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Reviews

Transposition and transduction of plasmid DNA in *Streptomyces* spp.

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SUMMARY

To expand the application of molecular genetics to many different streptomycete species, we have been developing two potentially widely applicable methodologies: transposon mutagenesis and plasmid transduction. We constructed three transposons from the *Streptomyces lividans* insertion sequence IS*493*. Tn*5096* and Tn*5097* contain an apramycin resistance gene inserted in different orientations between the two open reading frames of IS*493*. These transposons transpose from different plasmids into many different sites in the *Streptomyces griseofuscus* chromosome and into its resident linear plasmids. Tn*5099* contains a promoterless *xylE* gene and a hygromycin-resistance gene inserted in IS*493* close to one end. Tn*5099* transposes in *S. griseofuscus* giving operon fusions in some cases that drive expression of the *xylE* gene product, catechol deoxygenase, giving yellow colonies in the presence of catechol. We have also developed plasmid vectors that can be transduced into many streptomycete species by bacteriophage FP43. We describe the characterization of FP43 and mapping of several bacteriophage functions. The region of cloned FP43 DNA essential for plasmid transduction includes the origin for headful packaging.

INTRODUCTION

Recently, molecular genetic methods have been developed and applied to the analysis of biosynthesis of secondary metabolites in the actinomycetes. Many biosynthetic genes have been cloned and analysed in some detail [18,27]. From these studies, it is apparent that most antibiotic biosynthetic pathway genes are clustered and that at least several clusters respond to positive regulation [4,27]. Cloned antibiotic biosynthetic genes have been moved into heterologous hosts, resulting in the production of hybrid antibiotics [2,8,12]. However, there are several problems associated with molecular methods to introduce and stably express heterologous genes in streptomycetes. Transformation protocols often need to be developed for individual species [11,19,20], and heterologous DNA is often subject to restriction [20–23]. Certain streptomycete plasmids are unstable with heterologous DNA inserts, and some plasmids cannot be stably maintained without selection. Furthermore, autonomously replicating vectors can cause reduced production of secondary metabolites in highly developed production strains (E.T. Seno, personal communication). Methods to overcome these problems may require fairly detailed information about the genes, plasmids and strains being used, thus limiting application of some molecular methods to fairly well characterized strains.

Since many streptomycetes and other actinomycetes produce secondary metabolites having diverse biological activities [7], it would be advantageous to be able to exploit the genetic diversity by having the ability to rapidly construct stable recombinants of many species. To accomplish this, methods to rapidly introduce DNA into many hosts and to stably insert the DNA into different sites in the chromosome were needed. To approach these problems, we have begun to develop transposition and plasmid transduction systems for streptomycetes.

TRANSPOSITION

Transposons have a number of potential applications for improving production of secondary metabolites and for the construction of strains to produce hybrid secondary metabolites. Transposons could be used for gene disruption to identify and clone global regulatory genes, secondary metabolite genes, and strong or regulated promoters. They could also be used to block com-

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peting or unnecessary pathways for more efficient production of secondary metabolites, and to stably insert heterologous DNA into actinomycete genomes. We have constructed three transposons that might be useful for some of these applications.

Construction of transposons Tn5096, Tn5097 and Tn5099

The isolation of an insertion element, IS*493*, from *S. lividans* was recently reported [29]. IS*493* is 1.6 kb in size, contains homologous inverted repeats and two putative open reading frames (Fig. 1). Two transposons, Tn*5096* (Fig. 1) and Tn*5097*, were constructed by cloning the *E. coli* apramycin-resistance (Am^R) gene into the unique *Sty*I site in IS*493* [28]. Tn*5097* contained the apramycin-resistance gene in the opposite orientation from Tn*5096*. A promoter-probe transposon, Tn*5099*, which can form operon fusions in vivo, was constructed by cloning a *xylE* and hygromycin-resistance (Hm^R) gene cassette [13,15] into the *Sau*I site of IS*493* (Fig. 1) [10]. Since the unique *Sau*I site was located in the left inverted repeat, we used a synthetic oligonucleotide to recreate the inverted repeat.

Analysis of transposition in S. griseofuscus

The transposons were cloned into *E. coli* vectors unable to replicate in *Streptomyces* spp., into the unstable streptomycete vector pHJL401 [16] or into the temperature-sensitive vector pGM160 [25], and introduced into *S. griseofuscus* by transformation. Colonies containing transposon insertions were selected by three procedures: (i) by selecting directly for Am^R or Hm^R after transformation with the non-replicating vector; (ii) by curing pHJL401-derived transformants through two cycles of sporulation, then selecting for Am^R clones; and (iii) by growing pGM160-derived transformants for 2 days at 29 °C in the presence of Am or Hm, shifting the temperature to 39 °C for 7 days, then picking Am^R or Hm^R sectors [28]. The temperature shift method was the most useful since many independent sectors containing transpositions were readily obtained. Strains containing putative transpositions were screened for loss of plasmid markers phenotypically and by Southern hybridization. Strains which showed hybridization to transposon (Fig. 2) but not to plasmid DNA probes were confirmed to have Tn*5096*, Tn*5097* or Tn*5099* insertion into

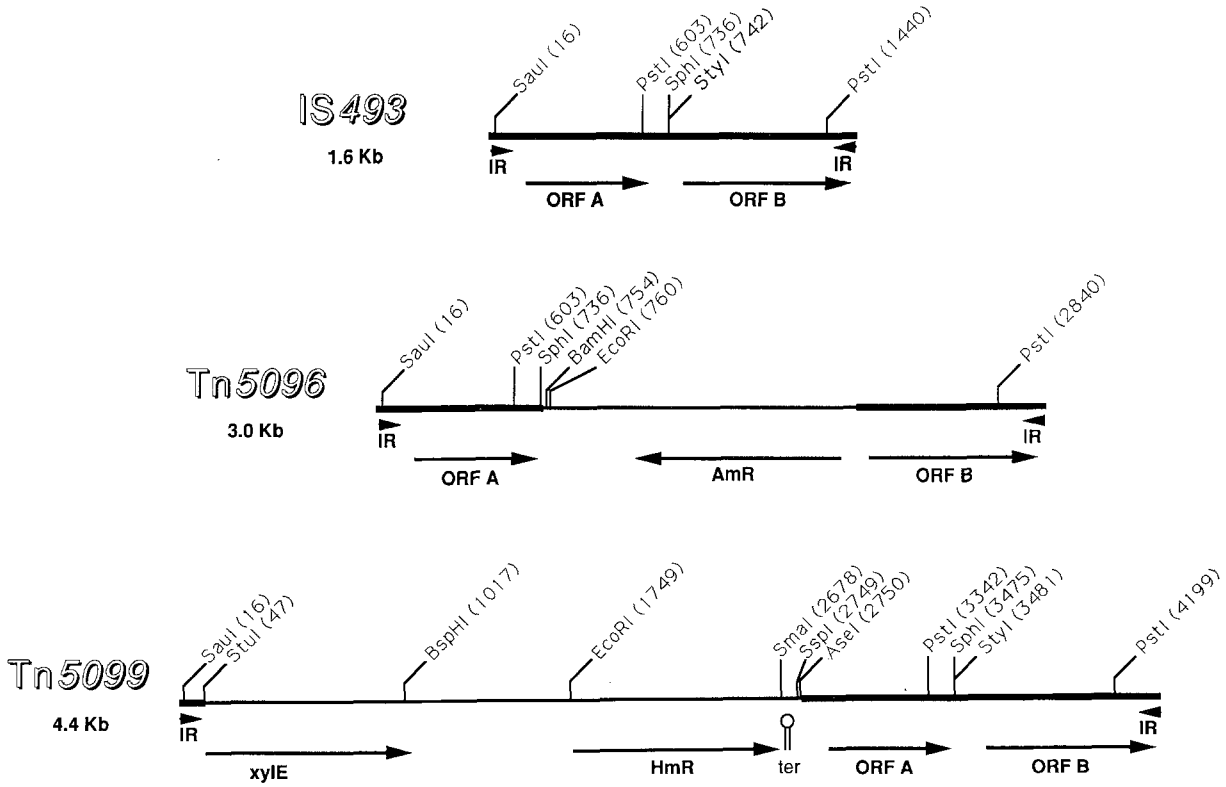

Fig. 1. Physical map of IS*493*, Tn*5096* and Tn*5099*. Tn*5096* was constructed by cloning an apramycin resistance gene (Am^R) into the unique *Sty*I site of IS*493* [28]. Tn*5099* was constructed by inserting a promoterless *xylE* gene, a hygromycin-resistance gene (Hm^R) and the *korB* transcription terminator (ter) [6] between the left end inverted repeat (IR) and ORF A (see Solenberg and Baltz [28] and Hahn et al. [10] for details of constructions and plasmid vehicles). Arrows indicate direction and approximate extent of open reading frames. Distance (base pairs) of each restriction site from the left end is indicated in parentheses.

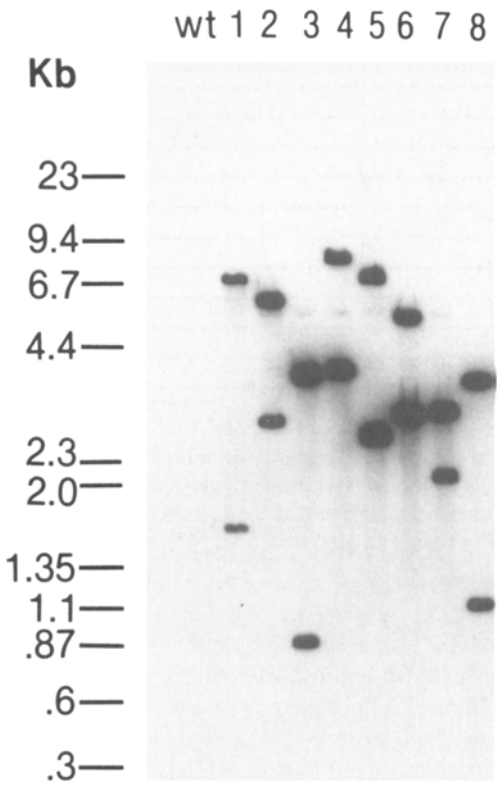

Fig. 2. Southern hybridization analysis of Tn*5096* and Tn*5097* transpositions in *S. griseofuscus* [28]. Total DNA from *S. griseofuscus* clones was digested with *Bam*HI and probed with [^{32}P]dCTP)-labelled Tn*5096* DNA. No hybridization to plasmid DNA was observed for these putative transpositions (data not shown). Numbers at the top of lanes correspond to different insertions. Lane 1 is a Tn*5097* insertion; all others are Tn*5096* insertions. Data from Solenberg and Baltz [28].

S. griseofuscus genomic DNA. Twenty-five independent clones containing Tn*5096* or Tn*5097* were analysed and 24 contained transposon insertions in different sites based on sizes of *Bam*HI fragments in Southern blots (Fig. 2). Two insertions (Ω3 and Ω16) were located on *Bam*HI, *Sph*I and *Sma*I fragments of similar size. DNA sequencing confirmed that these two insertions were in the same site [28].

The sequences of several insertion sites have been determined [28]. IS*493*-type elements appear to favor a CANTg target site and duplicate 3 bp of host DNA upon insertion.

To determine if Tn*5096* could transpose throughout the genome of *S. griseofuscus*, DNA from 21 *S. griseofuscus* strains containing Tn*5096* insertions was digested with *Ssp*I, and the fragments were separated by pulsed field gel electrophoresis [28]. At least 27 bands were evident in wild type DNA; two bands corresponded to linear plasmids (65 and 200 kb). The DNA was transferred to nylon membranes and probed with Tn*5096* DNA. Insertions have been observed in 12 *Ssp*I fragments, and 15 random insertions were located in 10 *Ssp*I fragments (Table 1). In addition, one insertion was located in the 65-kb plasmid and three insertions in the 200-kb plasmid. One-hundred-thirty *S. griseofuscus* strains containing Tn*5096* insertions were screened for particular phenotypes, and auxotrophic, sporulation-defective and antibiotic (lankacidin) blocked mutants have been identified [28]. Another transposon, Tn*5098* containing two tylosin genes inserted close to the apramycin-resistance gene [28] also transposed in *S. griseofuscus*. The frequency of auxotrophic mutations among strains containing transpositions was about 0.2% [28]. The data indicated that IS*493*-derived transposons transpose relatively randomly into plasmid and chromosomal sites.

The *xylE* gene in Tn*5099* was expressed in *E. coli* when Tn*5099* was cloned downstream of the *lac* promoter on a multicopy plasmid. Strains containing transpositions of Tn*5099* in *S. griseofuscus* showed high, low or no expression of the *xylE* gene product on plates [10]. Tn*5099* should, therefore, be useful to identify and clone strong and regulated promoters in streptomycetes.

Transposition and application in other streptomycetes

We have recently demonstrated that Tn*5096* can transpose in the tylosin producing *Streptomyces fradiae*. Experiments are underway to determine if Tn*5096* transposes in other species as well. We have demonstrated that two sites in IS*493*-derived transposons can accept heterologous DNA without abolishing transposition. We are in the process of cloning other segments of DNA into these transposons to determine if these elements can be used to construct stable recombinant strains containing new combinations of antibiotic biosynthetic genes.

TRANSDUCTION

McHenney and Baltz [22] have developed a method to transduce plasmid DNA using the streptomycete bacteriophage FP43 [9]. FP43 is a broad host range bacteriophage, plating on about 50% of streptomycete strains tested [5,20,23]. Because of its broad host range, gene transfer systems based on FP43 should be applicable to many streptomycete strains.

Bacteriophage FP43

FP43, a temperate bacteriophage, formed lysogens on *S. griseofuscus* [9]. In FP43 lysogens of *S. griseofuscus*, prophage DNA is inserted into the chromosome in a site-specific manner. A segment of FP43 DNA (*pin*) was able to block plaque formation of FP43 when cloned into

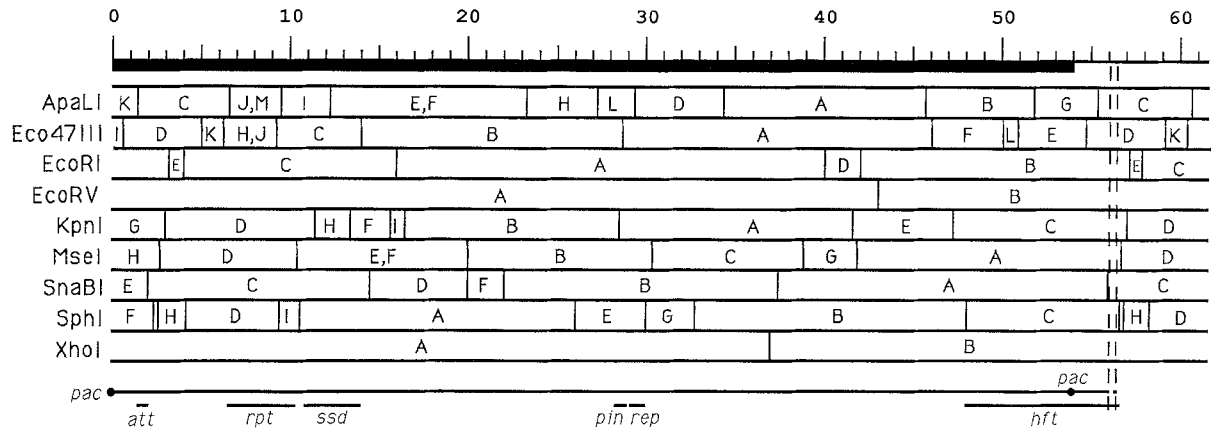

Fig. 3. Physical map of FP43 DNA. Zero corresponds to the site of initiation of DNA packaging and the numbering corresponds to the direction of DNA packaging. The bold line indicates the extent of the FP43 genome. The dashed lines indicate the approximate location of cleavage of the first headful. The approximate location of phage functions is indicated at the bottom of the map. With some digestions, submolar *pac* fragments were evident (eg. *Apa*L1 K; *Eco*47111 I; *Kpn*I G; *Mse*I H; *Sna*B1 E; and *Sph*I F). Data from Hahn et al. [9].

S. griseofuscus [9]. The FP43 genome was 54 kb in size and contained 65% G + C. The 56-kb FP43 DNA isolated from phage particles was terminally redundant and circularly permuted; FP43 packages its DNA by a headful packaging mechanism. FP43 does not share immunity or strong antisera cross-reactivity with any of nine streptomycete bacteriophage (FP4, FP22, FP46, FP50, FP55, FP60, FP61, VP11, R4) which were tested [9].

We constructed a restriction map of FP43 DNA (Fig. 3) starting at the site of initiation of DNA packaging (*pac*) and proceeding to the right in the direction of packaging. Six other bacteriophage functions have been mapped (Fig. 3). The bacteriophage attachment site (*att*) mapped at 2 kb and the *pin* function at 28 kb. A segment of FP43 DNA, *rep*, which mapped at 29 kb, can function as an origin of plasmid replication in *S. griseofuscus* and *S. ambofaciens* [1]. A region containing single-stranded DNA (ssd) was located between 10.6 to 14.5 kb on the map. A mutant (*rpt*) was isolated which contains a deletion of 3 kb between 6.2 and 10.5 kb on the map.

Transduction of plasmid DNA by FP43

A segment of FP43 DNA (*hft*) was cloned into pIJ702 [14], resulting in pRHB101 [22]. When FP43 was grown on a strain containing pRHB101, the plasmid was packaged into phage heads as a linear concatemer [22] and could be transduced into other strains. Under the conditions used, transduction was observed in about 80% of the streptomycete species tested, and in strains of at least three other genera, *Chainia*, *Streptoverticillium* and *Saccharopolyspora* [22,23].

We wanted to use FP43 to transduce plasmids containing cloned genes into different streptomycete strains. To test if heterologous DNA could be inserted into pRHB101 without affecting transducibility of the plasmid, the erythromycin resistance (*ermE*) gene of *Saccharopolyspora erythraea* was cloned from pKC488 [30] into the unique *Kpn*I site of pRHB101 [24]. The resulting plasmid, pRHB111, was transformed into *S. griseofuscus*. pRHB111 conferred high level resistance to erythromycin. FP43 transduced pRHB111 into wild type *S. griseofuscus* at a frequency of 1.6×10^{-4}/PFU when selecting for thiostrepton resistance [24]. This frequency was similar to the frequency of transduction of pRHB101 [22]. FP43 also transduced pRHB111 into *S. ambofaciens*, *S. thermotolerans*, *S. fradiae* and *S. albus* P at frequencies comparable to those observed with pRHB101, and high level resistance to erythromycin was expressed in all five species [24]. These experiments indicated that DNA can be inserted into pRHB101 without affecting transducibility of the plasmid, and that the cloned DNA can be readily expressed in heterologous hosts. To facilitate the cloning of heterologous genes for transduction, a derivative of pRHB101 (pRHB116), which contained an oligonucleotide with multiple restriction sites inserted at the *Kpn*I site was constructed. This vector might be useful to assess the transducibility of other cloned genes.

Transduction of plasmid DNA by FP43 has a number of advantages over transformation as a method of introducing DNA into streptomycetes. FP43 has a broad host range and can transduce plasmid DNA into many species. Transduction avoids the often difficult and tedious procedures of protoplast formation and regeneration required for standard transformations. Furthermore, since transduction uses cells rather than protoplasts, the cell growth conditions can be varied to minimize restriction [23].

TABLE 1

S. griseofuscus SspI bands with transpositions

Fragment size[a]	Transposon hits	Band	Strain[b]			
980/800	2	A	Ω10	Ω19		
605		B				
450		C				
440		D				
424	1	E	Ω7			
393	1	F	Ω17			
379*	3	G	Ω4	Ω11	Ω14	
327		H				
285		I				
252	1	J	Ω5			
233*	3	K	Ω3		Ω13	Ω16
211*		L				
197	1	M	Ω1			
197	3	M′[c]	Ω18	Ω20	Ω21	
175*		N				
150		O				
134		P				
109*	1	Q	Ω12			
97	1	R	Ω15			
81		S				
65		T				
65	1	T′[c]	Ω9			
48		U				
42	2	V	Ω2	Ω8		
36		W				
30		X				
24		Y				

[a] Approximate fragment size in kb based on standards (λ ladder and yeast chromosomes). Asterisks indicate heavy bands which may be doublets. Total size of the *S. griseofuscus* genome may be as low as 6400 kb or over 7500 kb if heavy bands are counted twice.

[b] Strains Ω1 to Ω16 were randomly selected. Other strains were selected for a spoecific phenotype (Ω19,20,21 = Bun$^-$; Ω17 = Phe$^-$; Ω18 = Spo$^-$). Ω3 and Ω16 are in *Bam*HI, *Sph*I and *Sma*I fragments of similar size.

[c] The M′ and T′ bands are linear plasmids that migrate into the gel in the absence of *Ssp*I digestion.

Also, in some cases, plasmids can be passaged through modifying hosts by transduction to help bypass restriction barriers [22]. Therefore, transduction of plasmid DNA by FP43 may simplify strain constructions in streptomycetes and broaden the utility of recombinant DNA technology to newly isolated and poorly characterized streptomycetes.

CONCLUSIONS

We have developed two molecular genetic methods to facilitate genetic manipulations in streptomycetes. We developed transposons which might be used to identify and clone genes or regulatory sequences of interest, and to stably insert genes into heterologous hosts. We also developed a plasmid transduction system to facilitate gene transfer between streptomycetes. We think that these methods will have applications for the improvement of secondary metabolite production and will aid in the discovery of novel or hybrid secondary metabolites in many streptomycetes.

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