Transposon Tn917PF1 mutagenesis in *Bacillus licheniformis*

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The plasmid pTnPF1 containing the transposon Tn917PF1 was introduced into the protoplasts of two *Bacillus licheniformis* strains in the presence of polyethylene glycol. Transpositions were produced at high temperature which inhibited plasmid replication and kanamycin was used for selection. Transposon Tn917PF1 was inserted randomly into the bacterial chromosome, producing different auxotrophic, prophage BLF and bacitracin-non-producing mutants. The auxotrophic mutant phenotypes were characterized by the Holliday-test and some mutations by hybridization with a transposon DNA probe. Insertions for the entire chromosome or for the prophage genophore were found at random, but preferred target sites were detected within limited regions, like the bacitracin synthetase or sulphate reductase genes. The partial physical map of the chromosomal region of bacitracin synthetase was constructed based on the hybridization patterns of insertion mutants.

Keywords: *Bacillus licheniformis*, transposon mutagenesis, auxotroph, prophage mutant, bacitracin synthetase

INTRODUCTION

Transposons are useful tools for genetic analysis. In bacilli, the enterococcal transposon Tn917 is the most frequently used for transposition mutagenesis (Youngman *et al.*, 1983; Bohall & Vary, 1986; Hartley & Paddon, 1986; Wati *et al.*, 1990). This transposon and its developed versatile derivatives are suitable for insertional mutagenesis (Vandeyar & Zahler, 1986; Sandman *et al.*, 1987), rapid cloning (Youngman *et al.*, 1984), the construction of transcriptional gene fusions (Perkins & Youngman, 1986; Zuberi *et al.*, 1990; Youngman, 1990) and the characterization of genes which are over-expressed (Zagorec & Steinmetz, 1991).

The peptide antibiotic bacitracin (Bt)-producing *Bacillus licheniformis* strain 19 carries three temperate bacteriophages: LP52 (Doskočil *et al.*, 1986), DLP10716 (Thorne & Kowalski, 1976) and BLF (Holczinger, 1986) integrated as prophages into the chromosome. Having a natural erythromycin (Em) resistance determinant on the chromosome, the *erm* gene of Tn917 is non-selectable in this strain. Therefore the Tn917PF1 derivative, carrying

an additional kanamycin (Km) resistance gene for selection, was used. Since the promoter of this *aphA'* gene is strong and outward-directed, the Tn917PF1 can also be used for the isolation and characterization of mutants with certain gene over-expression in *B. subtilis* (Zagorec & Steinmetz, 1991).

Transposon mutagenesis using Tn917PF1 is reported here for two *B. licheniformis* strains, to obtain different insertion mutants. Auxotrophic, Bt synthetase and BLF prophage mutants were selected and characterized by the Holliday-test, antibiotic or phage production. Insertion sites were determined by hybridization with the transposon DNA.

METHODS

Bacterial strains and plasmids. *B. licheniformis* 19 (our laboratory) is an industrial Bt-producing strain and is resistant to Em. Three temperate bacteriophages (LP52; DLP10716 and BLF) are integrated in its chromosome. *B. licheniformis* ATCC 9800 ($\equiv$ ATCC 9789) (OKI, Budapest, Hungary) is a Bt-non-producer and is sensitive to phage BLF. Both strains are prototrophic. *Micrococcus flavus* (*M. luteus*) ATCC 10240 (OKI, Budapest, Hungary) was used to test Bt production. Plasmid pTnPF1 (Zagorec & Steinmetz, 1991) contains the Km resistance gene *aphA'* from Tn1545 which was inserted into the transposon Tn917 without the putative terminator. The plasmid

Abbreviations: Bt, bacitracin; Cm, chloramphenicol; Em, erythromycin; EtBr, ethidium bromide; Km, kanamycin.

had a super-thermosensitive replication origin and a chloramphenicol (Cm) resistance marker outside the transposon sequence. The plasmid donor was *B. subtilis* GM479.

Media. For the transformation of *B. licheniformis* protoplasts, media as described by Prágai *et al.* (1994), YTA media described by Sík *et al.* (1980) and BMM minimal medium described by Hanlon & Hodges (1981) were used.

Determination of Tn917PF1 transposition frequency. The frequency of transposition was determined essentially as described by Youngman (1987). *B. licheniformis* carrying pTnPF1 was grown in YTA broth containing 20 µg Cm ml⁻¹ and 10 µg Km ml⁻¹ at 30 °C until the mid-exponential phase. Dilutions were plated in two series on YTA plates supplemented with Km (10 µg ml⁻¹) or Km and Em (10 and 1 µg ml⁻¹) and incubated at either permissive (30 °C) or non-permissive (50 °C) temperatures for plasmid replication. The transposition frequency was expressed as the ratio of c.f.u. at 50 °C and 30 °C.

Selection for Tn917PF1-generated chromosomal insertions.

A single colony of *B. licheniformis* carrying pTnPF1 was grown in YTA broth containing 20 µg Cm ml⁻¹ and 10 µg Km ml⁻¹ at 30 °C overnight (14–16 h). The culture was diluted 1000-fold into 5 ml of 50 °C YTA broth containing 10 µg Km ml⁻¹ and grown until the late exponential phase (10–12 h). The culture was then diluted and plated on YTA agar medium containing 10 µg Km ml⁻¹ and incubated at 50 °C overnight (14–16 h). After incubation, colonies were screened for Cm sensitivity on YTA agar medium containing 10 µg Km ml⁻¹ and 20 µg Cm ml⁻¹. Km-resistant and Cm-sensitive colonies were candidates to carry Tn917PF1 insertions and to eliminate the vector. Plasmid elimination was confirmed in 100 samples by the quick-test method (Prágai *et al.*, 1994).

Isolation and identification of auxotrophs. Colonies of *B. licheniformis* carrying Tn917PF1 insertions were transferred to BMM minimal medium and auxotrophs were detected as non-growing. Auxotrophic mutants were characterized by growth on BMM minimal medium supplemented with different combinations of amino acids, vitamins, purines and pyrimidines (Holliday, 1956) and then verified.

Isolation of Tn917PF1 insertions into prophage BLF. *B. licheniformis* 19 Tn917PF1 insertion mutants were tested on the BLF-sensitive strain ATCC 9800 for phage production. Mutants which did not produce a lysis halo around the colony on the lawn were selected.

Isolation of Bt-non-producing mutants. *B. licheniformis* 19 Tn917PF1 insertion mutants were tested on a *M. flavus* lawn for Bt production. Those having no inhibition zone around the colony after incubation for 24 h at 37 °C were selected.

DNA isolation. Plasmid pTnPF1 DNA was isolated from *B. subtilis* GM479 by a large-scale plasmid purification method using alkaline lysis and CsCl–EtBr centrifugation (Bron, 1990). Total DNA was extracted from the strains and insertion mutants as described by Bron (1990).

Restriction endonuclease digestion. The enzymes were purchased from Stratagene and used according to the manufacturer's specifications.

DNA–DNA hybridization. Endonuclease-treated DNAs were separated by agarose gel electrophoresis (0.7 %), denatured and blotted onto nitrocellulose as described by Perbal (1988). Denatured plasmid pTnPF1 or BLF phage DNA was radio-labelled with [³²P]dCTP (Amersham) using the T⁷QuickPrime Kit (Pharmacia) according to the manufacturer's specifications.

Prehybridization, hybridization and detection were as described by Sambrook *et al.* (1989).

RESULTS

Tn917PF1-generated chromosomal insertions

Plasmid pTnPF1 carrying transposon Tn917PF1 was isolated from *B. subtilis* GM479, and introduced into *B. licheniformis* 19 and ATCC 9800 protoplasts in the presence of 30 % (w/v) polyethylene glycol 6000. Transformants were selected with Cm and Km and their plasmid content was confirmed by the quick-test method (Prágai *et al.*, 1994).

The Tn917PF1 average transposition frequency, determined in three independent experiments (selection with Km), was 8.6×10^{-4} and 1.2×10^{-3} in *B. licheniformis* 19 and ATCC 9800, respectively. When both Km and Em were used for selection, the corresponding frequencies were 7.2×10^{-4} and 1.1×10^{-3} , respectively. The close similarity of these results indicated that Em did not induce transposition in *B. licheniformis* and therefore was omitted in further experiments.

The Tn917PF1-generated insertion mutants of two *B. licheniformis* strains were selected with Km at 50 °C. To obtain transposition results (Table 1) as accurately as possible, 190–200 colonies were tested from many independent experiments. In this way, the distorting effect of a possible 'jackpot' event of early transposition might be decreased. After heat-treatment at 50 °C, 99 % of the colonies became Cm-sensitive, proving effective plasmid elimination and showing that all Km-resistant colonies resulted from the transposition (the frequencies of spontaneous chromosomal Km-resistant mutations in *B. licheniformis* 19 and ATCC 9800 were 1×10^{-7} – 3×10^{-7}).

To determine whether random or site-specific Tn917PF1 insertions occurred in the chromosome of *B. licheniformis* 19, 13 independently isolated insertion mutants were examined by hybridization with the transposon probe. Since *Bam*HI cleaves Tn917PF1 to a 1.6 and 5.0 kb fragment, two hybridizing bands should have resulted from the *Bam*HI-digested chromosomal DNA of the

Table 1. The results of transposon Tn917PF1 mutagenesis for two *B. licheniformis* strains

	ATCC 9800	19
No. of independent experiments	30	29
No. of the tested colonies	5860	5620
Km-resistant colonies	100 %	100 %
Cm-sensitive colonies	98.8 %	99.3 %
Auxotrophic colonies	1.0 %	0.7 %
Colonies with defective BLF prophage	–	0.35 %*
Bt-non-producing colonies	–	0.6 %*

*Only 2000 of the 5620 colonies were tested for phage BLF or Bt production as described in Methods.

Fig. 1. Southern hybridization of *Bam*HI-digested *B. licheniformis* 19 chromosomal DNA samples containing Tn917PF1 insertions with 32 P-labelled pTnPF1 DNA probe. The molecular mass marker was *Hind*III-digested λ DNA. Lanes: A, wild-type *B. licheniformis* 19; B–N, *B. licheniformis* 19 Tn917PF1 insertion mutants.

Table 2. Identification of *B. licheniformis* auxotrophs by the Holliday-test

Fifty-eight and 39 auxotrophic mutants of *B. licheniformis* ATCC 9800 and *B. licheniformis* 19, respectively, were identified.

Requirements	Auxotrophs (%) of strain:	
	ATCC 9800	19
Arginine	5.2	2.6
Arginine or ornithine	6.9	7.7
Aspartic acid	–	5.1
Cysteine	–	5.1
Cysteine or thiosulphate or methionine	24.1	–
Histidine	1.7	–
Isoleucine	6.9	–
Methionine	3.5	10.3
Nicotinic acid	–	2.6
Proline	–	7.7
Riboflavin	–	5.1
Serine	3.5	10.3
Thiamine	10.3	15.3
Threonine	3.5	–
Tryptophan	12.0	5.1
Uracil	1.7	12.8
Undetermined	20.7	10.3

mutants. As shown in Fig. 1, only two hybridizing bands of different sizes were detected in every lane. This indicates a single copy of Tn917PF1 in each chromosome. Ap-

parently, different sites were used by the transposon in different chromosomes, confirming random insertion.

Isolation and characterization of auxotrophic mutants

A total of 58 (1%) auxotrophic mutants were detected from *B. licheniformis* ATCC 9800 and 39 (0.7%) from strain 19 (Table 1). For strain ATCC 9800, 11 different classes, and for strain 19, 12 different classes of nutritional requirements and one unspecified class could be distinguished (Table 2).

To determine reversion frequencies, a *B. licheniformis* ATCC 9800 sulphate reductase mutant and a *B. licheniformis* 19 thiamine auxotroph were grown for 20 generations in YTA broth without Km. The cells were then washed with sterile distilled water and the dilutions plated on YTA agar and BMM minimal medium. No prototrophic revertants were observed among 1.5×10^7 and 2.5×10^7 c.f.u., respectively, so the reversion rates were less than 10^{-9} per generation.

To examine whether site-specific or random insertions occurred within the classes of selected auxotrophic mutants, DNAs of 14 *B. licheniformis* ATCC 9800 sulphate reductase mutants (requiring cysteine, thiosulphate or methionine) were single- and double-digested with *Eco*RI and *Bam*HI, the fragments separated by agarose gel electrophoresis and hybridized with radiolabelled transposon DNA (Fig. 2). One hybridizing fragment was expected after *Eco*RI digestion and two after *Bam*HI single and *Eco*RI + *Bam*HI double digestion (no *Eco*RI sites and one *Bam*HI site exist within Tn917PF1). Twelve insertions, resulting in the same hybridization pattern, seemed to be identical. Since Southern hybridization can only distinguish insertions 100 bp apart, DNA sequencing of target sites will be needed to conclude that the 12 mutants have the same insertions. Two mutants had different patterns. In one mutant (Fig. 2, lane F) insertion was on the same *Eco*RI and *Bam*HI fragment, 2.3 kb apart. In the other (Fig. 2, lane B), two or four hybridization bands were found, indicating two insertions in the chromosome, which was rather unusual with Tn917. These latter insertions were on *Eco*RI or *Bam*HI fragments distinct from the previous 13.

Isolation of insertions in 'essential' DNA regions of prophage BLF

Two-thousand *B. licheniformis* 19 insertion mutants, isolated from 10 independent experiments, were tested for BLF phage production. Seven mutants (0.35%) did not produce phage BLF spontaneously. The bacteria were prototrophic, produced Bt and showed the same phage sensitivity as the original triple lysogenic strain. To determine the transposon insertion sites, Southern hybridizations were performed with 32 P-labelled pTnPF1 as probe. The transposon has two *Eco*RV cleavage sites, resulting in 0.4 (left end), 1.0 (middle) and 5.2 kb (right end) fragments. Thus, after *Eco*RV digestion, three fragments should hybridize with the pTnPF1 probe: an

Fig. 2. Southern hybridizations of *B. licheniformis* ATCC 9800 sulphate reductase mutants containing Tn917PF1 insertions with ^{32}P -labelled pTnPF1 DNA probe and the partial physical map of the sulphate reductase chromosomal region. The molecular mass marker was *Hind*III-digested λ DNA. The chromosomal DNA samples were digested by (a) *Eco*RI, (b) *Bam*HI and (c) *Eco*RI and *Bam*HI. Lanes: A, wild-type *B. licheniformis* ATCC 9800; B–O, *B. licheniformis* ATCC 9800 sulphate reductase auxotrophs. (d) Thirteen insertions were on the same *Eco*RI (Δ) and *Bam*HI ($\blacksquare$) fragment. One of them (Lane F) was 1.1 kb and the other 12 insertion sites (LHS, local hot spot) were 3.4 kb from the left-hand *Bam*HI site. Transposon Tn917PF1 is indicated by a box.

identical middle and the two flanking parts attached to the chromosomal fragments of different sizes. It was seen that, after hybridization with the pTnPF1 probe (Fig. 3a), the mutants exhibited different hybridization patterns, supporting random transposon insertions. The presence of prophage BLF in the mutants was confirmed by hybridization with radiolabelled phage DNA (Fig. 3b).

Isolation of Bt-non-producing mutants

The same 2000 *B. licheniformis* 19 insertion mutants were also tested for Bt production. No zone of inhibition was formed around 12 mutants (0.6%) on the lawn of *M. flavus*. These Bt non-producers were prototrophic with the same phage sensitivity and phage production as the original strain.

To determine the sites of transposon insertions, the

chromosomal DNAs of the 12 mutants were single- and double-digested with *Eco*RI, *Bam*HI, *Pst*I and *Sma*I, separated by agarose gel electrophoresis and hybridized with radiolabelled transposon DNA. Accordingly, five different insertion sites could be distinguished (Fig. 4). Three representatives were on sites C and D, four on site E and one on both A and B. Based on the hybridization patterns, the partial physical map of the chromosomal region of the Bt synthetase gene was constructed (Fig. 5). Four insertion sites (A, B, C and D) were found on the same *Pst*I or *Bam*HI fragment, while insertion site E was on another fragment. The beginning of the chromosomal part A-B-C-D overlapped the end of E. Therefore, the combined size of the two parts was about 47 kb. In this region the order of the five insertion sites was E-A-B-C-D, the distance between the outside insertions (E and D) was approximately 27.4 kb.

Fig. 3. DNA-DNA hybridization of *EcoRV*-digested *B. licheniformis* 19 chromosomal DNAs containing Tn917PF1 insertions, causing defective phage BLF phenotype. Probes were ^{32}P -labelled pTnPF1 (a) and BLF DNA (b). The molecular mass marker was *Hind*III-digested λ DNA. Lanes: A, wild-type *B. licheniformis* 19; B-H, *B. licheniformis* 19 insertion mutants carrying defective phage BLF. Only two bands were detected in lane C of (a), because the left-flanking part of the transposon attached to the chromosomal fragment did not separate from the 1.0 kb middle transposon part with a simple electrophoresis (result not shown).

Although Tn917PF1 was constructed to drive the transcription of chromosomal genes by its strong outward *aphA'* promoter (Zagorec & Steinmetz, 1991), Bt-overproducing mutants were not found among the 2000 insertions. About 10000 additional *B. licheniformis* 19 insertion mutants were tested for Bt production by replica plating. The Km-resistant colonies were tested with *M. flavus*. The inhibition zones were comparable with those of *B. licheniformis* 19, thus Bt-overproducing mutants were not found.

DISCUSSION

Transposon Tn917PF1, a derivative of Tn917, was successfully introduced into *B. licheniformis*. Due to the temperature-sensitive replication of the plasmid vector pTnPF1, it was possible to eliminate the freely replicating plasmid from the transformed cell and transposon mutants could be isolated as reported by Zagorec & Steinmetz (1991) for *B. subtilis*. For more efficient elimination of the vector, a higher temperature (50 °C) was used, as suggested by Wati *et al.* (1990) for *B. licheniformis*.

The frequency of Tn917PF1 transposition was nearly the same as that reported for *B. subtilis* (Zagorec & Steinmetz, 1991) and *B. licheniformis* NCIB6346 (Wati *et al.*, 1990). It was approximately tenfold higher than with the original Tn917 in *B. subtilis* (Youngman *et al.*, 1983). In contrast to a reported case in *Enterococcus faecalis* (Tomich *et al.*, 1980), Em was not needed to induce transposition in *B. licheniformis*.

The frequency of auxotrophic mutation was similar to that observed in *B. megaterium* (Bohall & Vary, 1986) and *B. amyloliquefaciens* (Hartley & Paddon, 1986), but was less

than the 3–8% in *B. subtilis* (Youngman *et al.*, 1983) and 9% in *B. licheniformis* NCIB6346 (Wati *et al.*, 1990). Based on the distribution of auxotrophs, no insertion 'hot spot' was found for transposon Tn917PF1 in the two *B. licheniformis* strains. This is in contrast with the hot spot regions observed in the *glt* locus in *B. subtilis* 168 (Youngman *et al.*, 1983), and the *argGH* region in *B. licheniformis* NCIB6346 (Wati *et al.*, 1990). Nevertheless, strain ATCC 9800 should be identical to NCIB6346. An explanation of these different results could be that the results reported by Wati *et al.* (1990) were based on a few experiments in which early transpositions may have been enriched in the *argGH* region. In the present work, 30 experiments were carried out with strain ATCC 9800 to decrease possible biased results. The distribution and phenotype of *B. licheniformis* 19 and ATCC 9800 auxotrophs were different (Table 1). This was not surprising, because the two strains are different in phage sensitivity and production, antibiotic sensitivity, Bt synthesis and colony morphology.

Considering the whole chromosome (Table 2 and Fig. 1) or larger DNA regions (Fig. 3) as potential targets for transposition, Tn917PF1 insertions occurred randomly, as observed in *B. megaterium* for Tn917 (Bohall & Vary, 1986). However, smaller chromosomal regions, such as particular genes (Fig. 2 and Fig. 5), may have had a single or a few preferred target sites for transposon Tn917PF1. Thus, the Bt synthetase gene of at least 26 kb [estimated by the molecular mass of the three-component enzyme complex (Ishihara *et al.*, 1989)] had three, and the smaller sulphate reductase gene had one target site in which more than one insertion was detected. It was interesting to observe that one of the sulphate reductase mutants had two chromosomal insertions, which is rather unusual for Tn917.

The partial physical map of the chromosomal region of the Bt synthetase gene (Fig. 5) was constructed based on hybridization patterns. Since the distance between the two outside insertions is about 27.4 kb and the estimated size of the Bt synthetase gene is at least 26 kb, it is very likely that the three structural genes are located in an operon or a cluster. A similar arrangement was observed for other cyclic peptide antibiotics of *Bacillus* origin, like the synthetases of gramicidin S, tyrocidine and surfactin (Krättschmar *et al.*, 1989; Mittenhuber *et al.*, 1989; Nakano *et al.*, 1991). Clustering has also been observed for antibiotic biosynthesis of some *Streptomyces* and filamentous fungi (MacCabe *et al.*, 1990; Malpartida *et al.*, 1990; Gutiérrez *et al.*, 1991; Raibaud *et al.*, 1991; Tobin *et al.*, 1991). Comparing this partial physical map with the physical map of Bt synthetase structural genes of *B. licheniformis* ATCC 10716 (Korsnes *et al.*, 1986; Ishihara *et al.*, 1989), there was little similarity. This may be explained by the difference in the two strains. *B. licheniformis* 19 was optimized for high industrial yield (600–660 IU Bt ml⁻¹), but ATCC 10716 is a low-yielding laboratory strain.

In about 12000 Tn917PF1 insertion mutants, Bt-overproduction was not found. The explanation can be that either there was no preferred target site for the

Fig. 4. DNA-DNA hybridization of five different *B. licheniformis* 19 Bt-non-producing Tn917PF1 mutants with 32 P-labelled pTnPF1 as probe. The chromosomal DNAs were single- and double-digested with *Bam*HI, *Eco*RI, *Pst*I and *Sma*I. The molecular mass marker was *Hind*III-digested λ DNA. Lanes: W, wild-type *B. licheniformis* 19; A-E, *B. licheniformis* 19 Bt-non-producers. Tn917PF1 has no *Eco*RI or *Pst*I sites, but has one *Bam*HI and two *Sma*I sites as seen in Fig. 5. For single and double *Sma*I digestions, too little chromosomal DNA was used in some lanes, so the third band, the transposon left-flanking part (0.3 kb) with a chromosomal fragment, could not be detected.

Fig. 5. Tn917PF1 insertions in the Bt synthetase gene. The chromosome is indicated by a horizontal line with restriction endonuclease cleavage sites (■, *Bam*HI; △, *Eco*RI; ○, *Pst*I; □, *Sma*I). Tn917PF1 is indicated by a box. The partial restriction map was constructed on the basis of Fig. 4. Insertion sites were marked A, B, C, D and E according to the lanes in Fig. 4.

transposon in the regulator region of Bt synthetase gene, or the transposon was inserted in the wrong orientation. Even if inserted in the right orientation, no overpro-

duction can be expected when the *aphA'* promoter is weaker than that of the Bt synthetase gene.

ACKNOWLEDGEMENTS

We are grateful to Michel Steinmetz for the generous gifts of *B. subtilis* GM479 (pTnPF1 donor). We would like to thank Lóránd Szabó for carrying out some of the tests, László Szabó, ABC, for taking video photographs and Mark Davies for his help in revising the manuscript. The work was supported by the Foundation for Hungarian Higher Education and Research grant No. 1019/92.

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Received 11 May 1994; revised 14 July 1994; accepted 20 July 1994.