JOURNAL OF BACTERIOLOGY, Sept. 1991, p. 5578–5581
0021-9193/91/175578-04$02.00/0

Vol. 173, No. 17

Transposition of Tn*5096* from a Temperature-Sensitive Transducible Plasmid in *Streptomyces* spp.

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Received 8 April 1991/Accepted 1 July 1991

Transposon Tn*5096* was inserted into a derivative of the temperature-sensitive plasmid pMT660 containing the bacteriophage FP43 *pac* site. The resulting plasmid, pRHB126, was transduced by FP43 into several *Streptomyces* species. Tn*5096* transposed from pRHB126 into different sites in the genomes of *Streptomyces ambofaciens*, *Streptomyces cinnamonensis*, *Streptomyces coelicolor* A3(2), *Streptomyces fradiae*, *Streptomyces griseofuscus*, and *Streptomyces thermotolerans*.

Tn*5096* is a transposon that contains an apramycin resistance gene [*aac(3)*IV or Am^r] inserted between two open reading frames in the *Streptomyces lividans* insertion sequence IS*493* (24, 25). Tn*5096* transposes from plasmids into many different sites in the *Streptomyces griseofuscus* chromosome and into two resident linear plasmids (24). The consensus target site for insertion, on the basis of a limited number of sequences, was CANTg. Sequence analysis of DNA flanking the target sites suggested that Tn*5096* transposes into protein coding regions and other sequences.

Two other derivatives of IS*493* also transpose in *S. griseofuscus*. Tn*5098* contains two antibiotic biosynthetic genes inserted next to the Am^r gene in Tn*5096* (24). Tn*5099* contains a promoterless *xylE* gene and a hygromycin resistance gene (15) inserted close to the left-end inverted repeat of IS*493*. Tn*5099* has been used to locate a phosphate-regulated promoter that drives *xylE* (catechol dioxygenase) expression in *S. griseofuscus* (12).

All three transposons derived from IS*493* transpose from a temperature-sensitive plasmid, pGM160 (21). A limitation of this delivery system is that pGM160 derivatives must be introduced into streptomycetes by protoplast transformation, and the plasmids containing the transposons are somewhat unstable (23). To further test the host range and utility of IS*493*-derived transposons, it would be advantageous to have a stable plasmid delivery vector that could be introduced into many *Streptomyces* spp. by transduction.

We recently cloned a segment of bacteriophage FP43 DNA into plasmid pIJ702 that caused the resulting plasmid (pRHB101) to be transducible by FP43 (18). The segment of DNA that mediates transduction contains a *pac* site for headful packaging (11), and transducing particles contain linear concatemers of pRHB101 (18), presumably generated by rolling circle replication of pRHB101 followed by headful packaging. Transduction has been demonstrated in 23 of 30 *Streptomyces* species tested (18, 19). The *ermE* gene from *Saccharopolyspora erythraea* was inserted into *Kpn*I site of pRHB101, and the resulting plasmid (pRHB111) was transducible in five species at efficiencies similar to those obtained with pRHB101 (3, 20). Transductants expressed high-level resistance to erythromycin, and the plasmid was stable, suggesting that this plasmid transduction system might be generally useful to transfer cloned DNA into many *Streptomyces* spp.

Birch and Cullum (5) described pMT660, a temperature-sensitive mutant of pIJ702 (14). If pMT660 expressed the temperature-sensitive phenotype in many *Streptomyces* spp., then an FP43-transducible derivative of pMT660 might be a suitable delivery vehicle for IS*493*-derived transposons. (These strains and other strains and genetic elements used in this work are given in Table 1.) To test this, we inserted an 8.8-kb *Sph*I fragment of FP43 DNA containing the *pac* site into the *Sph*I site of pMT660 by using standard cloning techniques (22) and transformed *S. griseofuscus* C581 as previously described (18). The resulting plasmid, pRHB106 (Fig. 1), was transducible by FP43 into *S. griseofuscus* BES2057 at frequencies similar to those observed with pRHB101 (10^{-4} transductants per PFU [18]; Table 2). We then inserted a *Kpn*I fragment from pCZA163 containing Tn*5096* into the *Kpn*I site of pRHB106 to construct pRHB126 (Fig. 1) and transformed *S. griseofuscus* C581. FP43 lysates prepared on *S. griseofuscus* MM30(pRHB126) and *S. griseofuscus* MM29(pRHB106) were used to transduce several different streptomycetes. Generally, pRHB106 and pRHB126 were transduced at similar frequencies in individual *Streptomyces* species under the conditions tested (Table 2). (The conditions tested were optimized for most of the strains in previous studies [17, 19].) An exception was *Streptomyces albus* P, which yielded 200-fold fewer transductants with pRHB126 than with pRHB106. *S. albus* P produces restriction endonuclease *Sal*PI, an isoschizomer of *Pst*I. pRHB106 has one *Pst*I site, whereas pRHB126 has four *Pst*I sites (Fig. 1). An FP43 lysate prepared on *S. albus* MM31(pRHB126) transduced *S. albus* P at a frequency of 10^{-4} per PFU. We showed previously that pRHB101 passaged through *S. albus* P became modified for *Pst*I and transduced *S. albus* P more efficiently than unmodified pRHB101 from *S. griseofuscus* (18). Thus, the very low transduction frequency (10^{-9}) in *S. albus* P observed with FP43 lysates prepared on *S. griseofuscus* MM30(pRHB126) was probably due to restriction. The general pattern that insertion of Tn*5096* into the *Kpn*I site of pRHB106 resulted in relatively minor or no alterations in transducibility of the plasmid in most streptomycetes tested is consistent with other experiments that demonstrated that insertion of the *ermE* gene into the *Kpn*I site of pRHB101, a plasmid very similar to pRHB106 but lacking the *ts* mutation in plasmid replication, did not disrupt transducibility of the plasmid (20). Therefore, pRHB126 appears to be a suitable vector to introduce Tn*5096* into many streptomycetes by transduction.

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TABLE 1. *Streptomyces* strains, plasmids, bacteriophage, and transposon

Strain or genetic element	Relevant characteristics	Source or reference
S. albus P	Produces *Sal*PI (*Pst*I); transducible[a]	7, 18
S. albus MM31	*S. albus* P containing pRHB126	This report
S. ambofaciens BES2281	Nonrestricting; produces spiramycin; transducible	10, 18
S. aureofaciens NRRL8092	Produces narasin; transducible	6, 18
S. cinnamonensis ATCC 15413	Produces monensin; transducible	9, 18
S. coelicolor A3(2)	Produces several antibiotics; transducible	13, 18
S. fradiae M1	Produces tylosin; transducible	4, 17
S. fradiae T59235	Produces tylosin; transducible	4, 17
S. fradiae GS88	Produces demycinosyltylosin; transducible	4, 17
S. griseofuscus C581 (ATCC 23917)	Nonrestricting; produces lankacidin; transducible	8, 18
S. griseofuscus MM29	C581 containing pRHB106	This report
S. griseofuscus MM30	C581 containing pRHB126	This report
S. thermotolerans NRRL 15270	Mutant blocked in carbomycin biosynthesis; transducible	10, 18
S. griseofuscus BES2057	C581 containing plasmid pKC684 integrated in the chromosome; pKC684 expresses FP43 *pin* and blocks FP43 plaque formation	1, 11
pIJ702	Broad-host-range streptomycete vector; *tsr*	13, 14, 16, 18
pMT660	*ts* derivative of pIJ702	5
pRHB106	Transducible derivative of pMT660; contains FP43 *pac*	This report
pRHB126	pRHB106 containing Tn*5096*; transducible	This report
pCZA163	pUC19 derivative containing Tn*5096* flanked with linkers	24
FP43	Broad-host-range streptomycete bacteriophage; transduces plasmid containing FP43 *pac*	8, 11, 18
Tn*5096*	IS*493* containing Am^r gene; transposes in *S. griseofuscus* and other streptomycetes	24; this report

[a] Transducible with plasmid pRHB101 by bacteriophage FP43.

To determine whether Tn*5096* could transpose from pRHB126 in different *Streptomyces* spp., transductants of different strains were selected directly either on modified R2 agar (2) plus apramycin (Am) at concentrations of 25 or 50 μg/ml or on modified R2 agar (2) plus thiostrepton (Ts) at a concentration of 25 μg/ml. Transductants selected for Am^r were incubated at 29 to 34°C for 3 to 5 days and then shifted to 39°C for 6 to 13 days. Alternatively, individual transductants selected for Ts^r or Am^r were grown in TS broth (2) plus 25 μg of Ts per ml and 25 μg of Am per ml, sonicated, and plated on modified R2 agar plus 25 μg of Am per ml. Colonies were grown for 3 to 5 days at 29°C and then shifted to 39°C for 6 to 13 days (24). Sectors of colonies growing at 39°C were tested for the Am^r Ts^s phenotype, and transpositions were confirmed by Southern hybridization to transposon but not to plasmid vector sequences. Figure 2 shows the Southern hybridization data obtained with *Streptomyces cinnamonensis*. All 10 mutants derived from Am^r Ts^s sectors contained Tn*5096* inserted in different sites, and none of the strains contained sequences that hybridized to plasmid pRHB106. Southern hybridizations with *Bam*HI-cleaved DNAs from *S. griseofuscus*, *Streptomyces ambofaciens*, *Streptomyces coelicolor*, *Streptomyces fradiae*, and *S. thermotolerans* mutants obtained from Am^r Ts^s sectors also

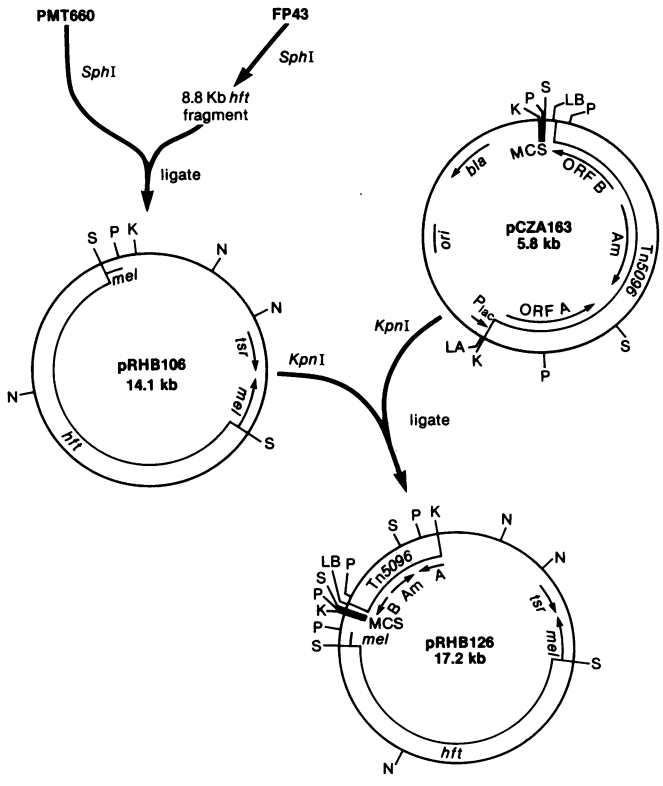

FIG. 1. Construction of plasmids pRHB106 and pRHB126. Bacteriophage FP43 was digested with *Sph*I, and an 8.8-kb fragment was gel purified (22) and cloned into the unique *Sph*I site of pMT660 to give pRHB106. pRHB106 was digested with *Kpn*I and ligated with a gel-purified *Kpn*I fragment from pCZA163 containing Tn*5096*, giving pRHB126. Abbreviations: K, *Kpn*I; N, *Nco*I; P, *Pst*I; S, *Sph*I; LA, linker A containing *Hin*dIII, *Bgl*II, *Nsi*I, and *Kpn*I sites (24); LB, linker B containing *Ksp*632I, *Xba*I, *Nsi*I, *Bgl*II and *Hin*dIII sites (24); MCS, multiple cloning site from pUC19 (only sites for *Kpn*I, *Pst*I and *Sph*I are shown); Am, apramycin resistance gene; *bla*, β-lactamase gene; *hft*, fragment containing FP43 *pac* site that mediates high-frequency transduction (11, 18); *mel*, melanin production; *tsr*, thiostrepton resistance gene. Only pertinent restriction sites are shown; more extensive restriction maps are available upon request.

TABLE 2. Transduction of plasmids pRHB106 and pRHB126 in *Streptomyces* spp.

Strain[a]	Transduction frequency by[b]:	
	pRHB106	pRHB126
S. albus P	2×10^{-7}	1×10^{-9}
S. albus P[c]		1×10^{-4}
S. ambofaciens BES2281	4×10^{-6}	2×10^{-6}
S. aureofaciens	2×10^{-6}	2×10^{-6}
S. coelicolor A3(2)	8×10^{-9}	6×10^{-8}
S. fradiae T59235	3×10^{-5}	4×10^{-6}
S. fradiae GS88	1×10^{-5}	4×10^{-5}
S. fradiae M1	2×10^{-6}	2×10^{-6}
S. griseofuscus BES2057	1×10^{-4}	1×10^{-4}
S. thermotolerans	2×10^{-6}	3×10^{-7}

[a] Cells of all strains except *S. coelicolor* A3(2) were grown in TS broth (2) and transductions to Ts[r] were carried out as previously described (18). The *S. fradiae* strains, *S. aureofaciens*, and *S. thermotolerans* were all grown at 39°C to mid-exponential phase; *S. albus* P was grown at 29°C to mid-exponential phase; and *S. ambofaciens* and *S. griseofuscus* were grown at 29°C to stationary phase before transductions (17, 19). *S. coelicolor* spores, prepared by growing cells on modified R2 agar (2) at 29°C, were used for transduction.

[b] Transducing lysates were prepared on *S. griseofuscus* MM29(pRHB106) or *S. griseofuscus* MM30(pRHB126), except as noted in footnote *c*.

[c] Transducing lysate prepared on *S. albus* MM31(pRHB126).

demonstrated that these strains contained transpositions in different sites of their respective genomes (not shown). This is consistent with data from a previous study which indicated that Tn*5096* and Tn*5098* can insert into many different sites in the genome of *S. griseofuscus* with few site or regional preferences (24).

Streptomyces albus P and *Streptomyces aureofaciens* did not yield transposition mutants by the temperature shift method. *S. aureofaciens* yielded Am[r] Ts[r] sectors that contained free plasmid DNA. This suggested that the mutation in pRHB126 causing temperature sensitivity may revert at significant frequencies in *S. aureofaciens* or that *S. aureofaciens* mutates to suppress the temperature-sensitive phenotype. An FP43-transducing lysate was prepared on an *S. aureofaciens*(pRHB126) strain derived from a temperature-resistant sector, and pRHB126 was transduced into *S. griseofuscus*. The transductants expressed the normal temperature-sensitive phenotype of the original plasmid, suggesting that *S. aureofaciens* can mutate to suppress the temperature sensitivity of pRHB126. Alternatively, reversion of the mutation in the plasmid to temperature resistance might suppress the original mutation in a mixed population of plasmids. If the temperature sensitive-allele remained predominant, then it might account for the temperature-sensitive phenotype observed after transduction of the plasmid back into *S. griseofuscus*. Further work is needed to clarify this issue.

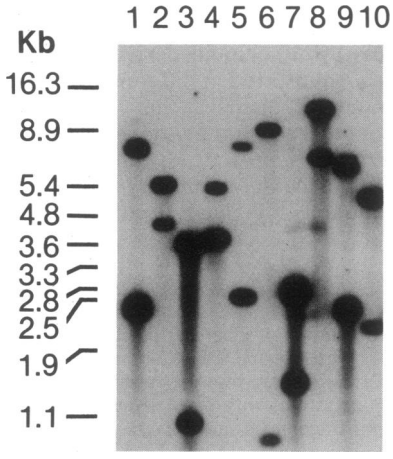

FIG. 2. Southern hybridization analysis of Tn*5096* insertions in *S. cinnamonensis*. DNA was isolated from 10 colony-purified Am[r] Ts[s] sectors from *S. cinnamonensis*(pRHB126). DNA was digested with *Bam*HI, and then fragments were separated by agarose gel electrophoresis and probed with radiolabeled Tn*5096*, which contains one *Bam*HI site (24). Tn*5096* did not hybridize to untransduced *S. cinnamonensis* ATCC 15413 DNA. Radiolabeled pRHB106 did not hybridize to DNA from any of the strains (not shown).

In summary, we have used a broad-host-range plasmid and a broad-host-range bacteriophage to develop a transducible, temperature-sensitive delivery vehicle for Tn*5096*. We have shown that the delivery vehicle, pRHB126, can be transduced into all of the streptomycetes tested, including one that produced a restriction endonuclease that can cleave pRHB126 at four different sites. By using this transduction system, we have shown that Tn*5096* has broad host specificity in *Streptomyces* spp. This combination of broad-host-range genetic elements should allow rapid application of transposon mutagenesis to many species of this important genus. A possible limitation of this system is the apparent inability to observe Am[r] Ts[s] sectors after temperature shift with two of the eight species tested. It is not yet clear whether this is an inherent problem with the plasmid that cannot be overcome by modifying the conditions to identify Am[r] Ts[s] derivatives or whether the transposon does not hop frequently enough in these species to be detected by this method. For species such as these, the use of a different delivery system, such as pCZA159 or pCZA168 (24), may provide an alternative method to test for Tn*5096* transposition if protoplast transformation can be achieved.

We thank J. Cullum for pMT660, P. Solenberg for fruitful discussions and comments on the manuscript, and B. Fogleman for typing the manuscript.

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