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Host-Dependent Transposon Tn*5*-Mediated Streptomycin Resistance

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Transposon Tn*5* encodes streptomycin resistance in addition to kanamycin-neomycin resistance. This resistance was not detectable in *Escherichia coli* but was efficiently expressed in *Rhizobium meliloti* and certain other strains. By analysis of cloned Tn*5* restriction endonuclease fragments, the streptomycin resistance (*str*) gene was located in the right-hand side of the central region as the transposon is conventionally drawn. Transcription of *str* appeared to originate at p_L, the promoter for the *neo* gene (neomycin phosphotransferase type II). Expression of streptomycin resistance in *E. coli* was obtained after cloning of the *neo-str* region downstream of a strong *E. coli* promoter. A construct in which p_L was deleted also showed differential expression of streptomycin resistance.

Tn*5* is a transposon used widely for insertion mutagenesis in the genera *Escherichia*, *Klebsiella*, *Pseudomonas*, *Agrobacterium*, and *Rhizobium* (for reviews see references 3 and 27). Tn*5* consists of a 2,700-base-pair (bp) core region flanked by two nearly identical copies of the insertion sequence IS*50* in opposite orientations (4, 19).

The right-hand inverted repeat (IS*50*R) codes for two proteins involved in both the transposition of Tn*5* and the regulation of transposition (1, 17, 29). The left-hand inverted repeat (IS*50*L) carries an ochre mutation which results in the synthesis of two truncated proteins (1, 28, 29). In addition, this mutation appears to be in the promoter region for the neomycin phosphotransferase (*neo*) gene and results in ca. 10-fold increase in neomycin resistance in *Escherichia coli* over the IS*50*R promoter sequence (28, 29).

The 2,700-bp core region of Tn*5* contains a 1,200-bp coding sequence for a neomycin phosphotransferase type II (2, 19) which confers resistance to the aminoglycoside antibiotics kanamycin and neomycin. There remains a 1,500-bp region of DNA.

Tn*5*-mediated streptomycin resistance (Sm^r) has been reported by Mazodier et al. in *Methylobacterium organophilum* (21) and Forrai et al. in *Rhizobium meliloti* Rm41 (15). This Sm^r phenotype, however, is not expressed in *E. coli*. At about the same time, we detected this Tn*5* Sm^r marker independently in *R. meliloti* SU47. In the course of characterizing the general *R. meliloti* transducing phage φM12 (13), we observed cotransduction of Nm^r and Sm^r for several different Tn*5* insertions, suggesting that the Sm^r was Tn*5*-encoded. We have confirmed this and localized a *str* gene at the right-hand side of Tn*5*, in agreement with results of others published subsequently (22, 26; G. Selvaraj and V. N. Iyer, Abstr. 9th N. Am. Rhizobium Conf. 1983, P35, p. 18). In addition, we have found that Tn*5*-encoded Sm^r can, in fact, be expressed in *E. coli* when *str* is cloned downstream from a stronger *E. coli* promoter, which suggests that the lack of Sm^r expression in *E. coli* is at the level of transcription.

Bacterial strains and plasmids are listed in Table 1. Rich medium was LB (25), with 2.5 mM $MgSO_4$ and 2.5 mM $CaCl_2$ added for *R. meliloti*. Minimal medium was M9 (25) supplemented with 0.8% glucose and 0.5% Casamino Acids for selection of trimethoprim-resistant *E. coli* or 0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 μg of biotin per ml, and 0.2% glucose for *R. meliloti*. *Rhizobium leguminosarum* and *Rhizobium japonicum* were grown in TY medium (6) or minimal medium as described previously (14).

Antibiotics were obtained from Sigma Chemical Co., St. Louis, Mo., except for tetracycline which was obtained from Calbiochem-Behring, La Jolla, Calif. Concentrations for *E. coli* were (micrograms per milliliter): ampicillin, 50; chloramphenicol, 25; tetracycline, 10; trimethoprim, 25; and kanamycin, 25. Concentrations for *R. meliloti* were (micrograms per milliliter): neomycin, 200; streptomycin, 100; and chloramphenicol, 15. Restriction enzymes and T4 DNA ligase from New England Biolabs were used according to the specifications of the manufacturer. Transcription from the *tac*II promoter in pSE181, pGS215, and pGS216 was induced with 5 mM isopropyl-β-D-thiogalactoside.

Plasmids were transferred in triparental "patch"-mating with exconjugants selected on minimal agar containing antibiotics (for pSUP104 and derivatives, 15 μg of chloramphenicol per ml; for pGM102 and pGS150, 10 μg of tetracycline per ml).

Plasmid DNA isolation and agarose gel electrophoresis were essentially as described by De Picker et al. (10). All restriction fragments used for cloning were isolated from an agarose gel after restriction with the appropriate enzyme(s), essentially as described by Thuring et al. (33). We used pGS170 (Table 1) as a source for Tn*5* restriction fragments. After transformation of the ligation mixture into *E. coli* MM294A, recombinant plasmids pGS201 through pGS204 were obtained by selection on LB-chloramphenicol, pGS200 and pGS210 were obtained on LB-kanamycin, and pGS205 was obtained on M9-trimethoprim agar. Recombinant plasmids pGS215 and pGS216 were obtained by cloning of the internal *Bgl*II(1)-*Bgl*II(2) restriction fragment of Tn*5* into the *Bgl*II site of pSE181 (Fig. 1 and 2) after transformation into *E. coli* GS1004 and selection on LB-ampicillin agar. Plasmids in individual colonies were subsequently analyzed in small-scale preparations (7) by restriction endonuclease analysis on 0.8% agarose gels.

Labeling of proteins in *E. coli* maxicells was performed essentially as described by Sancar et al. (30), except with *E. coli* RB901 irradiated with 35 J/m^2, and electrophoresis was done as described by Elledge and Walker (11).

Tn*5* encodes streptomycin resistance in *Rhizobium* spp. Transduction experiments (13) have suggested that Tn*5* encodes Sm^r. To confirm this, we first introduced Tn*5* into pGM102 (23), a derivative of the broad host range plasmid RP4, and used the resulting plasmid pGS150 to introduce Tn*5* into a variety of hosts. Sm^r was efficiently expressed in

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties[a]	Source or reference
Rhizobium meliloti		
SU47		34
102F34		D. Helinski
Rm41		26
L26		K. LeMieux
R. leguminosarum		
RL300		18
R. japonicum		
201		20
Escherichia coli		
MM294A	*pro-82 thi-1 endA1 hsdR17 supE44*	G. Walker
RB901	F⁻ *thr-1 leu-6 lacY? rpsL31 mtlA* Δ(*recA-srl*)*21 lexA51* (Def) *lexA3* (Ind⁻) *sulA11*	R. Brent
GS1004	F⁻ *lacI*^{q}	S. Elledge
Klebsiella aerogenes		
CG566	*metB4 rha-1 nadB1*	L. Reitzer
Plasmids		
pGM102	Ap^r Tc^r, Km^s RP4 deletion mutant	23
pGS150	pGS102 Ω::Tn*5*	This work
pWB6	Ap^r Tp^r	W. Buikema
pRK2013	Km^r Nm^r, helper plasmid for mobilization of pSUP104	12
pSUP104	Tc^r Cm^r	31
pBR322	Ap^r Tc^r	8
pGS170	pBR322 Ω::Tn*5*	This work
pGS200	Cm^r Km^r Nm^r, Sm^r in *R. meliloti* SU47	This work
pGS201	Cm^r Km^r Nm^r	This work
pGS202	Cm^r	This work
pGS203	Cm^r Km^r Nm^r	This work
pGS204	Cm^r Km^r Nm^r, Sm^r in *R. meliloti* SU47	This work
pGS205	Cm^r Km^r Nm^r Tp^r	This work
pGS210	Cm^r Km^r Nm^r, Sm^r in *R. meliloti* SU47	This work
pSE181	Ap^r Km^r Nm^r	S. Elledge
pGS215	Ap^r Km^r Nm^r, Sm^r is isopropyl-β-D-thiogalactoside inducible	This work
pGS216	Ap^r Km^r Nm^r	This work

[a] Abbreviations used for drugs are: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Nm, neomycin; Sm, streptomycin; Tc, tetracycline; and Tp, trimethoprim.

R. meliloti SU47, 102F34, RM41, and L26, *R. leguminosarum* RL300, and *R. japonicum* 201. However, no resistance could be detected in *E. coli* MM294A or *Klebsiella aerogenes* CG566.

We cloned a number of different restriction fragments of Tn*5* in the broad host range vector pSUP104 (31). This plasmid replicates stably in a variety of gram-negative bacteria, including rhizobia, and can be mobilized by complementation in *trans* with a second plasmid that contains an IncP1 transfer region, such as pRK2013 (12). Restriction maps of recombinant plasmids pGS200 to pGS204 are shown in Fig. 1.

Plasmid pGS200 contains the entire *Hin*dIII(1)-*Hin*dIII(2) fragment of Tn*5* and expressed Km^r in *E. coli*. In addition, both pGS201 and pGS203, which contain DNA from the left-hand side of Tn*5*, expressed Km^r in *E. coli*, in accord with published data (1, 2, 19). In *E. coli* MM294A, none of the hybrid plasmids pGS200 to pGS204 expressed Sm^r. After mobilization into *R. meliloti* SU47, plasmids pGS200 and pGS204 expressed Sm^r, but plasmids pGS201 to pGS203 did not. The level of resistance expressed by pGS204 (100 μg/ml), however, was ca. eightfold lower than that by pGS200 (800 μg/ml). Comparison of these results with Fig. 1 indicates that Sm^r of Tn*5* is encoded in the *Sal*I-*Hin*dIII(2) fragment.

In addition, since pGS202 did not express Sm^r in SU47, the region between the *Sal*I and *Bam*HI sites (ca. 350 bp) appears to be essential for expression. To confirm this, we cloned a *Bam*HI fragment that carries the trimethoprim resistance of R751 (24) into the *Bam*HI site of pGS204. This 3.05-kilobase fragment (35) was obtained from pWB6 (Table 1). The hybrid plasmid, designated pGS205 (Fig. 1), was isolated in *E. coli* and subsequently mobilized into *R. meliloti* SU47. As expected, transconjugants carrying pGS205, unlike those carrying pGS204, were no longer Sm^r.

Expression of the *str* gene under control of different promoters. To determine the direction of transcription of the Tn*5* *str* gene, we cloned the central region of Tn*5* in an *E. coli* expression vector under control of the high-level *tac*II promoter (9). The vector plasmid pSE181 (S. E. Elledge and G. C. Walker, unpublished data) is a pBR322 (8) derivative containing the *tac*II promoter followed by a polylinker fragment containing several restriction endonuclease sites (B. Seed, personal communication). The Tn*5* *Bgl*II(1)-*Bgl*II(2) fragment (Fig. 1) was cloned in both orientations in the *Bgl*II site of the polylinker (Fig. 2) to yield the recombinant plasmids pGS215 and pGS216. In *E. coli* GS1004, after induction with 5 mM isopropyl-β-D-thiogalactoside, Sm^r (15 μg/ml) was expressed from pGS215 but not from pGS216 or the control, pSE181. Thus, transcription of the *str* gene proceeds from left to right on the standard Tn*5* map. Since the *neo* gene is transcribed in this direction as well, it is possible that transcription of *str* initiates at the p_L promoter in IS*50*L (Fig. 1). The level of expression is not directly comparable to that in pGS200 to pGS205 because the latter have a lower copy number than pSE181 and derivatives.

With *E. coli* maxicells (30), no major differences (attributable to the *tac-str* constructs) were observed on sodium dodecyl sulfate-polyacrylamide gels, except for a dramatic increase of the neomycin phosphotransferase band with pGS215 (data not shown). Therefore the protein or proteins responsible for Sm^r either are not strongly expressed or are obscured by other bands.

In plasmid pGS210 (Fig. 1), the internal *Bgl*II(1)-*Bgl*II(2) fragment of Tn*5* has been inverted, so that the *neo* and *str* genes are transcribed from p_R rather than p_L. In *R. meliloti* SU47, this plasmid showed no difference in the level of Nm^r compared with pGS200, the normal orientation (Fig. 1). However, the level of Sm^r was slightly reduced (Table 2). Thus, p_L appears stronger than p_R in *R. meliloti*, as is the case of *E. coli* (29).

The results described above confirm the previously published observation (22, 26) that in *R. meliloti*, streptomycin resistance is encoded by a gene (*str*) located to the right of *neo* in Tn*5*. The gene is transcribed from left to right, presumably from the p_L promoter of IS*50*L, and is expressed in *R. meliloti* but not in *E. coli*. A *neo-str* operon is compatible with the results of Berg et al. (5) in *E. coli* which indicate that transcription initiating at the p_L promoter of

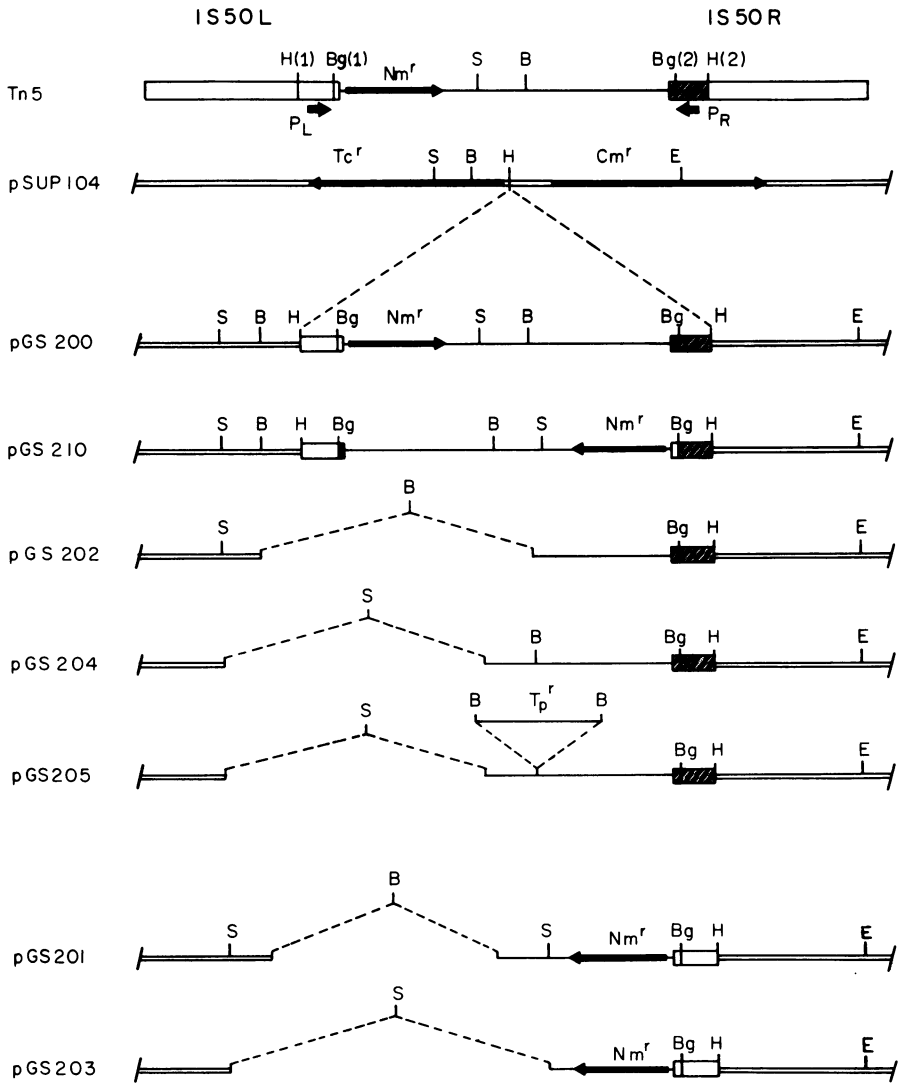

FIG. 1. Schematic map of Tn*5* (19) and different constructs in the vector pSUP104 (31). Abbreviations used for the restriction enzymes are: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; and S, *Sal*I. The *Hin*dIII and *Bgl*II restriction sites in Tn*5* are labeled (1) or (2) for clarity when referred to in the text. pSUP104 sequences are drawn as a double line in the constructs. Abbreviations for the drug markers are: Cm, chloramphenicol; Tc, tetracycline; and Nm, neomycin. The end of IS*50*L containing p_L is drawn open, the end of IS*50*R containing p_R is drawn hatched. Plasmids pGS200 to pGS204 were constructed by cloning of the indicated Tn*5* fragments directly into pSUP104; pGS205 was derived from pGS204 by insertion of a *Bam*HI-*Bam*HI fragment from pWB6. pGS210 was obtained by inversion of the *Bgl*II-*Bgl*II fragment of pGS200. All plasmids are drawn relative to the vector plasmid pSUP104.

IS*50*L extends beyond the unique *Sal*I site of Tn*5* (Fig. 1).

Tn*5*-encoded Sm^r is expressed in rhizobia and the other bacteria but not in *E. coli*. However, it is expressed in *E. coli* when the stronger *tac*II promoter is provided. This suggests that the differential expression of Sm^r is due to differential transcription from p_L in IS*50*L. That interpretation is reinforced by pGS204, which also shows differential expression of Sm^r, albeit at a lower level (Table 2). Since p_L is deleted in pGS204, in this plasmid transcription must originate either from a promoter in the vector or from a previously unrecognized Tn*5* promoter downstream of the unique *Sal*I site. In either case, this is a second example of host-dependent transcription.

A large open reading frame of at least 335 bp starts 23 bp downstream from the *neo* gene and extends beyond this *Sal*I site (2). However, this probably does not represent the *str* gene, because plasmid pGS204 expresses Sm^r even though it lacks the first 324 bp of this open reading frame. This result might indicate that in the construction of pGS204 (Fig. 1), this open reading frame was connected to an open reading frame in the vector to form a protein fusion, but this is unlikely for the following reason. The tetracycline resistance region of pSUP104, in which this fragment is cloned, is derived from pBR322. In pBR322 (32), two open reading frames start downstream from the *Sal*I site in the tetracycline resistance gene and are directed against the major tetracycline promoter, but in pGS204, these two reading frames are out of phase with the open reading frame which spans the unique *Sal*I site of Tn*5*.

The fact that pGS204 expresses Sm^r would indicate that

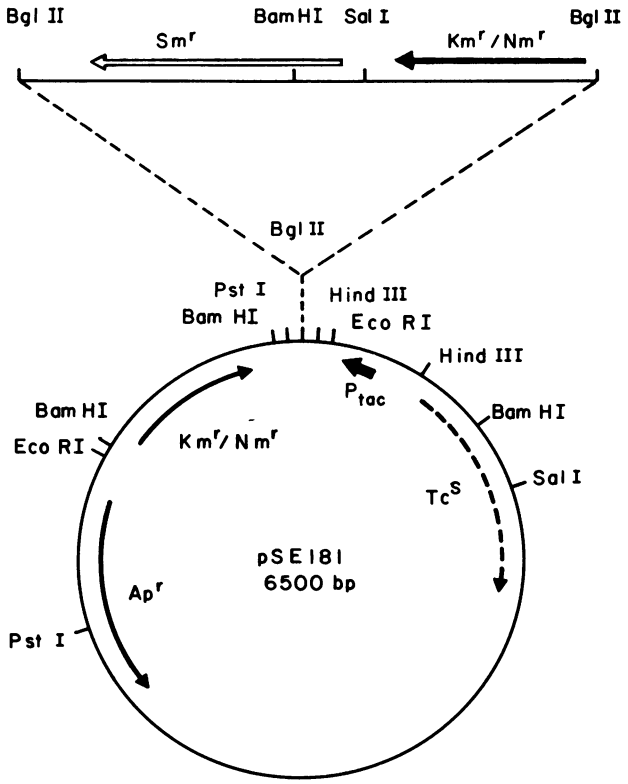

FIG. 2. Schematic restriction map of pSE181 showing the localization and orientation of the insert present in pGS215. Abbreviations are: Ap^r, ampicillin resistance; Km^r Nm^r, kanamycin-neomycin resistance. The tetracycline resistance region is indicated Tc^s since resistance is not expressed. The location of the *tac*II-promoter (P_{tac}) and the putative location of the streptomycin resistance determinant (Sm^r) are indicated. pGS216 is the same as pGS215, except that the *BglII-BglII* restriction fragment is present in the opposite orientation; Km^r Nm^r in pSE181 (16).

the *str* gene begins downstream of the unique *SalI* restriction site (Fig. 1). However, from deletion analysis, Putnoky et al. (26) position the start of *str* slightly upstream of this *SalI* site. Until the protein can be characterized, this discrepancy will remain unresolved.

TABLE 2. MICs of different antibiotics for *E. coli* and *R. meliloti* harboring different plasmids

Plasmid	MIC (μg/ml) required to inhibit the following strain[a]:			
	E. coli MM294A		*R. meliloti* SU47	
	Kanamycin	Streptomycin	Neomycin[b]	Streptomycin
None	<5	<2	<5	<2
pSUP104	<5	<2	<5	<2
pGS200	200	<2	>600	800
pGS210	50	<2	>600	600
pGS201	200	<2	>600	<2
pGS202	<5	<2	<5	<2
pGS203	200	<2	>600	<2
pGS204	<5	<2	<5	100
pGS205	<5	<2	<5	<2

[a] Concentrations were determined by plating out dilutions of log-phase cultures on LB-agar containing increasing concentrations of the antibiotics. Steps were: 50 μg/ml for kanamycin, 200 μg/ml for neomycin, and 100 μg/ml for streptomycin. The highest concentration at which growth was observed, overnight for *E. coli* or after 4 days for *R. meliloti*, is reported.

[b] Concentrations of neomycin could not be determined accurately due to precipitation of the antibiotic in LB at concentrations higher than 600 μg/ml.

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