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Mu*d*SacI, a Transposon with Strong Selectable and Counterselectable Markers: Use for Rapid Mapping of Chromosomal Mutations in *Salmonella typhimurium*

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The transposable bacteriophage Mu and its mini-Mu derivatives are useful tools for the genetic analysis of many bacteria. A variety of antibiotic-resistant Mu derivatives have been constructed, allowing direct selection for cells which contain the transposon. However, in many cases a counterselection against the transposon would greatly facilitate further genetic analysis. In this paper we report the construction of Mu*d*SacI, a mini-Mu derived transposon containing the *sacB* (secretory levansucrase) gene of *Bacillus subtilis*, which confers sucrose sensitivity upon gram-negative bacteria. We describe the use of this transposon as a tool for rapid genetic mapping of chromosomal genes in *Salmonella typhimurium*. Simple modifications of this approach should facilitate rapid mapping in many other bacteria as well.

Initial genetic mapping of mutations on the chromosome of *Salmonella typhimurium* has historically been done by conjugation crosses (9). More recently the development of the hybrid Mu*d*P22 specialized transducing phage has led to faster, easier mapping methods (3, 39). Ordered sets of Mu*d*P22 lysates which span the chromosomal map can be used to rapidly map mutations with a counterselectable phenotype (e.g., auxotrophy) to a chromosomal interval of 1 to 5 min (3). Alternatively, mutations which do not confer a counterselectable phenotype can be mapped with plates which select for tetracycline sensitivity (3, 5, 23). To do this, a Tn*10* insertion linked to the mutation of interest is isolated from a random pool of transposon insertions. The map location of the Tn*10* insertion is then determined by selecting for colonies which lose Tn*10* by recombination with transducing DNA from one of the regions amplified by Mu*d*P22. Cells which retain the *tetA* (tetracycline efflux) gene of Tn*10* display increased sensitivity to fusaric acid and thus do not grow on tetracycline sensitivity plates. However, tetracycline sensitivity selection does not work well under certain growth conditions (for example, a low growth temperature); furthermore, in certain strains, many colonies which have lost Tn*10* by transduction are killed by the severity of the selection, and the instability of Tn*10* itself can lead to a high spontaneous background of growth. Thus, the tetracycline sensitivity selection is often inadequate.

An alternative counterselection relies on the sucrose sensitivity of gram-negative bacteria carrying the *sacB* gene from *Bacillus* spp. Plasmid clones of the *sacB* gene have been used in a variety of gram-negative species to select for allelic exchange (4, 6, 10, 18, 25, 28, 29, 33), to select for plasmid clones containing inserts (27), and to capture insertion sequences (6, 12). Previously, Tn*5* derivatives containing *sacB* have been constructed and used to promote plasmid curing and deletion formation (17) and to study intramolecular transposition (36) and genetic rearrangements of the chromosome (24). However, Tn*5* derivatives carry transposase and hence are unstable—a feature which limits their usefulness as counterselectable markers for genetic mapping.

In contrast, derivatives of phage Mu can be used to isolate stable insertions or to generate pools of random chromosomal insertions which can be screened for linkage to genes of interest (14, 16). Therefore, in order to map temperature-conditional mutations of *S. typhimurium* which confer resistance to phage P22 (a phenotype which is not counterselectable) (20), we constructed a new Mu derivative which carries the *sacB* gene (Fig. 1). A restriction map of the Mu*d*SacI element is shown in Fig. 2.

Although *sacB* is a useful counterselectable marker, the reasons why *sacB* expression is lethal for gram-negative bacteria are not fully understood. When the *sacB* structural gene is expressed from its own regulatory region, its transcription is attenuated (1, 2, 34, 35). The presence of sucrose leads to antitermination, resulting in toxic overexpression of the *sacB* gene (1, 34, 35). Simply overexpressing *sacB* from regulatory sites that are not affected by sucrose is also toxic, but the toxicity is still much greater when sucrose is present (21, 27). Thus, full toxicity seems to require SacB enzyme activity. The SacB protein catalyzes a transfructosylation reaction that hydrolyzes sucrose, releasing glucose and fructosylating an acceptor molecule (13). The product of the fructosylation reaction, the polymer levan, may be toxic to gram-negative bacteria.

Stable transposition by Mu*d*SacI. The strains and plasmids used in this study are listed in Table 1. Plasmid pPC99 was electroporated into strain TT10309 to make strain MST3279, which carries both the Mu*d*SacI plasmid and a Mu*cts62*hP1 prophage. To confirm that the Mu*d*SacI element was competent for transposition, strain MST3279 was heat shocked to induce the chromosomal Mu*cts62*hP1 prophage (14). If Mu*d*SacI is proficient for transposition, transposase from the induced helper phage will act in *trans* upon the left (MuL) and right (MuR) ends of Mu*d*SacI to carry out multiple rounds of replicative transposition. Lytic growth of the phage ultimately leads to packaging of Mu*cts62*hP1 chromosomal insertions, Mu*d*SacI chromosomal insertions, or plasmid pPC99 within MuhP1 phage particles. The MuhP1 lysate produced was used to transduce strain TR5878 to Kan^r. The resulting transductants were then screened for Amp^r and sucrose sensitivity. Kan^r Amp^r colonies could result from the transduction of the pPC99 plasmid (without transposition), while Kan^r Amp^s colonies could result from transduction of chromosomal Mu*d*SacI

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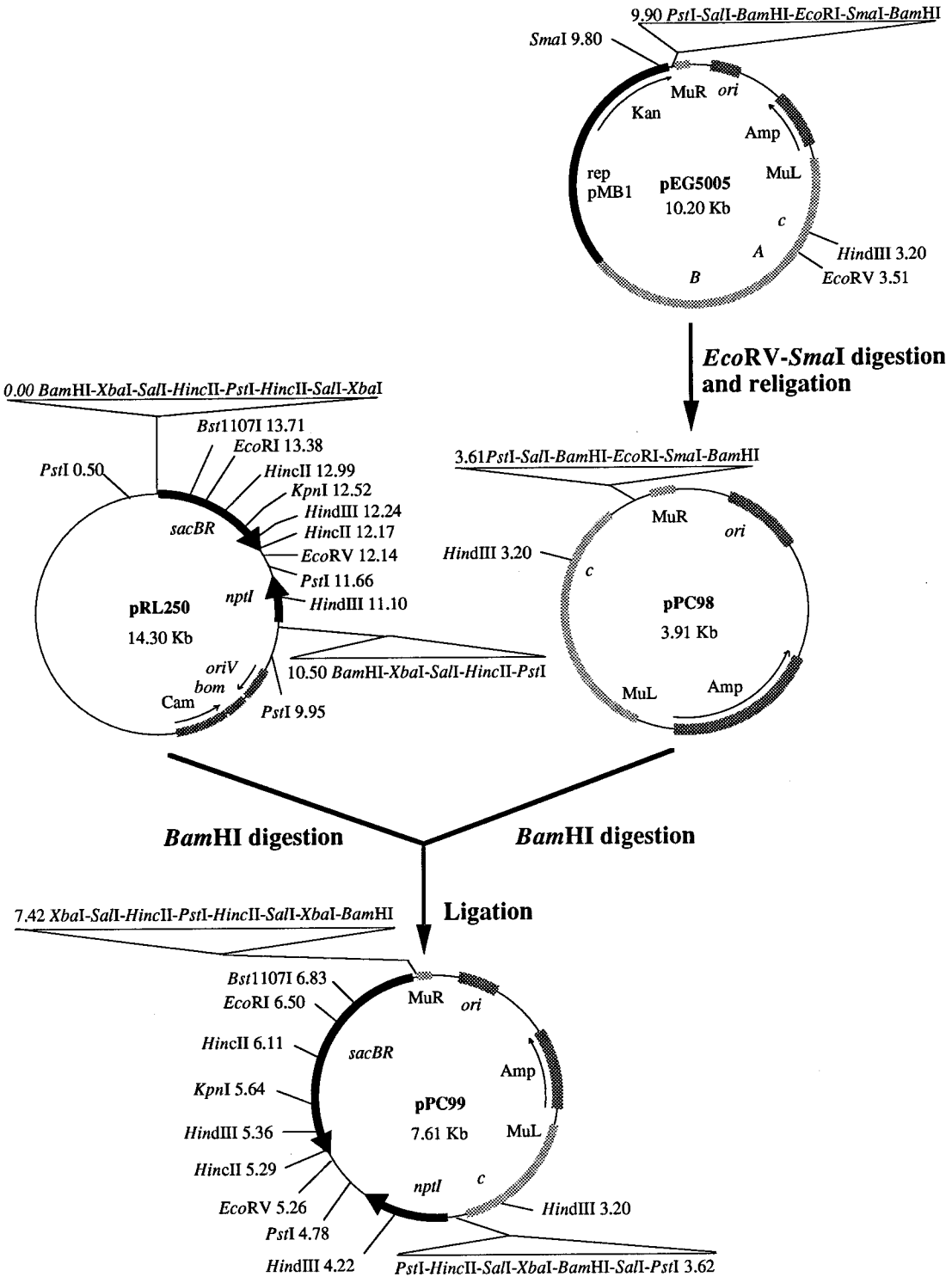

FIG. 1. Construction of Mu*d*SacI. The mini-Mu cloning vector pEG5005 (15) was used as a source for the MuL and MuR ends required for transposition. First the transposase-encoding genes *A* and *B*, the pMB1 replicon, and the Kan^r gene were deleted via *Eco*RV and *Sma*I digestion of the plasmid; the genes and replicon were then blunt end religated and electroporated into strain VJSS120. The resulting transformants were selected as resistant to 100 mg of ampicillin per ml and were replica plated to screen for kanamycin sensitivity. The structures of the Amp^r Kan^s plasmid derivatives were confirmed by restriction endonuclease digestion, and one of the plasmids was designated pPC98. Next an *nptI-sacBR* cassette (29) was prepared by digestion of plasmid pRL250 (6) with *Bam*HI. Plasmid pPC98 was also digested with *Bam*HI, and the DNA fragments were ligated with T4 DNA ligase (17). The ligated DNA was electroporated into MS1363, and Kan^r Amp^r colonies were selected. The colonies were then screened for Cam^s and sucrose sensitivity. The structure of plasmid isolated from Kan^r Amp^r Cam^s Suc^s colonies was confirmed by restriction digests. The *nptI-sacBR* cassette was cloned within the ends of the mini-Mu transposon in the order MuL-*cts62-nptI-sacBR*-MuR. A restriction map of the Mu*d*SacI element is shown in Fig. 2.

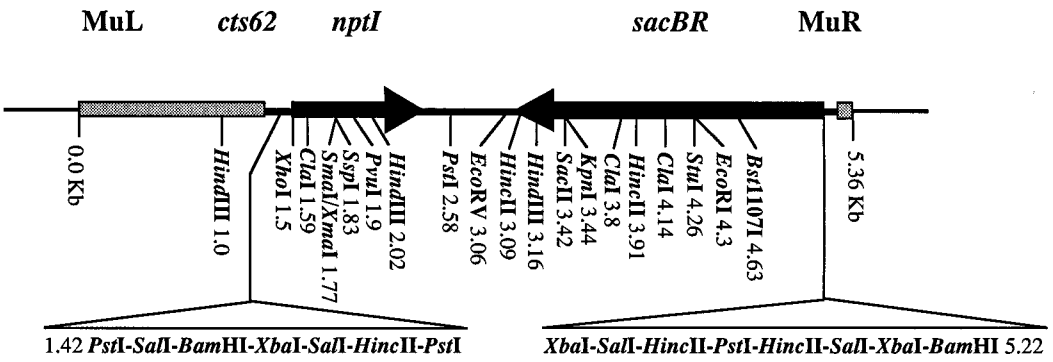

FIG. 2. Restriction map of Mu*d*SacI. MuL, left end of Mu required for transposition; *cts62*, temperature-sensitive allele of the Mu repressor; *nptI*, kanamycin resistance gene from Tn*903*; *sacBR*, secretory levansucrase operon from *B. subtilis*; MuR, right end of Mu required for transposition. Relative to MuL the *nptI* gene is transcribed rightwards from its own promoter and the *sacBR* operon is transcribed leftwards from its own promoter. In the absence of sucrose the *sacBR* operon is not activated. Unlike many Mu derivatives, Mu*d*SacI fully expresses the Mu repressor because *cis*-regulatory sequences have not been removed (15).

insertions which then recombine with the recipient genome. To confirm that Kan^r Amp^r colonies arose from transposition, the Kan^r Amp^s colonies were replica plated to screen for auxotrophic insertions (22). A variety of auxotrophs were obtained, and in each case tested the auxotrophy was 100% linked to the Kan^r phenotype (data not shown). This result indicates that Mu*d*SacI had transposed from the pPC99 plasmid and inserted randomly into the *S. typhimurium* chromosome when transposase was provided in *trans.*

Transposition of Mu*d*SacI via transitory *cis* complementation. Transitory *cis* complementation of defective Mu phage or defective Tn*10* transposons and their derivatives is the most efficient manner in which to generate single transposition events and pools of random insertions (14, 16, 19, 22). Successful transitory *cis* complementation of defective mini-Mu phage requires that two conditions be met (16). First, the two Mu elements must be close; however, phage P22 packaging constraints (44 kb) do not allow both the defective transposon (e.g., Mu*d*SacI, Mu*d*Cam [11], or Mu*d*J [8]) and the transposase donor (Mu*d*I [7]) to be completely packaged within one phage head. Second, both the MuL and MuR ends of the defective transposon must be present within the same phage particle as the MuL end of the transposase donor. Therefore, we constructed a transitory *cis*-complementation system for transposon Mu*d*SacI. One of the auxotrophic insertions described above (strain MST3280) was within the *S. typhimurium his* operon (*his*::Mu*d*SacI). Three-factor crosses with characterized *his* mutations indicated that the *his*::Mu*d*SacI insertion lay between the *hisD* and *hisA* genes, in the *hisCBH* region of the operon (data not shown). From these crosses we saved a *his*::Mu*d*SacI *hisA9944*::Mu*d*I (Kan^r Amp^r Cam^s) mutant (strain MST3281) and confirmed its sucrose sensitivity.

Phage P22HT105/1 *int-201* (32) was grown on strain MST3281 to yield a donor lysate for transitory *cis* complementation. The donor lysate was used to infect MS1363, and Kan^r colonies were selected (22). About 25,000 colonies were screened for transposition: the resulting colonies remained Amp^s, indicating that they did not coinherit Mu*d*I, and the majority of the colonies were His$^+$, indicating that the Mu*d*SacI was not simply inherited by homologous recombination into the *his* locus. A variety of auxotrophs were obtained, and in each case tested, the auxotrophy was 100% linked to the

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or comments	Source or reference
S. typhimurium		
MS1363	*leuA414*(Am) *supE* (Fels2$^-$)	M. Susskind
TE3461	*hisD9953*::Mu*d*Cam *hisA9944*::Mu*d*I	11
TR5878	*galE550 ilv-542 metA22 trpB2 metE551 xyl-404 rpsL120 fla-66 hsdL6 hsdSA29* (Fels2$^-$)	J. Roth
TT418	*glyA540*::Tn*10*	J. Roth
TT10309	*galE542 zxx-1826*::Mu*cts62*hP1	J. Roth
VJSS120	*hsdL6 hsdSA29 hsdSB121 galE856 xyl-404 leu-3121 metA22 metE551 trpC2 ilv-452 rpsL120 zxx-7201*::Mu*cts62*hP1	V. J. Stewart
MST3279	*galE542 zxx-1826*::Mu*cts62*hP1/pPC99	This study
MST3280	*galE550 ilv-542 metA22 trpB2 metE551 xyl-404 rpsL120 fla-66 hsdL6 hsdSA29* (Fels2$^-$) *his*::Mu*d*SacI	This study
MST3281	*his*::Mu*d*SacI *hisA9944*::Mu*d*I	This study
MST3282	*leuA414*(Am) *supE* (Fels2$^-$) *pur*::Mu*d*SacI (Ade$^-$ Thi$^-$)	This study
MST3283	*galE550 ilv-542 metA22 trpB2 metE551 xyl-404 rpsL120 fla-66 hsdL6 hsdSA29* (Fels2$^-$) *pur*::Mu*d*SacI (Ade$^-$ Thi$^-$)	This study
Plasmids		
pEG5005	Mu*d*5005 in vivo cloning vector	15
pRL250	Broad-host-range conjugative plasmid carrying the *nptI sacBR* cassette	6
pPC98	*Eco*RV-to-*Sma*I deletion derivative of pEG5005	This study
pPC99	pPC98 with *nptI sacBR* cassette cloned into *Bam*HI sites	This study

TABLE 2. Location of the *pur*::Mu*d*SacI insertion by using Mu*d*P22 lysates

Lysate strain	Relevant genotype[a]	Map position and packaging direction[b]	Sucrose-resistant transductants[c]	Prototroph transductants[c]
TT15254	*cysKAM1586*::Mu*d*P (A)	50′ clockwise	+	+
TT15255	*guaAB5641*::Mu*d*P (B)	52′ counterclockwise	−	−
TT15632	*guaAB5641*::Mu*d*Q (A)	52′ clockwise	+++	+++
TT15257	*purG2149*::Mu*d*Q (B)	54′ counterclockwise	−	−
TT15256	*purG2149*::Mu*d*P (A)	54′ clockwise	++	++
TT15263	*cysHIJ1547*::Mu*d*P (B)	60′ counterclockwise	+	+
TT15239[d]	*putA1019*::Mu*d*Q (A)	21.5′ clockwise	−	−

[a] A, clockwise packaging; B, counterclockwise packaging.
[b] Packaging of chromosomal DNA in Mu*d*P22 particles occurs processively for five to six headfuls in one direction from the point of insertion, with each sequential headful of DNA packaged at a decreasing frequency. Each headful carries approximately 1 min (44 kb) of DNA.
[c] −, 0 transductants; +, 0 to 3 transductants; ++, 4 to 10 transductants; +++, >10 transductants.
[d] The lysate grown on TT15239 is distant from all of the *pur* loci and was used as a representative negative control.

Kan^r phenotype (data not shown). These results indicate that Mu*d*SacI transposed to random sites in the chromosome at high efficiency via transitory *cis* complementation from the *his*::Mu*d*SacI *hisA9944*::Mu*d*I donor.

Mapping chromosomal Mu*d*SacI insertions via Mu*d*P22. To demonstrate that sucrose sensitivity could be used for counterselection, we compared the genetic mapping of an auxotrophic Mu*d*SacI insertion by transduction to prototrophy with mapping by transduction to sucrose resistance. Strain MST3282 contains a *pur*::Mu*d*SacI insertion and requires both adenine and thiamine to supplement its purine auxotrophy. This phenotype could be due to an insertion in any of four different loci, *purD*, *purF*, *purG*, and *purI* (22), which map at four different locations on the *S. typhimurium* chromosome (31). The auxotrophic mutation was mapped by spot transduction with Mu*d*P22 lysates with selection for prototrophy on minimal medium (37) or with selection for sucrose resistance on nutrient broth with 5% sucrose (without added NaCl).

The selection for sucrose resistance indicated that the Mu*d*SacI insertion mapped near 54 min on the *S. typhimurium* chromosome, and selection for prototrophy yielded the same result (Table 2). The map position of and phenotype conferred by the Mu*d*SacI insertion were consistent with those obtained with a *purG* mutation at 54 min. To confirm this, MST3282 was transduced to tetracycline resistance with a lysate grown on MST300 (*glyA*::Tn*10*). The *glyA* gene is closely linked to *purG* on the genetic map (31), so if the Mu*d*SacI insertion is in the *purG* gene, a fraction of the Tet^r transductants obtained from this cross would be expected to lose the Kan^r phenotype conferred by the Mu*d*SacI insertion by cotransduction. Of the resulting Tet^r transductants, about 10% (69 of 699) simultaneously became Kan^s and no longer required adenine and thiamine supplements on minimal medium.

The above-described results indicate that Mu*d*SacI provides a useful counterselectable phenotype for genetic mapping. We are currently using the Mu*d*SacI transposon and sucrose sensitivity mapping to locate conditional P22^r mutants of various classes on the *S. typhimurium* genetic map. This approach may be extended to other bacterial species that are sensitive to phage P22, Mu, or P1 (14, 20, 26, 30, 38, 40) or any other gram-negative bacteria with a suitable delivery system.

A caveat about sucrose sensitivity medium. There have been previous reports that the temperature and salinity of the medium affect the sucrose sensitivity phenotype conferred by *sacB* in *Escherichia coli* (4). We noticed that the sucrose sensitivity phenotype of *S. typhimurium* was less leaky on nutrient agar plates containing 5% sucrose if NaCl was omitted from the medium and cells were washed with salt-free medium before plating. In addition, we found that the sucrose sensitivity phenotype was best scored within 16 h on rich medium to avoid growth of spontaneous sucrose-resistant colonies. Finally, we observed that the sucrose sensitivity phenotype was less leaky at 42 or 30°C than at 37°C; however, as long as the above-mentioned precautions were followed, sucrose sensitivity was still easily and rapidly scored at all three temperatures.

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