Construction of a derivative of Tn917 containing an outward-directed promoter and its use in *Bacillus subtilis*

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Engineered variants of the transposon Tn917 have been widely used to obtain insertion mutations and transcriptional fusions in *Bacillus subtilis* and other Gram-positive bacteria. We have developed a novel Tn917-based methodology useful for isolation and characterization of mutants resulting from gene over-expression. A Tn917 variant was constructed which contains a strong out-facing promoter near one end, able to promote transcription of genes in the vicinity of its insertion target. This transposon, designated Tn917PFI, was tested in model conditions. Three Tn917PFI mutants of *B. subtilis*, with phenotypes presumed to result from gene over-expression, were analysed. Their phenotypes were shown to be due to transcription from the transposon promoter. In one mutant the promoter activated a *deg* gene, probably *degQ*. The other two contained different insertions decryptifying a *B. subtilis* gene encoding β -galactosidase.

Introduction

Transposons are powerful tools for the genetic study of both bacteria and eukaryotes. A large number of transposon derivatives, most currently based on Mu, Tn5 or Tn10, have been adapted for use in *Escherichia coli* and several Gram-negative bacteria (reviewed by Berg *et al.*, 1989). Youngman and co-workers have constructed several variants of the *Streptococcus faecalis* transposon Tn917 which were shown to be efficient in *Bacillus subtilis* and other Gram-positive bacteria for the identification and cloning of genes and for analysis of their transcription (reviewed by Youngman, 1987). Here we describe the construction of a Tn917 derivative containing a strong outward-directed promoter. This transposon (Tn917PFI) was tested in model situations showing that it could be used for isolation of insertions resulting in gene over-expression.

Methods

Bacterial strains and plasmids. The *E. coli* strain TG1 [*supE thi Δ(lac-pro) hsdD5 (F':traD36 proAB lacZΔM15)*] was used for plasmid construction and preparation, except where specified. The *B. subtilis*

strains used in this work are listed in Table 1. Strain GM122 contains a *sacB'-lacZ* fusion inserted at the *sacB* locus and a partial deletion of *sacB* (Steinmetz *et al.*, 1989).

The following plasmids have been previously described. pAT21 (Trieu-Cuot *et al.*, 1985) contains the kanamycin resistance gene (*aphA*) of Tn1545 on a 1.5 kb *Clal* fragment; a *RsaI* site lies between the stop codon and the putative terminator of *aphA*. pJM783 (Perego *et al.*, 1988) contains the following elements on a 5 kb *SmaI-StuI* fragment: (i) the *lacZ* coding sequence fused to translational signals functional in *B. subtilis*, (ii) the *AvaI-PvuII* fragment of pBR322 (Bolivar *et al.*, 1977), and (iii) the *Cm^R* gene (*cat*) of pC194 (Horinouchi & Weisblum, 1982) minus the last 6 codons; as previously shown (Trach & Hoch, 1989), this truncated *cat* gene is still functional. pTV8, pTV21Δ2, and pTV32ts have been described by Youngman (1987). The latter contains a super-thermosensitive origin of replication.

Construction of pMZ20, pMZ21, pMZ23, and pLC28 is described in Fig. 1. pMZ27 is a pSL42 derivative (Crutz *et al.*, 1990) containing the *sacX* gene. The *SmaI-StuI lacZ-cat* cassette of pJM783 was inserted into pMZ27 at the *NcoI* site located within *sacX* (Zukowski *et al.*, 1990), leading to pMZ28, in which *lacZ* and *sacX* were in opposite orientations. The construction of pTnPF1 is described in Fig. 2.

Culture media and genetic techniques. LB medium (Miller, 1972) supplemented with appropriate antibiotics was used for selection of transformants of *B. subtilis* and *E. coli*. DS medium was described by Schaeffer *et al.* (1965). Preparation of competent cells and transformation were performed as previously described (Aymerich *et al.*, 1986). Growth and selection were at 37 °C, except when specified. Competent cells of PY350 were prepared at 34 °C, in the presence of Em (0.4 µg ml⁻¹). Km^R, Cm^R and Em^R transformants of *B. subtilis* were selected on plates supplemented with the relevant antibiotic (5, 4 and 0.4 µg ml⁻¹, respectively). Ap^R, Cm^R and Km^R transformants of *E. coli* were selected on plates supplemented with the relevant antibiotic (50, 15 and 15 µg ml⁻¹, respectively).

Phenotypic characterization of *B. subtilis* strains expressing a *lacZ* fusion was performed on C mineral solid medium (Aymerich *et al.*,

Abbreviations: Ap, ampicillin; *bla*, gene coding for β -lactamase; *cat*, gene coding for *Cm* acetyltransferase; *Cm*, chloramphenicol; *erm*, gene conferring resistance to Em; Em, erythromycin; Km, kanamycin; LS, levansucrase; X-Gal, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside; XPrt, extracellular proteases.

Fig. 1. Construction of pMZ21, pIC28, and pMZ23. (a) To construct pMZ21, strain PY197(pTV8) was transformed with pTV21Δ2 linearized by *Xba*I. pMZ20 was a recombinant plasmid containing the *Sma*I–*Bam*HI–*Sma*I (S–B–S) linker present in pTV8 upstream from the *erm* gene. pMZ21 was generated from pMZ20 by an *Eco*RI–*Hpa*I deletion (H/E, ligation site). pMZ21 and its derivatives described below were constructed in *E. coli* TG1. (b) pIC28 was obtained by inserting the *Ca*II–*Rsa*I fragment (*aphA'*) of pAT21 conferring Km^R into the pMZ21 *Bam*HI site; a *Bam*HI site was reconstituted upstream from *aphA'*. (c) pMZ23 was obtained by substitution of the *Bam*HI–*Eco*RV fragment containing the promoter and the major part of *aphA'* in pIC28 by the *cat* gene of pTV21Δ2 (*Eco*RI–*Xho*II fragment).

Table 1. *B. subtilis* strains

Strain	Genotype	Source or reference*
BG4138	<i>trpC2 thr5 hisA1 degQ::cat degR::erm</i>	D. Henner, Genentech, San Francisco, USA
PY197	<i>trpC2 thr5</i> (pTV8)	P. Youngman, University of Pennsylvania, Philadelphia, USA
PY350	<i>trpC2 thr5</i> (pTV32ts)	P. Youngman
GM122	<i>trpC2 sacB'–lacZ</i>	Steinmetz <i>et al.</i> (1989)
GM275a	<i>sacA321 sacX::Tn917</i>	Le Coq <i>et al.</i> (1991)
GM479	<i>trpC2 sacB'–lacZ</i> (pTnPF1)	pTnPF1 tf GM122
GM480	as GM275a plus <i>lacZ–cat::sacX</i>	pMZ28 tf GM275a
GM481	as GM480 plus <i>sacX::Tn917PF1</i>	pIC28 tf GM480
GM483	as GM122 plus <i>Tn917PF1::(lac1X)</i>	Transposition of <i>Tn917PF1</i> in GM122
GM484	as GM122 plus <i>Tn917PF1::(degQ)</i>	
GM485	as GM122 plus <i>Tn917PF1::(lacX)2</i>	
GM663a	<i>trpC2 thr5</i> (pTnPF1)	pIC28 tf PY350

* tf, transformation (the indicated donor plasmid or chromosomal DNA was used to transform the indicated recipient strain).

1986) supplemented with glucose (10 g l⁻¹) and X-Gal (20 mg l⁻¹) (CGX medium). CGXS is CGX containing sucrose (20 g l⁻¹). For quantitative phenotypic characterization of strains, cultures in LB or DS medium were inoculated from fresh isolates on LB plates as described previously (Steinmetz *et al.*, 1989). Cell extracts were

prepared, and β -galactosidase activities were assayed, as described by Aymerich *et al.* (1986). Screening for the XPrt phenotype was done by transferring bacteria onto LB plates supplemented with 1% (w/v) skimmed milk powder (Difco).

The transposition frequency in GM479(pTnPF1) was determined

essentially as described by Youngman (1987), except that lincomycin was omitted. GM479 was grown at 30 °C in LB medium supplemented with Cm and Em (4 and 5 µg ml⁻¹, respectively). Aliquots were plated on LB plates supplemented with Em (0.4 µg ml⁻¹) and incubated at either permissive (30 °C) or non-permissive (46 °C) temperature for replication of pTV32ts and derivatives. The transposition yield was expressed as the ratio of Em^R colony-forming units at 46 °C to those at 30 °C.

In vitro DNA techniques. Standard DNA techniques were used. The alkaline lysis method of Birnboim & Doly (1979) was used for extraction of plasmid DNA. DNA fragments with incompatible termini were made blunt with the Klenow (large) fragment of *E. coli* DNA polymerase I, in the presence (5'-protruding ends) or in the absence (3'-protruding ends) of the four deoxyribonucleotides before ligation as appropriate.

Results

Construction of Tn917PF1

Our aim was to insert a strong promoter into Tn917, near one end. Perkins & Youngman (1986) showed that insertion of sequences into a *Hpa*I site located 277 bp from the left extremity of Tn917 (Shaw & Clewell, 1985) did not affect the transposition of the element. A *Sma*I–*Bam*HI–*Sma*I linker was inserted at this site and plasmid pTV8 (10 kb) containing this Tn917 variant was used as a starting point for the construction of several Tn917 derivatives (Youngman, 1987). However, pTV8 itself is not suitable because it is large and must be prepared from *B. subtilis*. Therefore, we constructed a small pBR322 derivative, pMZ21, which contains the left end of Tn917, including the *Sma*I–*Bam*HI–*Sma*I linker (Fig. 1a).

The promoter we chose was that from the Km^R gene (*aphA*) of Tn1545. It is a strong promoter expressed in both *B. subtilis* and *E. coli*. The fragment used carried the promoter and the coding sequence of *aphA*, but not its putative terminator (Trieu-Cuot *et al.*, 1985). The terminator-less gene (hereafter designated *aphA'*) was cloned into the pMZ21 *Bam*HI site (Fig. 1b). pIC28, a recombinant plasmid in which *aphA'* was oriented towards the left extremity of the transposon, was used to insert *aphA'* by homologous recombination into a plasmid containing a functional Tn917 (Fig. 2). The resulting plasmid and transposon were designated pTnPF1 and Tn917PF1, respectively.

Tn917PF1 promotes transcription near its insertion site

Vandeyar *et al.* (1986) isolated and characterized several insertions of Tn917 into the *B. subtilis* *ilvBC-leu* operon, and showed that Tn917 is strongly polar when inserted with its *erm* gene and the operon in opposite orientations. Therefore it was important to show that transcription

Fig. 2. Construction of the transposon Tn917PF1. PY350 containing pTV32ts was transformed by plasmid pIC28 linearized with *Pst*I. pTnPF1 resulted from the double cross-over shown and was selected by Km^R. The resulting transposon was designated Tn917PF1. LE and RE, left and right extremities, respectively, of Tn917 (Shaw & Clewell, 1985).

initiated at the *aphA'* promoter could proceed through the 277 bp DNA segment separating *aphA'* from the left extremity of Tn917PF1 (Fig. 2) and out into neighbouring sequences. To check this, we constructed strains which contained both a Tn917 element (wild-type or Tn917PF1) and a promoter-less *lacZ* gene inserted side by side into the chromosome and oriented in such a way that *lacZ* expression would measure transcription emerging from the transposon through the left extremity (Fig. 3). These constructs were inserted into the *sacX* gene of *B. subtilis*, which is very weakly transcribed (A. M. Crutz and others, unpublished); furthermore *sacX* and the reporter gene had opposite orientations. As shown in Fig. 3, there was a strong expression of the promoterless *lacZ* gene when it was downstream from Tn917PF1.

To check whether *aphA'* was still active after sporulation commitment, the strains described above were grown in DS medium (Schaeffer *et al.*, 1965) and *lacZ* expression was measured during growth and stationary phase. β -Galactosidase activity stopped increasing less than 1 h after the end of exponential growth. A similar expression pattern and similar β -galactosidase activities were observed in LB medium and in mineral medium supplemented with glucose (data not shown).

Fig. 3. Measurement of the strength of the Tn917PF1 promoter in the *B. subtilis* chromosome. GM257a, a *sacX*::Tn917 mutant, was transformed to *Cm*^R with linearized pMZ28 plasmid, giving GM480, which contains a promoter-less *lacZ* reporter gene downstream from the left end of Tn917. (b) Linearized pIC28 was used to transform GM480 to *Km*^R, giving GM481, which contained Tn917PF1 upstream from the reporter. β-Galactosidase activity was measured in GM480 and GM481 growing in DS medium, and is expressed in Miller units. LE, left end of Tn917.

Utilization of Tn917PF1 to isolate *deg* hyperproducer mutants

The *B. subtilis deg* genes (reviewed by Steinmetz & Aymerich, 1990) encode activators of synthesis of several degradative enzymes, including levansucrase (LS) and extracellular proteases (XPrt). The over-expression of at least two *deg* genes, *degQ* (Yang *et al.*, 1986; Amory *et al.*, 1987) and *degR* (Nagami & Tanaka, 1986; Yang *et al.*, 1987), results in over-expression of both LS and XPrt. Deletions of these genes where they have been replaced by resistance markers are available (Yang *et al.*, 1986, 1987) which could facilitate the identification of newly isolated *deg* mutations. This system therefore constituted a good model to test Tn917PF1 as a tool for obtaining over-expression mutants. pTnPF1 was introduced by transformation into strain GM122, which carries a fusion of the LS (*sacB*) gene to *lacZ*. Transposition frequencies were measured in six independent cultures of the transformant, as described in Methods. The average transposition yield was 5×10^{-4} . GM122 mutants over-expressing the *sacB*'-*lacZ* fusion were screened for in the presence of the β-galactosidase indicator X-Gal on CGXS plates supplemented with both Em and Km (0.4

Fig. 4. *In vivo* deletion of the *aphA'* promoter in a Tn917PF1 mutant. The mutant was transformed to *Cm*^R with pMZ23 linearized with *Pst*I. This resulted in a *Km*^S transformant.

and $5 \mu\text{g ml}^{-1}$ respectively) and incubated at 46°C . About 10^5 Tn917PF1 mutant colonies from each culture were screened and three independent β-galactosidase overproducer mutants, designated GM483, GM484 and GM485, were isolated.

It was important to verify that the phenotype of Tn917PF1 insertions was due to increased transcription of a gene located downstream from *aphA'*. Therefore, we constructed pMZ23, a pIC28 derivative in which a *Cm*^R gene was substituted for the *aphA'* promoter (Fig. 1c). pMZ23 could be used to delete this promoter *in vivo*, as shown in Fig. 4. The β-galactosidase overexpression phenotype was suppressed by this substitution in all three mutants.

GM484 was probably a *deg* mutant since it was also a hyperproducer of XPrt, as revealed by tests on solid medium. We showed that it contained an insertion near *degQ* as follows. The strain was transformed to *Cm*^R with DNA from strain BG4138 (*degQ*::*cat*). All transformants lost the GM484 pleiotropic phenotype. Two classes of recombinants could be distinguished on the basis of their resistance to antibiotics. The majority had lost both *Em*^R and *Km*^R. This showed that the insertion was strongly linked to *degQ*. A minority of transformants (about 1%) retained both resistances and were therefore double mutants resulting from a cross-over between *degQ*::*cat* and the inserted transposon. Thus *degQ*::*cat* was epistatic to the insertion. This epistasis and the tight linkage between the mutations strongly suggested that the phenotype of GM484 was due to transcription of *degQ* from the *aphA'* promoter present within Tn917PF1. However, it cannot be excluded that the insertion activated another gene, linked to *degQ* and whose function required *degQ*.

The insertions present in GM483 and GM485 did not

affect *deg* genes since synthesis of XPrt was not altered in these strains. Furthermore, over-expression of β -galactosidase activity was observed even when the relevant insertions were transferred by transformation into a strain which did not contain the *sacB'*-*lacZ* fusion. Since this phenotype was abolished when *aphA'* was deleted, this strongly suggested that the insertions activated the expression of a gene encoding an endogenous β -galactosidase or an activator of the synthesis of such a gene.

Cloning of a *B. subtilis* β -galactosidase gene

The chromosomal regions flanking Tn917PF1 in GM483 and GM485 were cloned using the pTV21 Δ 2-based methodology (Youngman, 1987). Analysis of the resulting plasmids showed that the insertions were 0.7 kb apart and in the same orientation. These insertions directly activated the gene coding for the β -galactosidase activity, since a chromosomal fragment located downstream from *aphA'* insertions expressed β -galactosidase activity in an *E. coli* Δ *lacZ* mutant (data not shown).

Mutations affecting the synthesis of the endogenous β -galactosidase of *B. subtilis* were recently isolated and mapped in the *hisA*-*thrA* region of the chromosome by Errington & Vogt (1990). A PBS1 transducing lysate was prepared from strain GM483 and used to transduce a *hisA1 thrA5* double mutant. No linkage was observed between the Tn917PF1 insertion and the auxotrophic markers, suggesting that the gene activated by the insertion was different from that previously mapped.

Discussion

We constructed Tn917PF1, a Tn917 derivative containing a strong promoter inserted 277 bp from the left end of the transposon and facing out (Fig. 2). Tn917PF1 strongly activated expression of chromosomal genes located downstream from this promoter (Fig. 3a). As the phenotype of Tn917PF1 insertions could have been due to more classical effects such as gene disruption, a simple methodology was developed to establish that the phenotype resulted directly from the transcription of genes from the transposon promoter (Fig. 4).

Some of the genes which could be over-expressed and identified using Tn917PF1 mutagenesis could be identified by alternative methods, for example by cloning on a multicopy plasmid. The latter method was used to identify the *degR* gene (Nagami & Tanaka, 1986). However, Tn917PF1 mutagenesis could be used to over-produce genes which are naturally under the control of limiting (or transiently expressed) transcription activators or induced by an unknown inducer. Mutagenesis by

Tn917PF1 could be useful for genetic studies of *B. subtilis* sporulation, which involves a cascade of positive regulators, including several sigma factors (Losick *et al.*, 1986). This prompted us to measure the efficiency of the Tn917PF1 promoter in sporulation conditions. The promoter appeared inefficient after the end of exponential growth, whereas an ideal system for such studies would be a transposon containing a promoter that is both inducible and expressed in the stationary phase.

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