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Tn*10*-Derived Transposons Active in *Bacillus subtilis*

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Small derivatives of the *Escherichia coli* transposon Tn*10*, comprising IS*10* ends and a chloramphenicol resistance gene, were introduced in *Bacillus subtilis* on a thermosensitive plasmid, pE194. In the presence of the Tn*10* transposase gene fused to signals functional in *B. subtilis*, these derivatives transposed with a frequency of 10^{-6} per element per generation. They had no highly preferred insertion site or region, as judged by restriction analysis of the chromosomal DNA, and generated auxotrophic and sporulation-deficient mutants with a frequency of about 1%. These results suggest that Tn*10* derivatives might be a useful genetic tool in *B. subtilis* and possibly other gram-positive microorganisms.

Several transposons derived from gram-positive bacteria were successfully introduced and shown to be functional in *Bacillus subtilis*. Among these are Tn*916* and Tn*1545*, which are conjugative transposons (2, 6), and Tn*917*, a Tn*3*-like transposon that has been extensively used for mutagenesis and cloning in this host (22, 23). The characteristics of Tn*10*, which is one of the best-known *Escherichia coli* transposons (11), make it a good candidate for development as an alternative to Tn*917*. It is a composite transposon, consisting of a central tetracycline resistance (Tc^r) marker and two IS*10* elements (left and right) in inverted orientation, which are delimited by an outside and an inside end, relative to the Tc^r marker (5). Short IS*10* extremities, 23 bp of the inside end or 42 bp of the outside end, and the product of the transposase gene, encoded by the IS*10* right element, are needed for transposition. In vitro experiments indicate that the *E. coli*-encoded proteins IHF and HU are necessary for outside-end but not for inside-end transposition (14). Numerous Tn*10* derivatives that transpose at a high frequency and generate useful gene fusions have been constructed (20); these derivatives represent convenient tools for insertional mutagenesis. Tn*10* has no particular hot spot for insertion, although a consensus target sequence has been determined (8, 13).

In this report, we describe the construction and characterization of Tn*10* derivatives active in *B. subtilis*. Their transposition occurs with a rather high frequency and is essentially random. They should therefore be interesting alternatives to Tn*917* in *B. subtilis* and possibly in other gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *B. subtilis* strains used were 168 (*trpC2*) and MI112 (*leuA8 arg15 thrA recE4*; 19). *E. coli* MT102 (*strA araD139 leu lacX74 galU galK hsdR*; 1) was used for plasmid constructions. Plasmids pNK861 (20), pTV1ts (23), pC194 (10), and p602-22 (S. F. J. Le Grice, Methods Enzymol., in press) are described elsewhere. pE194ts and pNK1250 were kindly provided by A. Gruss and N. Kleckner, respectively. pHV1248, pHV1249, pHV1263, and pHV1264 were constructed as described below.

Media and transformation. Competent *E. coli* and *B. subtilis* cells were prepared and transformed as previously described (3, 16). Selection of *E. coli* transformants was made on Luria-Bertani solid medium (LB) supplemented with ampicillin (100 μg/ml). Selection of *B. subtilis* transformants was made on LB supplemented with 3 μg of chloramphenicol per ml; 5 μg/ml was used for further growth. When more than 10^7 cells were plated on chloramphenicol at 51°C, residual growth of sensitive cells did not allow detection of chloramphenicol-resistant (Cm^r) colonies. These were detected after replica plating at 51°C, using 5 μg of chloramphenicol per ml. Erythromycin was added when necessary to a concentration of 0.3 μg/ml.

Auxotrophic mutants were detected on minimal medium supplemented with tryptophan (10 μg/ml) and chloramphenicol (5 μg/ml), further characterized on plates containing mixtures of four or five different amino acids or vitamins, and finally identified on plates with a single amino acid or vitamin (plus tryptophan) from the mixtures that gave positive results. Sporulation mutants were identified as colonies lacking brown pigment on Difco nutrient broth supplemented with 0.5 μM calcium nitrate and 1 μM ferrous sulfate.

Plasmid constructions. Plasmid pHV1249 was constructed from pNK1250, which consists of (i) mini-Tet Tn*10*, comprising a Tc^r marker flanked by 307-bp inverted repeats derived from IS*10*, which include 23-bp inside ends; (ii) the transposase gene fused to the *tac* promoter; and (iii) the pBR322 replicon. The Tc^r marker, flanked by two *Bam*HI sites, was replaced by a Cm^r marker (*Nae*I-*Mbo*I fragment of pC194) functional in *B. subtilis*, which gave a 1.1-kb Tn*10* derivative. Between the two *Cla*I sites of the resulting plasmid was inserted the larger *Taq*I fragment of plasmid pE194ts, containing thermosensitive replication functions active in *B. subtilis* and an erythromycin resistance (Em^r) gene. To express the transposase gene in *B. subtilis*, its ribosome-binding site (RBS) was replaced by the RBS of the expression vector p602-22. The sequences upstream of the transposase gene and the resulting plasmid, named pHV1249, are depicted in Fig. 1. A similar plasmid, pHV1248, was built starting form pNK861; it carried a mini-Tn*10* containing outside rather than inside ends and pE194ts replication functions in the opposite orientation relative to pHV1249. Its map is presented in Fig. 1. Com-

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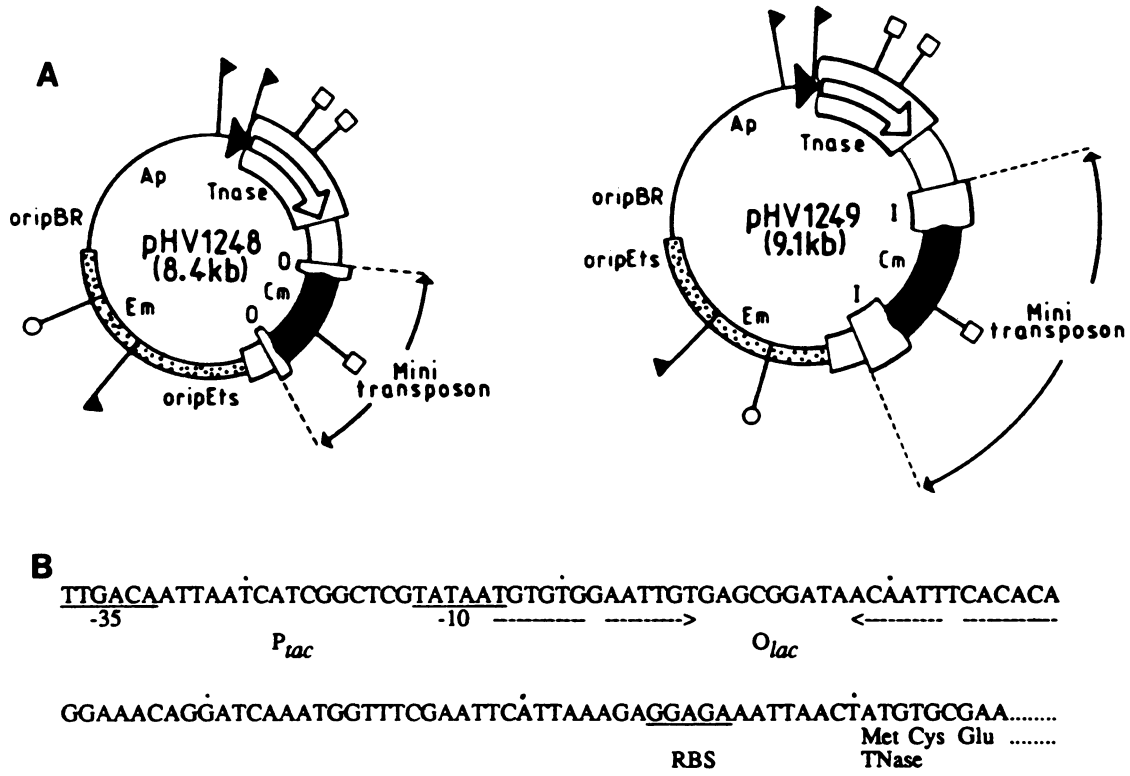

FIG. 1. Structures of pHV1248 and pHV1249. (A) Symbols: ——, pBR322 sequence; ▒, pE194ts sequence; □, Tn*10* sequence; ■, pC194 sequence. ori denotes replication origin. Ampicillin (Ap), erythromycin (Em), and chloramphenicol (Cm) resistance genes are indicated. The transposase (Tnase)-coding sequence is represented by an arrow, preceded by a black triangle for promoter and RBS fused to it. Outside (O) and inside (I) ends of the transposon are indicated. Restriction sites for *Nco*I (□), *Eco*RI (▲), and *Sst*I (○) are depicted. (B) Sequence upstream of the transposase gene. The *tac* promoter P_{tac}, *lac* operator O_{lac}, and RBS (derived from *B. subtilis* expression vector p602-22; Le Grice, in press) are indicated. Complete plasmid sequences are available upon request.

plete sequences of pHV1248 and pHV1249 are available upon request. Plasmids pHV1263 and pHV1264 were constructed as controls; they are related to pHV1248 and pHV1249, respectively, but lack a 1.9-kb segment carrying the transposase gene.

Detection of transposition events. A transposon-carrying plasmid was introduced in *B. subtilis* by transformation, selecting for Cm^r. The transformants were colony purified at 30°C on the selective medium. An entire colony, formed overnight from a single cell, was inoculated in 10 ml of rich medium containing chloramphenicol and incubated at 30°C. When the culture reached mid-logarithmic phase (optical density at 650 nm of 0.1 to 0.2), it was shifted to 51°C for 2 to 3 h (six to nine generations) to allow dilution of the plasmid. Samples were then plated at 51°C on medium containing chloramphenicol (5 μg/ml for the wild-type strain; 3 μg/ml for a *recE4* mutant) to detect transposition events and at 30°C, on a medium without antibiotic, to determine the viable cell count. It took about 30 generations for the single cell deposited on solid medium to yield the 10 ml of culture from which samples were plated at 51°C.

DNA analysis. Chromosomal DNA was purified from cell lysates (9) by phenol-chloroform extraction, followed by isopropanol precipitation and RNase treatment. To detect transposition, purified DNA was restricted, segments were separated by electrophoresis, and the transposon was revealed by Southern hybridization (18). For *Sfi*I restriction analysis, DNA was purified in agarose plugs as described by Smith et al. (17). DNA fragments were separated by field inverted gel electrophoresis (DNAstar, London, England) under 10 V/cm for 18 h at 18°C with a switching ramp of 0.3 to 15 s in the forward direction and 0.1 to 5 s in the backward direction.

RESULTS

Plasmid structures. To test Tn*10* transposition in *B. subtilis*, we used plasmids containing (i) a ≈1-kb mini-Tn*10*, composed of a Cm^r gene flanked by the inside or outside ends of IS*10*; (ii) the Tn*10* transposase gene fused to transcription and translation signals active in *B. subtilis*; and (iii) the pE194ts replicon, which is thermosensitive in *B. subtilis*. Construction of these plasmids, named pHV1248 and pHV1249, is described in Materials and Methods, and their structures are shown in Fig. 1. Two control plasmids, pHV1263 and pHV1264, lacked the transposase gene. All plasmids conferred Cm^r and Em^r upon *B. subtilis*.

Detection of Tn*10* transposition events. Transposition of mini-Tn*10* from the thermosensitive plasmids into the chromosome of *B. subtilis* can be revealed by selection of Cm^r cells able to grow at a temperature restrictive for plasmid replication (see Materials and Methods for experimental procedure). A similar strategy was previously used with a gram-positive transposon, Tn*917* (23). With pHV1248 and pHV1249, the proportion of thermoresistant Cm^r cells was about 10^{-4} (Table 1). In contrast, with the control plasmids pHV1263 and pHV1264, which lack the transposase gene, this proportion was 10^4-fold lower. These results suggest that transposition of mini-Tn*10* did occur in *B. subtilis*. Among the Cm^r colonies growing at 51°C, 90% were Em^s

TABLE 1. Transposition in *B. subtilis* 168

Plasmid (relevant genotype)	Expt[a]	Viable cell count (10^8)/ml	Resistant cell count/ml[b]	Proportion of resistant cells (avg ± SD)
pHV1248 (Tn*10* Tnase^{+c})	1	5	5×10^4	$(1.7 \pm 0.7) \times 10^{-4}$
	2	4.5	8×10^4	
	3	8.5	10^5	
	4	7	2×10^5	
pHV1263 (Tn*10* Tnase$^-$)	1	1	<10	$\leq 10^{-8}$
	2	2.5	<10	
	3	2.5	<10	
	4	3.6	<10	
pHV1249 (Tn*10* Tnase$^+$)	1	5.5	6×10^4	$(1.5 \pm 1) \times 10^{-4}$
	2	3.5	3×10^4	
	3	6	5×10^4	
	4	6	2×10^5	
pHV1264 (Tn*10* Tnase$^-$)	1	7.5	10	2.6×10^{-8}[d]
	2	6.5	<10	
	3	2.4	40	
	4	2.9	<10	
pTV1ts (Tn*917*)	1	5.3	7.5×10^2	$(1.2 \pm 0.5) \times 10^{-6}$
	2	1.9	1.3×10^2	

[a] Numbers refer to independent experiments.
[b] Selection was at 51°C for Cm^r or Em^r in the case of Tn*10* or Tn*917*, respectively.
[c] Tnase, Transposase.
[d] Results of four experiments were pooled to calculate the proportion.

and 10% were Em^r. The former phenotype is expected from transposition; the latter might be due to integration of the entire thermosensitive plasmid into the chromosome, as previously reported for Tn*917* (23). Transposition from dimeric (or multimeric) plasmids, which involves transposon ends residing on different monomeric units, could generate such integrants.

To compare the transposition efficiency of mini-Tn*10* with that of Tn*917*, our experimental procedure was applied to plasmid pTV1ts, a pE194ts derivative carrying Tn*917* (23). Control experiments have shown that pTV1ts and pHV1248 or pHV1249 are maintained at similar copy numbers (≈10). Selection of clones in which transposition occurred was carried out on erythromycin instead of chloramphenicol, since Tn*917* carries an Em^r gene. Transposition was 100 times more frequent with mini-Tn*10* than with Tn*917* (Table 1).

Physical analysis of transposition events. To confirm that the thermoresistant Cm^r Em^s cells resulted from transposition, their DNAs were analyzed by restriction. The transposons used contain a single *Nco*I and no *Eco*RI site (Fig. 1), and a double digestion of DNA that carries them should generate two segments homologous to pHV1248. Analysis of 14 clones, obtained in a single experiment with pHV1248, is presented in Fig. 2. With 12 of the 14 clones, two bands of

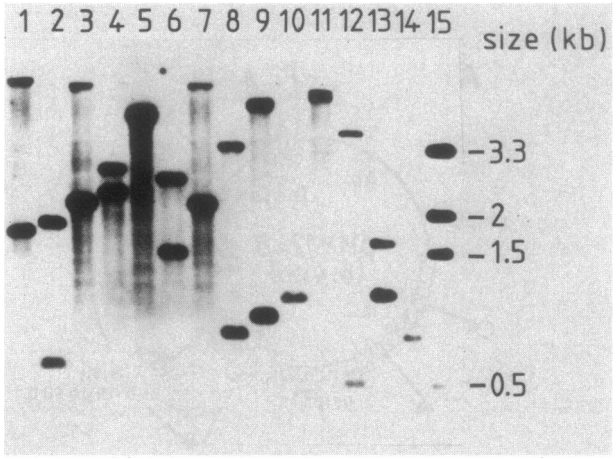

FIG. 2. Restriction analysis of transposition events. DNA was extracted from 14 thermoresistant Cm^r Em^s clones and restricted by *Eco*RI and *Nco*I; the segments were then separated by gel electrophoresis and hybridized to pHV1248 (lanes 1 to 14). Plasmid pHV1248 restricted with *Eco*RI and *Nco*I was loaded in lane 15. Its smallest fragment (300 bp) migrated out of the gel. Sizes were estimated by comparison with a Raoul (Appligène, Illkirsch, France) marker.

different sizes were detected. In the remaining two cases (Fig. 2, lanes 5 and 11) a single band was visible, which may have been due to the identical sizes of the two expected segments or to migration of the shorter segment out of the gel. These data confirm that mini-Tn*10* transposition occurred in *B. subtilis*. The site of transposon insertion was different for each clone, as deduced from the size variability of the hybridizing segments.

Tn*10* transposition is random. To examine whether a particular region of the chromosome was a preferential target for transposition, DNA from 12 of the 14 clones described above was purified in agarose plugs and restricted with *Sfi*I. The segments were separated by field inverted gel

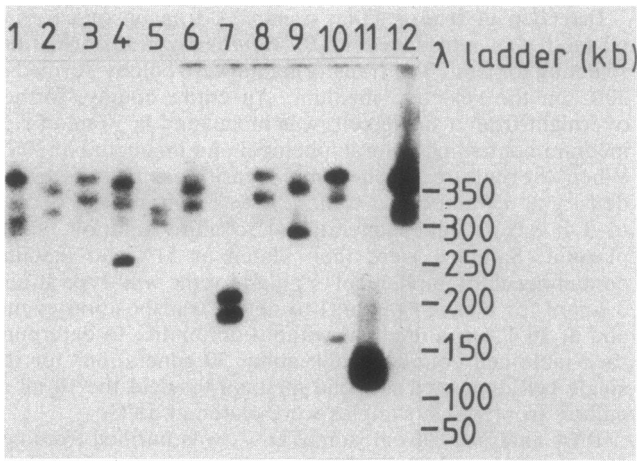

FIG. 3. Regional analysis of transposition events. DNA was extracted in agarose plugs from 12 Cm^r clones, restricted with *Sfi*I, separated by field inverted gel electrophoresis (see Materials and Methods for conditions used), and hybridized to a Tn*10* probe (the smaller *Eco*RV segment of pHV1248). Clones analyzed in lanes 1 to 12 correspond to clones analyzed in lanes 1 to 11 and 14 in Fig. 2, respectively. Sizes were estimated relative to those of a lambda ladder.

TABLE 2. Transposition of Tn*10* in a *B. subtilis recE4* mutant

Plasmid	Expt[a]	Viable cell count/ml	Resistant cell count/ml[b]	Proportion of resistant cells (avg ± SD)
pHV1248	1	3.5×10^7	2×10^2	$(3.5 \pm 1.3) \times 10^{-6}$
	2	6×10^7	2×10^2	
	3	10^8	2×10^2	
	4	10^8	3×10^2	
pHV1264	1	2×10^7	≤10	4×10^{-8}[c]
	2	2×10^7	≤10	
	3	10^8	≤10	
	4	10^8	10	

[a] Numbers refer to independent experiments.
[b] Selection was for Cm^r at 51°C.
[c] Results of the four experiments were pooled to calculate the proportion.

electrophoses, transferred to a nitrocellulose membrane, and hybridized with a mini-Tn*10* probe (Fig. 3; lanes 11 and 12 were overloaded). Two to three hybridizing segments were observed in each lane. This result was probably due to partial *Sfi*I digestion, since the chromosomal *sacA* gene probe hybridized to several bands on the same membrane (not shown). The fastest bands, presumably generated by complete restriction, were compared with a lambda ladder. Their sizes corresponded to those of nine different *Sfi*I segments (S2 in three cases; S3 in two cases; S1, S4, S5, S7, S9, S11, and S12) previously identified by P. Piggot (personal communication). From the known positions of these segments on the *B. subtilis* chromosome (Piggot, personal communication), we conclude that the insertions were scattered and that Tn*10* had no strong region preference.

Tn*10*-mediated insertional mutagenesis. Insertion of Tn*10* in the *Salmonella typhimurium* chromosome produced 1 to 2% auxotrophic mutants (12). To test whether this was also the case in *B. subtilis*, we screened 2,142 Cm^r colonies for auxotrophs. Thirteen clones unable to grow on minimal medium supplemented with tryptophan (recipient strain marker) and chloramphenicol (transposon marker) were recovered, which corresponds to a proportion of 0.6%. Among the 13 auxotrophs, 4 were His^-, 4 were Leu^-, 1 was Ilv^-, 1 was Glu^-, 1 was Cys^-, and 1 was Thi^-. One auxotroph responded to one of the amino acids Ile, Leu, Thr, and Lys. A regulation function affecting these different biosynthetic pathways might have been affected by Tn*10* insertion. Hence, seven different phenotypes were recovered, among which the two most frequent were observed in 4 of the 13 cases (30%). In contrast, Tn*917* gives ≈90% of *gltA* auxotrophs (23). Sporulation-deficient mutants were also identified by the absence of brown pigmentation on sporulation medium. Among 2,142 clones screened, 15 Spo^- were detected (0.7% of the population). Similar results were obtained with Tn*917* (1 to 2%). Tn*10* therefore appears to be a convenient tool for mutagenesis in *B. subtilis*.

Transposition in a *recE4* strain. Tn*917* transposition frequency is not affected by *recE4* mutation (23). A transposition test was performed with pHV1248 in a *recE4* strain (Table 2). Unexpectedly, the proportion of thermoresistant Cm^r clones was a 100-fold lower than in the wild-type strain. Similar results were obtained with pHV1249 (not shown). The difference was not due to plasmid monomer copy number, structural stability, or segregational instability at 51°C, all of which were similar for the wild-type and *recE4* mutant strains.

DISCUSSION

Miniderivatives of Tn*10*, a transposon isolated from *E. coli*, were shown to be functional in *B. subtilis*, provided that the transposase gene was fused to appropriate expression signals. The best results were obtained with the *E. coli tac* promoter and a *B. subtilis* RBS, derived from the expression vector p602-22 (Le Grice, in press). This fusion contains the *lac* operator, which offers the possibility of controlling transposase expression. Replacement of the p602-22 RBS by the transposase RBS decreased transposition efficiency in *B. subtilis* 100-fold, whereas fusion of the p602-22 promoter and RBS to the transposase gene did not allow detection of any transposition in *B. subtilis* (not shown). We have no explanation for the latter observation.

Cells carrying mini-Tn*10* in their chromosomes reached a proportion of 10^{-4} in cultures grown exponentially for 30 generations. Since the average copy number of pE194ts is about 10 (21), a transposition frequency of 0.5×10^{-6} per element and per generation can be calculated for pHV1248 and pHV1249. Tn*917*, a natural gram-positive element, transposed in our hands with a frequency of 0.7×10^{-8} when the same experimental procedure and mode of calculation were used. This low frequency relative to that of mini-Tn*10* could reflect a naturally controlled expression of the transposase. In *E. coli*, the transposition frequency of nonmodified Tn*10* is 10^{-7} per element per generation (15) but could be increased up to one event per cell and per generation when the transposase was overexpressed (20).

The frequency of transposition was similar with either the outside-end transposon, which requires *E. coli* IHF and HU proteins in vitro, or the inside-end transposon, which does not (14). It is possible that the functional equivalents of these proteins are present in *B. subtilis* but that they do not affect transposition frequency greatly. It has been proposed that IHF and HU proteins are highly conserved among species, from archaebacteria to eubacteria (4).

Surprisingly, when the *B. subtilis recE* gene (the *E. coli recA* equivalent) was inactive, the transposition frequency decreased 100-fold. A direct involvement of RecE in transposition is not likely, as there is no evidence for a role of RecA in Tn*10* transposition in *E. coli*. Possibly, RecE plays an indirect role by its control of SOS functions, provided that one of the SOS-controlled genes is required for transposition. A different, indirect role for RecE can also be postulated. The plasmid that we used generates high-molecular-weight multimers, which are 10-fold more abundant in the wild-type *B. subtilis* than in a *recE4* mutant (7). If these plasmid forms were a preferred transposon donor, the transposition process could be less efficient in the *recE4* strain.

Small Tn*10* derivatives appear to be a convenient tool for insertional mutagenesis in *B. subtilis*. They generated auxotrophic and sporulation-deficient mutants at frequencies of 0.6 and 0.7%, respectively, and had no highly favored targets for transposition. They were carried on a thermosensitive delivery plasmid that contains the transposase gene separated from the minitransposon. The use of this plasmid is simple, and once the host cell is cured of it, further movement of the transposon cannot occur. These results encourage the use of Tn*10* derivatives as genetic tools in studies of *B. subtilis* and possibly other gram-positive microorganisms.

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