, Bnt	$< 2.8 \times 10^{-7}$
			50:50	R2, AS-1, Bnt	$< 5.6 \times 10^{-8}$
			90:10	R2, AS-1, Bnt	$< 3.1 \times 10^{-8}$
	pRHB304	Spores	10:90	R2	1.7×10^{-4}
			50:50	R2	2.2×10^{-4}
			90:10	R2	1.1×10^{-5}
<i>S. virginiae</i> A41030.4	pOJ436	Mycelia	10:90	R2	5.6×10^{-5}
			50:50	R2	1.1×10^{-4}
			90:10	R2	$< 5.0 \times 10^{-7}$
	pRHB304	Spores	10:90	R2, AS-1, Bnt	$< 1.0 \times 10^{-7}$
			50:50	R2, AS-1, Bnt	$< 1.0 \times 10^{-7}$
			90:10	R2, AS-1, Bnt	$< 5.6 \times 10^{-8}$
		Spores	ND	ND	ND

ND, Not determined, since *S. virginiae* did not sporulate on seven different media.

formation and plasmid transformation (McHenney & Baltz, 1988, 1991; Matsushima *et al.*, 1989). pRHB126 was introduced into '*S. toyocaensis*' at a frequency of 1×10^{-6} (p.f.u.)⁻¹ when selections were made for Am^R or Ts^R. This frequency is similar to those obtained in other streptomycetes (McHenney & Baltz, 1991). These data confirm that pRHB126 replicates in '*S. toyocaensis*', and that the lack of protoplast transformation by pRHB126 is likely due to restriction.

Conjugal mating of streptomycetes with *E. coli* S17-1

Several cloning vectors capable of conjugal transfer from *E. coli* to actinomycetes are now available (Baltz, 1994; Bierman *et al.*, 1992; Mazodier *et al.*, 1989). Some of these (e.g. pOJ436) can integrate site-specifically to form very stable recombinants. The process of conjugation has the added benefit that it can circumvent restriction barriers (Baltz, 1994; Bierman *et al.*, 1992; Matsushima *et al.*, 1994). We carried out conjugal matings between *E. coli* S17-1 harbouring pOJ436 and mycelia of '*S. toyocaensis*' and *S. virginiae* under several conditions, and none yielded exconjugants (Table 2). However, when we mated *E. coli* S17-1(pOJ436) with spores of '*S. toyocaensis*', exconjugants were obtained at very high frequencies. A 50:50 volume ratio of *E. coli* cells to '*S. toyocaensis*' spores yielded 2.2×10^{-4} exconjugants per '*S. toyocaensis*' recipient. We also carried out conjugation studies with *E. coli* S17-1 harbouring pRHB304. pRHB304 is a non-replicating cosmid derived from pOJ436 and contains a 37 kb insert of *Sacch. spinosa* DNA. pRHB304 formed exconjugants at frequencies very similar to those obtained with pOJ436, indicating that the size of pRHB304 did not pose a significant problem for transfer, integration or host restriction. Since the transformation and transduction experiments indicated that '*S. toyocaensis*' expresses some level of restriction, the conjugation data are consistent with previous observations that conjugation from *E. coli* to actinomycetes is an efficient means for bypassing restriction barriers (Bierman *et al.*, 1992; Matsushima *et*

al., 1994). It appears that '*S. toyocaensis*' cells in the process of germination may be most susceptible to efficient conjugal mating. We attempted to prepare spores of *S. virginiae* on several different media, including AS-1, Bnt, NC, TSA, R2 and R6, but were unsuccessful.

Integration of pOJ436 and pRHB304 into a unique site in the chromosome

pOJ436 and pRHB304 do not contain replication functions for streptomycetes, but contain the bacteriophage ϕ C31 integration functions. ϕ C31 has a unique integration site in *Streptomyces coelicolor* (Lomovskaya *et al.*, 1971) and *Streptomyces ambofaciens* (Kuhstoss *et al.*, 1991), and two sites in *Sacch. spinosa* (Matsushima *et al.*, 1994). We cleaved DNA from '*S. toyocaensis*' exconjugants containing pOJ436 or pRHB304 with *Eco*RI and carried out Southern hybridization analysis of the fragments separated by agarose gel electrophoresis using pOJ436 as a probe. Fig. 1 shows that cosmid pOJ436 has two *Eco*RI fragments of about 10.0 and 1.2 kb. The 1.2 kb fragment contains ϕ C31 *attP* (Bierman *et al.*, 1992), so integration at *attB* should eliminate the 1.2 kb fragment, and generate two new junction fragments. Since the *attP* site is ~100 bp from one of the *Eco*RI sites in pOJ436, only one of the new junction fragments is readily observed (Matsushima *et al.*, 1994). Fig. 1 shows that all of the exconjugants containing pOJ436 lost the 1.2 kb *Eco*RI fragment and gained a new fragment of ~1.5 kb. Two of the exconjugants contained the 9.0 kb vector sequence, whereas the other three contained a fragment of lower molecular mass. These data indicate that pOJ436 integrates into a unique site in '*S. toyocaensis*', but that the plasmid can undergo partial deletion in some cases.

Fig. 1 also shows the Southern blot analysis of five exconjugants containing pRHB304. All five contained the 1.5 kb *Eco*RI fragment observed in the pOJ436 exconjugants, indicating that integration occurred at the same *attB* site. Each of the exconjugants contained two other

Fig. 1. Southern analysis of '*S. toyocaensis*' exconjugants containing pOJ436 or pRHB304. Total DNA digested with *Eco*RI was separated by gel electrophoresis and probed with digoxigenin-labelled pOJ436.

Table 3. Antibiotic production in '*S. toyocaensis*' recombinants containing pOJ436, pRHB304 or pIJ702

Duplicate fermentations were run for experiments I and III; results are presented as mean $\pm$ standard deviation. Single fermentations were run in experiment II.

Experiment	Strain	Cosmid insert/plasmid	A47934 ($\mu\text{g ml}^{-1}$)	Percentage of control
I	A80934	—	640 $\pm$ 8	100 $\pm$ 1.3
	PM317	pOJ436	512 $\pm$ 13	80 $\pm$ 2.0
	PM318	pOJ436	634 $\pm$ 4	99 $\pm$ 0.6
	PM319	pOJ436 Δ 1	467 $\pm$ 72	73 $\pm$ 11.2
	PM320	pOJ436 Δ 2	435 $\pm$ 35	68 $\pm$ 5.5
	PM321	pOJ436 Δ 3	429 $\pm$ 8	67 $\pm$ 1.3
II	A80934	—	440	100
	PM322	pRHB304	97	22
	PM323	pRHB304	334	76
	PM324	pRHB304	172	39
	PM325	pRHB304	383	87
	PM326	pRHB304	304	69
III	A80934	—	610 $\pm$ 61	100 $\pm$ 10
	PM327	pIJ702	< 122	< 20
	PM328	pIJ702	< 122	< 20

large *Eco*RI fragments that hybridized to pOJ436, indicating that the $\sim$ 37 kb insert in pRHB304 contains at least one *Eco*RI site. Analysis of *Eco*RI-cleaved plasmid DNA indicated that pRHB304 actually contains three *Eco*RI sites. Since the sizes of the large pOJ436-hybridizing *Eco*RI fragments were the same in the five exconjugants, the results suggest that pRHB304 does not undergo substantial deletion as observed with pOJ436. It

is possible that the apparent deletions of vector sequences in exconjugants containing pOJ436 are associated with the regions containing three *cos* sites. This region was partially or completely lost during *in vitro* packaging to prepare the cosmid library from which pRHB304 was isolated. The data suggest that large inserts of heterologous DNA can be stably inserted into the '*S. toyocaensis*' chromosome at the ϕ C31 *attB* site.

Antibiotic production in recombinants containing pOJ436, pRHB304 or pIJ702

Table 3 shows that recombinants containing pIJ702 produced < 20% of control antibiotic yields. This is consistent with observations in other streptomycetes that the presence of self-replicating plasmid vectors can have negative effects on secondary metabolite production (Asturias *et al.*, 1994; Cox & Seno, 1990; Decker *et al.*, 1994; Thomas *et al.*, 1991). The exconjugants containing pOJ436 or pOJ436 with deletions produced 67–99% of the control amount of A47934. Exconjugants containing pRHB304 produced 22–87% of control yields. The cause of the variability between different clonal isolates is not known. The data indicate, however, that insertion of plasmids into the ϕ C31 *attB* site can be associated with modest to no reduction in antibiotic productivity, so the conjugation and chromosomal insertion system provided by pOJ436 should be a useful method to explore the effects of cloned genes on glycopeptide production.

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