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Genetics

Genetic transposition and insertional mutagenesis in *Bacillus subtilis* with *Streptococcus faecalis* transposon Tn*917*

(interspecific gene expression/phage SPβ/sporulation mutants)

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ABSTRACT **The *Streptococcus faecalis* transposon Tn*917* was introduced into *Bacillus subtilis* by transformation of competent cells with the plasmid pAMα1::Tn*917* and was tested for transposition activity by selection for insertions into the temperate phage SPβ. Insertions were obtained at a frequency indicating relatively efficient movement of the element, and Southern hybridization analysis of a particular insertion confirmed it to be the result of a genuine transposition event. A restriction fragment from pAMα1::Tn*917* containing the transposon sequences was ligated into a temperature-sensitive plasmid (pBD95), and transpositions into the *B. subtilis* chromosome were selected by requiring the transposon drug resistance to be maintained at temperatures nonpermissive for plasmid replication. Insertions have been recovered at many chromosomal sites, including ones that produced auxotrophy of different kinds and ones that produced various different sporulation-defective phenotypes, indicating good prospects for the use of Tn*917* as a tool for insertional mutagenesis in *B. subtilis*.**

Tn elements (transposable drug resistance elements, transposons) have come into increasing prominence as important tools for genetic analysis, particularly in some of the Gram-negative species, such as *Escherichia coli*, in which indigenous elements have been extensively characterized and adapted for special applications (1). In species with less developed genetics, transposon manipulations clearly offer even greater advantages over existing possibilities for conventional genetic analysis and even greater improvements over available gene cloning methods, a fact that has motivated recent efforts to introduce some of the well-characterized enteric bacterial elements into such Gram-negative bacteria as *Myxococcus* (2), *Caulobacter* (3), and *Anacystis* (4).

Until recently, it might have been hoped that Gram-negative transposons would also function in Gram-positive bacteria, such as *Bacillus subtilis*; indeed, this remains a possibility in most of the Gram-positives, for which little or no information is available concerning the expression of foreign genes. For *B. subtilis*, however, it has already been established that genes introduced from Gram-negative bacteria are not ordinarily active (5), and some of the genes tested have included the drug resistance determinants of transposons (A. L. Sonenshein, personal communication). Our own efforts to develop a system for using transposons in *B. subtilis* have therefore been restricted to examining the few elements of Gram-positive origin thus far identified (Tn*551*, Tn*554*, Tn*916*, and Tn*917*), about which relatively little is yet known. We have successfully introduced the *Streptococcus faecalis* transposon Tn*917* into *B. subtilis*, and we present evidence here that Tn*917* functions efficiently in a *B. subtilis* host. More significantly, we have been able to devise methods to select for Tn*917* insertions into the *B. subtilis* chromosome, and preliminary results of experiments of this kind indicate very good prospects for the use of Tn*917* as a tool to obtain insertional mutations of a type useful for the study of bacterial sporulation.

MATERIALS AND METHODS

Plasmids, Phage, and Bacterial Strains. Plasmids pAMα1 and pAMα1::Tn*917* (6) were obtained from D. Clewell. Plasmid pBD95 (7) and *B. subtilis recE4* mutant strain BD224 (8) were obtained from D. Dubnau. *B. subtilis* strain CU1050 (9), which we used as lawn bacteria to visualize SPβ phage plaques, and strain CU1147 (10), which we used as a source of SPβ*c2* phage, were obtained from S. A. Zahler. The Dedoner "kit" of mapping strains (11) was obtained from A. L. Sonenshein. All other strains were generated in this work and are described in the text.

Culture Media. PAB is Bacto Penassay broth (Difco antibiotic medium no. 3); LB medium is Luria–Bertani medium, prepared according to Levine (12); DSM is Difco sporulation medium [Difco nutrient broth, with 0.5 ml of 1 M NaOH, 10 ml of 1.2% $MgSO_4$, and 10 ml of 10% KCl per liter, and supplemented after autoclaving with 1 ml of 0.1 M $MnCl_2$/1 M $Ca(NO_3)_2$/0.001 M $FeSO_4$ per liter]; TSS is Tris–Spizizen salts medium [Spizizen minimal medium (13) with 60 g of Tris base per liter, titrated with HCl to pH 7.5, and supplemented after autoclaving with 10 ml of 0.4% $FeCl_3$/0.4% sodium citrate per liter].

Transformations and Transductions. Transformation-competent *B. subtilis* bacteria were prepared, stored, and transformed as described by Dubnau and Davidoff-Abelson (14). Standard methods were used for the preparation of PBS1 phage lysates and in performing transductions (15).

Selection and Scoring of Genetic Markers. Chloramphenicol resistance (Cm^r) was scored or selected on LB plates with Cm at 5 μg/ml. The phenotype of resistance to macrolides, lincosamides, and streptogramin B antibiotics (MLS^r) conferred by Tn*917* is inducible by erythromycin (Em) (6). In LB broth, Em was added to a concentration of 0.15 μg/ml and cultures were incubated 2 hr at 37°C or 3 hr at 33°C before selecting with Em at 1 μg/ml and lincomycin (Lm) at 25 μg/ml. For direct platings of transductants or transformants, cell suspensions were added to LB soft agar with 0.1 ml of a 10-μg/ml Em solution and plated as an overlay. After 2 hr at 37°C or 3 hr at 33°C, a second LB soft agar overlay was added containing 0.1 ml per plate of a 400-μg/ml Em solution and a 10-mg/ml Lm solution.

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Abbreviations: kb, kilobase pair(s); MLS, macrolide, lincosamide, and streptogramin B antibiotics; Cm, chloramphenicol; Em, erythromycin; Lm, lincomycin; Tc, tetracycline; ^{r}, resistance; ^{s}, sensitivity.

RESULTS

Introduction of Tn*917* into *B. subtilis*. Tn*917*, which confers in *S. faecalis* the pleiotropic MLS^r phenotype (16), was available as an insertion into the plasmid pAMα1, a 9.7-kilobase-pair (kb) *S. faecalis* replicon, which carries an amplifiable tetracycline-resistance (Tc^r) determinant (17) and which was thought to be capable of replication in *B. subtilis*. Many (but not all) plasmids of *Staphylococcus* (18, 19) and *Streptococcus* (20) origin have been found able to function as stable, autonomous replicons after being introduced into *B. subtilis* by transformation, and pAMα1 was believed to be among them because pAMα1 plasmid DNA could be used to transform competent *B. subtilis* cells to Tc^r (albeit at very low frequency) (21). In work to be published elsewhere we have shown that Tc^r transformants of *B. subtilis*, obtained with pAMα1 DNA, actually contain a form of the plasmid, only 4.6 kb in size, which we refer to as pAMα1Δ1, that has undergone a deletion; this apparently reflects the necessity for a large portion of pAMα1 to be eliminated (by an intramolecular recombination event) for the remaining portion (pAMα1Δ1) to replicate efficiently. This may explain, in part, the observation that pAMα1::Tn*917* cannot be used to transform a plasmidless *B. subtilis* recipient to either Tc^r or MLS^r. Nevertheless, it was possible to transform a pAMα1Δ1-containing *B. subtilis* recipient to MLS^r with pAMα1::Tn*917*, and the plasmid species thereby established (presumably by recombination between pAMα1Δ1 and pAMα1::Tn*917*) was pAMα1Δ1::Tn*917*, a 10-kb plasmid, the structure of which is shown in Fig. 1. Interestingly, the replication functions used by pAMα1::Tn*917* in *Streptococcus* and by pAMα1Δ1::Tn917 in *Bacillus* are different (unpublished data).

Isolation and Analysis of Tn*917* Insertions into SPβ. Because other kinds of insertion or recombination events may bear a misleading resemblance to transposition, we sought to test Tn*917* for transposition activity by selecting for insertions into a target molecule that could readily be isolated and subjected to restriction and Southern hybridization analysis, in order to determine the nature of any apparent transpositional insertions we might obtain. We chose as the target the large (130-kb) temperate phage of *B. subtilis* called SPβ (22). This phage was known to contain relatively extensive regions of "nonessential" DNA (S. Zahler, personal communication), which should be able to receive a transposition without inactivating the phage, and was known to accommodate easily extra DNA of at least transposon size without causing packaging problems (23). Thus, we reasoned that it should be possible to test for transposition by inducing a pAMα1Δ1::Tn*917*-containing SPβ lysogen, and using the lysate to superinfect another lysogen (or to infect a phage-sensitive strain to produce lysogens), with a selection for MLS^r expected to identify phage-borne transposition products. To obtain a nondefective transposon-carrying phage, lysates from a pAMα1Δ1::Tn*917*-containing lysogen were used to transduce MLS^r, many transductants were combined, induced once again, and the resulting lysate was used again for transduction of MLS^r. This mixture of transductants was then induced and plated for plaques on a MLS^s lawn. When the turbid centers of such plaques were picked and transferred to selective plates, greater than 90% were found to contain MLS^r bacteria. One nondefective, MLS^r-transducing SPβ phage identified in this way was purified for further study.

To determine whether the transposon-carrying phage contained an insert the same size as *Tn*917, DNA samples from it and from a phage containing no insert were digested with *Eco*RI (which does not cut within Tn*917*), and the resulting fragments were separated by electrophoresis on a 0.8% agarose gel. A photograph of such a gel, Fig. 2, shows that *Eco*RI cuts the phage

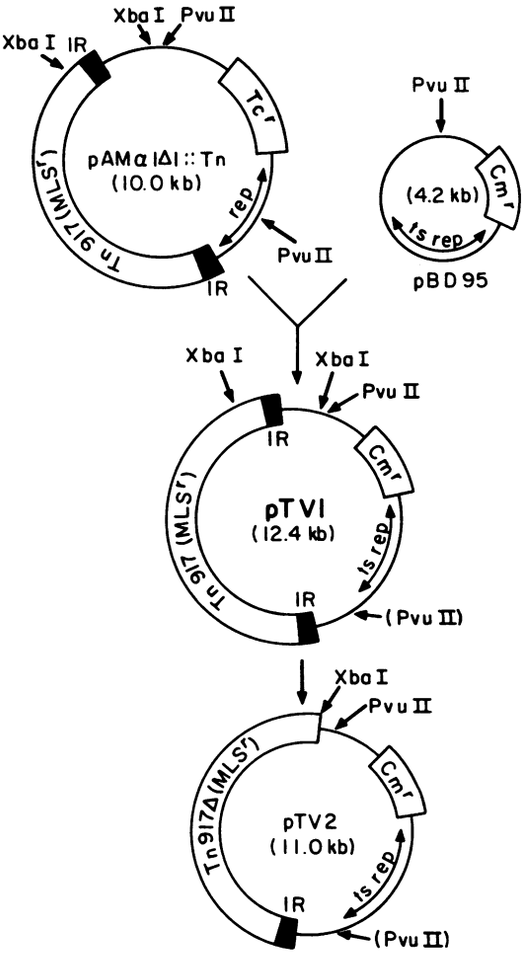

FIG. 1. Structures and origins of plasmid species. Competent *B. subtilis* cells were transformed with the 15.1-kb *S. faecalis* plasmid pAMα1::Tn*917*, selecting for MLS^r (see text), which established a 10.0-kb form of the plasmid with a deletion, called pAMα1Δ1::Tn*917*. The approximate region of this plasmid that is essential for replication is indicated by a double-headed arrow labeled "rep"; temperature-sensitive replication functions of other plasmids in the figure are denoted by "ts rep." The Tn*917*-containing *Pvu* II restriction fragment from pAMα1Δ1::Tn*917* was ligated into the unique *Pvu* II site in the Cm^r-carrying plasmid pBD95 (7), which is temperature-sensitive for replication, generating a plasmid called pTV1. This plasmid has only a single *Pvu* II recognition site, apparently because one of the sites, at one of the ligation junctions (the one shown in parentheses), was not restored by the blunt-end joining. To produce pTV2, one of the terminal inverted repeats (IRs) of Tn*917* was deleted by removing a small *Xba* I fragment from pTV1 that straddles one insertion junction.

DNA with no insert at least 35 times. In the digest of the insert-containing phage DNA, a single band is missing and a new band has appeared, which is larger than the missing band by 5.4 kb (transposon size).

To determine whether the transposon-carrying phage was free of pAMα1 sequences, Southern strips prepared with *Eco*RI-cut insert-containing phage DNA and with *Eco*RI-cut DNA from a phage with no insert were incubated with denatured pAMα1 or pAMα1::Tn*917* DNA that had been radiolabeled with ^{32}P by nick-translation. The results, also presented in Fig. 2, show that only strips prepared with transposon-carrying phage DNA reacted with the pAMα1::Tn*917* hybridization probe, and that no strips reacted with the pAMα1 probe (other controls not shown confirmed that both probes were appropriately labeled); the positive reaction of the nick-translated pAMα1::Tn*917* probe

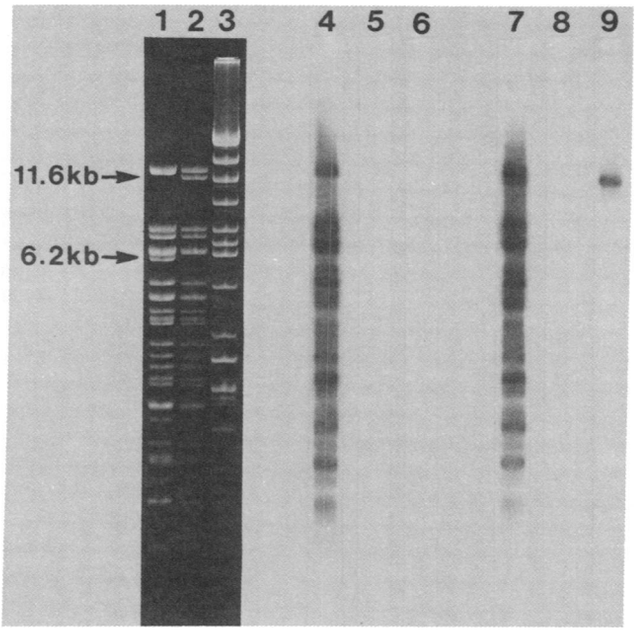

FIG. 2. Size and sequence composition of a MLS^r-carrying insert in a nondefective MLS^r-transducing SPβ phage. Lanes 1–3 show DNA restriction fragments separated by electrophoresis on 0.8% agarose. Wild-type SP$\beta c2$ DNA digested with *Eco*RI (which does not cut Tn*917*) was run in lane 1. *Eco*RI-digested DNA from a MLS^r-transducing SP$\beta c2$ phage (see text) was run in lane 2. An *Eco*RI* digest of SPO1 phage DNA (24), in lane 3, was used as a molecular weight reference. Lanes 4–9 show autoradiographs of similar agarose-separated *Eco*RI digests of SP$\beta c2$ (lanes 4–6) and MLS^r-transducing phage (lanes 7–9) DNAs that were denatured, blotted onto nitrocellulose (25), and incubated with denatured SP$\beta c2$ (lanes 4 and 7), pAMα1 (lanes 5 and 8), or pAMα1::Tn*917* (lanes 6 and 9) DNA that had been radiolabeled with ^{32}P by nick-translation (26).

with the transposon-carrying phage DNA strips was specific for the new band enlarged by transposon size.

A Selection for Transpositions into the *B. subtilis* Chromosome. Most useful applications of Tn*917* in *B. subtilis* would require some general means of selection for transposon insertions into chromosomal material. One common approach, which has been used with several of the Gram-negative transposons (27, 28) and also to select for Tn*551* insertions into the *Staphylococcus aureus* chromosome (29), is to place the transposon on a plasmid vector that is temperature sensitive for replication. Chromosomal insertions are then selected for by shifting to the nonpermissive temperature while maintaining selection for the transposon drug resistance. To attempt such an approach with Tn*917*, we have made use of the plasmid replication functions of pE194, the only *B. subtilis* plasmid (originally from *S. aureus*) known to be tightly temperature-sensitive for replication (30), and we have taken advantage of a derivative of this plasmid, called pBD95, constructed in the Dubnau laboratory, in which the region of pE194 normally carrying a MLS^r gene has been partially deleted and replaced with a Cm^r-carrying restriction fragment from pBD64 (30). The *Pvu* II fragment from pAMα1Δ1::Tn*917* that contains the transposon was ligated into a unique *Pvu* II site in pBD95 to produce a plasmid approximately 12 kb in size, which carries a Cm^r determinant in addition to the transposon and which depends upon the temperature-sensitive replication functions of pE194 for replication. The structure of this plasmid, which we refer to as pTV1, is diagrammed in Fig. 1.

To test pTV1 as a Tn*917* transposition selection vector, the pTV1-containing protoprophic strain PY143 was grown to mid-logarithmic phase at 33°C in LB broth containing Cm at 5 μg/ml, Em at 1 μg/ml, and Lm at 25 μg/ml and then plated on LB plates containing the same levels of Em and Lm, but no Cm, which were incubated at 48°C. MLS^r colonies appeared on these plates at a frequency of 2×10^{-5} relative to MLS^r colony-forming units at 33°C. In subsequent experiments, selections at any temperatures between 45°C and 49°C were indistinguishably effective, and selected colonies have consistently emerged at a frequency of $2–9 \times 10^{-5}$. When selected colonies were regrown at 48°C under MLS^r selection and then tested at 33°C for Cm^r, over 90% were found to be Cm^s. The approximate map positions of two inserts chosen at random from colonies selected in independent experiments were determined by phage PBS1 transduction of the Dedoner "kit" strains (11) to MLS^r, scoring in sequence of map order for linkage to markers contained in these strains. One insert was discovered to be near *hisA* and the other near *gltA*. More precise map positions were then determined from a three-factor analysis of transduction crosses, and the results placed the inserts as shown in Fig. 3. When chromosomal DNA containing the two inserts was digested with *Hin*dIII, blotted onto nitrocellulose, and tested for hybridization with nick-translated pBD95, pAMα1, and pAMα1::Tn*917* probes, only the pAMα1::Tn*917* probe gave a positive reaction (data not shown).

To test whether the production of Tn*917* inserts in this manner depended upon the ability of the cell to execute intermolecular homologous recombination, pTV1 was used to transform strain BD224, a *recE4* mutant known to be highly recombination deficient (8). This pTV1-containing *recE4* strain was subjected to a growth and selection protocol identical to that described for PY143, and selected colonies were recovered at a frequency of 2×10^{-5} (equivalent to that observed in a recombination-proficient strain). All 300 selected colonies tested as Cm^s at 33°C. To determine whether insert production required both ends of Tn*917* to be intact, one end of the transposon was deleted by removing a small *Xba* I restriction frag-

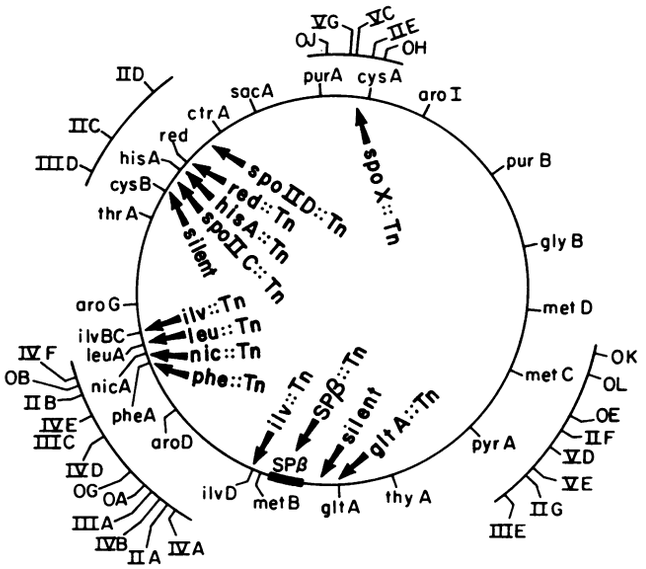

FIG. 3. Genetic map of the *B. subtilis* chromosome, showing determined positions of mapped Tn*917* insertions (the silent inserts, SPβ::Tn*917*, *red*::Tn*917*, and insertional sporulation mutants) and inferred probable positions of several (unmapped) insertional auxotrophic mutations in relation to known sporulation loci (outer perimeter) and certain "landmark" auxotrophic markers (inner circle). All mapped positions were determined from three-factor analysis of PBS1-mediated transduction crosses (data not shown).

ment from pTV1 (as shown in Fig. 1). In experiments with a strain containing the deletion plasmid, pTV2, transpositions were less than 1/100th as frequent.

Chromosomal Tn*917* Insertions Produce Different Kinds of Auxotrophic and Different Kinds of Sporulation-Defective Mutants. If the sites of chromosomal transposon insertions are distributed with a relatively high degree of randomness, populations of insert-containing cells include many kinds of insertional mutants. For example, when Tn*10* is used to "mutagenize" the chromosome of *Salmonella typhimurium*, insertional auxotrophs are produced at a level of approximately 1–2% (31). To determine whether pTV1-generated Tn*917* inserts in the *B. subtilis* chromosome were sufficiently random to permit the recovery of auxotrophs, a culture of PY143 grown to mid-logarithmic phase at 33°C was diluted 1:100 into 48°C broth containing MLS drugs, grown (overnight) to stationary phase, and plated for single colonies (on MLS-containing LB plates incubated at 48°C), and the single colonies were picked and transferred onto glucose-minimal plates to screen for auxotrophs. In four independent experiments, the selected colonies were found to include auxotrophs at levels of 3–8%. The growth requirements of several auxotrophs from each of the four selections were determined, revealing that glutamate-requiring mutants were the predominant class in each selected population, accounting for 90–95% of all auxotrophs (thus accounting for approximately 3–5% of all selected colonies); auxotrophs that were not glutamate requirers were of many different kinds, however, including some with phenylalanine, isoleucine and valine, histidine, nicotinamide, and leucine requirements (some determinations were made by S. Zahler, personal communication), as well as others with different but undetermined requirements. None, with the obvious exception of the glutamate requirers, appeared to be represented with a very strongly disproportionate abundance. Although none of the insertional auxotrophs have yet been mapped (except for the glutamate requirers, which we know to be at or near *gltA*), the probable map positions of several may be guessed on the basis of their growth requirements, and these probable positions are indicated in Fig. 3. The reversion level of one of the glutamate requirers was determined and found to be approximately 10^{-9}; when this insert was introduced into other strains by transformation and transduction, the MLS^r and the auxotrophic phenotype were 100% cotransferred.

In one of our four selected populations that were prepared for the identification of auxotrophs, a very striking nonauxotrophic mutant type appeared as a relatively abundant class (approximately 1%): it made a distinctly red or red-purple colony on several kinds of media (including LB); on sporulation-inducing substrates, such as DSM-agar plates, it was somewhat more pigmented than normal and did not appear to be at all defective in spore formation. The insert producing this phenotype was mapped, and it was found to be very close to *hisA* (85% cotransduced by PBS1), on the *ctrA* side in three-factor crosses (the map position is shown in Fig. 3). The affected gene, which we refer to as *red*, does not correspond with any previously characterized genetic locus in *B. subtilis*.

Mutations of *B. subtilis* that affect the process of spore formation (*spo* mutations) are a type of more direct relevance to the primary research interests of our laboratory. To determine whether Tn*917* might be useful for the isolation of insertional *spo* mutants, a MLS^r population of PY143 cells selected at 48°C (prepared as described above for the auxotroph analysis) was plated on DSM-agar plates, where the colonies of sporulation-defective mutants (particularly mutants blocked at early stages of sporulation) may be readily identified by their failure to produce normal amounts of a sporulation-associated brown pigment (32). After 48–72 hr of incubation at 37°C, 1–2% of the colonies on the DSM plates had the unpigmented or translucent appearance associated with sporulation-defective phenotypes. Of these, several failed to grow on lactate-minimal plates (the number varying from population to population), indicating that they were either auxotrophs or mutants defective in citric acid cycle enzymes, and thus sporulation-defective as an indirect consequence (33). Excluding these, *spo* mutants represented approximately 0.1–0.5% of the selected populations.

Three particularly white, but not very translucent insertional *spo* mutants [judged likely by their appearance to be tight "stage II" or "stage III" mutants (32)], recovered from three independent experiments, were mapped to approximate positions by PBS1 transduction using the Dedoner strains, and then positioned by three-factor crosses (deduced map locations are indicated in Fig. 3). Among the products of all crosses, the sporulation defects were 100% linked with the MLS^r phenotype, confirming that these were insertion-generated lesions. As shown in Fig. 3, the three insertional *spo* mutations mapped to three different chromosomal locations, two probably corresponding with the *spo* loci *spoIIC* and *spoIID*, which have been previously identified after conventional mutagenesis. The third (*spoX*::Tn*917* on the map in Fig. 3) was found to be in the *purA–cysA* region of the chromosome, which contains several *spo* loci. Three-factor transformation crosses (data not shown) indicate that *spoX*::Tn*917* is within or near a cloned cluster of sporulation-specific genes whose transcriptional regulation has been extensively studied in our laboratory (34) and that the insertion may identify a previously uncharacterized gene.

DISCUSSION

Tn*917* was discovered as a MLS^r determinant associated with the nonconjugative *S. faecalis* plasmid pAD2 (22 kb in size) (6). It was observed that this MLS^r determinant could move from pAD2 to several different conjugative plasmids in *S. faecalis* at a frequency of approximately 10^{-6}, and it is now known that Tn*917* is able to transpose to at least four different sites in a particular conjugative plasmid, pAD1 (approximately 50 kb in size) (35). No attempts have yet been made to select for Tn*917* insertions into the *Streptococcus* chromosome, and it has not been determined whether Tn*917* movements in *Streptococcus* are independent of host homologous recombination functions.

After its introduction into *B. subtilis* on the plasmid pAMα1::Tn*917*, we were able to detect transposition of Tn*917* into the temperate phage SPβ at a frequency that suggested efficient activity of the element in a *Bacillus* host. Moreover, when a restriction fragment from pAMα1Δ1::Tn*917* that contained the transposon was ligated into a temperature-sensitive plasmid (pBD95), to produce a transposition selection vector called pTV1, Tn*917* insertions into the chromosome were recovered by selection at 48°C at a frequency of $2–8 \times 10^{-5}$, indicating that plasmid-to-chromosome transpositions of Tn*917* in *B. subtilis* must occur at a frequency not greatly different from that of plasmid-to-plasmid transpositions in *S. faecalis* (with pAD2 as donor and pAD1 as target molecules), estimated on a transposition per vector basis, normalized for target size, and assuming copy numbers for pAD1, pAD2, and pTV1 of 1–2, 1–2, and 5–10, respectively [approximate figures based on plasmid yields (D. Clewell, personal communication, and unpublished data)]. The frequency of Tn*917* transposition from pTV1 into the *B. subtilis* chromosome was found to be unaltered in a host highly deficient for homologous recombination (a *recE4* mutant), and transposition was virtually abolished (reduced to less than 1/100th by a deletion that removed a portion of one end of Tn*917* (to produce the plasmid pTV2).

Several pTV1-generated chromosomal Tn*917* transpositions were characterized, and the results revealed that Tn*917* can insert at many different sites, to produce different kinds of insertional auxotrophs and different kinds of insertional sporulation-defective mutants, as well as phenotypically silent insertions at different locations, despite indications of "hot spots" of high target preference (such as the one within *gltA*). It was especially encouraging to find that three insertional *spo* mutations, which conferred similar phenotypes, mapped at three different locations. We are thus optimistic that Tn*917* insertions into virtually any (nonessential) *B. subtilis* gene may be obtained, providing there exists a sufficiently sensitive screening procedure to identify the phenotype of the resulting mutation.

The discovery that Tn*917* can transpose efficiently in a *B. subtilis* host raises the interesting question of whether other potentially useful insertion elements, such as the *Streptococcus* transposon Tn*916*, might also function in *Bacillus*. This unusual 15-kb element, which carries a Tc^r gene(s), can apparently catalyze its own conjugative transfer from cell to cell, as well as its transposition from replicon to replicon (36). The successful use of *Streptococcus* transposons in *Bacillus* also raises the question of whether these elements might function in other Gram-positive bacteria of increasing interest to both basic researchers and industrial microbiologists, such as *Streptomyces* or *Corynebacterium*, for which the lack of characterized indigenous insertion elements hinders many kinds of genetic manipulations.

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