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 Tn917PF1, was tested in model conditions. Three Tn917PF1 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 (Tn917PF1) 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) hisD5 (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 Tn5/45 on a 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–Stul fragment: (i) the lacZ coding sequence fused to translational signals functional in B. subtilis, (ii) the VanA–PouII fragment of pBR322 (Bolivar et al., 1977), and (iii) the CmR 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, pTV21A2, 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 Smal–Stul lacZ–cat cassette of pJM783 was inserted into pMZ27 at the Neol site located within sacX (Zukowsky 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). KmR, CmR and EmR transformants of B. subtilis were selected on plates supplemented with the relevant antibiotic (5, 4 and 0.4 μg ml⁻¹, respectively). ApR, CmR and KmA 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., 1986).
Fig. 1. Construction of pMZ21, pIC28, and pMZ23. (a) To construct pMZ21, strain PY197(pTV8) was transformed with pTV21Δ2 linearized by XbaI. pMZ20 was a recombinant plasmid containing the Smal–BamHI–Smal (S–B–S) linker present in pTV8 upstream from the *erm* gene. pMZ21 was generated from pMZ20 by an EcoRI–HpaI deletion (H/E, ligation site). pMZ21 and its derivatives described below were constructed in *E. coli* TG1. (b) pIC28 was obtained by inserting the *CaI–RsaI* fragment (*aphA*) of pAT21 conferring KmR into the pMZ21 BamHI site; a BamHI site was reconstituted upstream from *aphA*. (c) pMZ23 was obtained by substitution of the BamHI–EcoRV fragment containing the promoter and the major part of *aphA* in pIC28 by the *cat* gene of pTV21Δ2 (EcoRI–XhoII fragment).

Table 1. *B. subtilis* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference*</th>
</tr>
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<tbody>
<tr>
<td>BG4138</td>
<td><em>trpC2 thr5 hisAl degQ::cat degR::erm</em></td>
<td>D. Henner, Genentech, San Francisco, USA</td>
</tr>
<tr>
<td>PY197</td>
<td><em>trpC2 thr5</em> (pTV8)</td>
<td>P. Youngman, University of Pennsylvania, Philadelphia, USA</td>
</tr>
<tr>
<td>PY350</td>
<td><em>trpC2 thr5</em> (pTV32ts)</td>
<td>P. Youngman</td>
</tr>
<tr>
<td>GM122</td>
<td><em>trpC2 sacB'::lacZ</em></td>
<td>Steinmetz et al. (1989)</td>
</tr>
<tr>
<td>GM275a</td>
<td><em>sacA321 sacX::Tn917</em></td>
<td>Le Coq et al. (1991)</td>
</tr>
<tr>
<td>GM479</td>
<td><em>trpC2 sacB'::lacZ (pTnPFl)</em></td>
<td>pTnPFl tf GM122</td>
</tr>
<tr>
<td>GM480</td>
<td>as GM275a plus <em>lacZ::sacX</em></td>
<td>pMZ28 tf GM275a</td>
</tr>
<tr>
<td>GM481</td>
<td>as GM480 plus <em>sacX::Tn917PF1</em></td>
<td>pIC28 tf GM480</td>
</tr>
<tr>
<td>GM483</td>
<td>as GM122 plus <em>Tn917PF1::(lac1X)</em></td>
<td>Transposition of Tn917PF1 in GM122</td>
</tr>
<tr>
<td>GM484</td>
<td>as GM122 plus <em>Tn917PF1::degQ</em></td>
<td></td>
</tr>
<tr>
<td>GM485</td>
<td>as GM122 plus <em>Tn917PF1::(degQ)</em></td>
<td></td>
</tr>
<tr>
<td>GM653a</td>
<td><em>trpC2 thr5</em> (pTnPFl)</td>
<td>pIC28 tf PY350</td>
</tr>
</tbody>
</table>

* tf, transformation (the indicated donor plasmid or chromosomal DNA was used to transform the indicated recipient strain).
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 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 *HpaI* site located 277 bp from the left extremity of Tn917 (Shaw & Clewell, 1985) did not affect the transposition of the element. A *SmaI*–*BamHI*–*SmaI* 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 *SmaI*–*BamHI*–*SmaI* 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\(^{\prime}\*)) was cloned into the pMZ21 *BamHI* site (Fig. 1b). pIC28, a recombinant plasmid in which *aphA\(^{\prime}\)* was oriented towards the left extremity of the transposon, was used to insert *aphA\(^{\prime}\)* by homologous recombination into a plasmid containing a functional Tn917 (Fig. 2). The resulting plasmid and transposon were designated pTnPFl 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 initiated at the *aphA\(^{\prime}\)* promoter could proceed through the 277 bp DNA segment separating *aphA\(^{\prime}\)* 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\(^{\prime}\)* 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).
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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 $5 \times 10^{-4}$. GM122 mutants over-expressing the sacB'-lacZ fusion were screened for in the presence of the $\beta$-galactosidase indicator X-Gal on CGXS plates supplemented with both Em and Km (0.4 and 5 $\mu$g ml$^{-1}$ respectively) and incubated at 46°C. About $10^5$ Tn917PF1 mutant colonies from each culture were screened and three independent $\beta$-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 CmR 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 $\beta$-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 CmR 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 EmR and KmR. 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

![Fig. 4. In vivo deletion of the aphA' promoter in a Tn917PF1 mutant. The mutant was transformed to Cm^R with pMZ23 linearized with PstI. This resulted in a Km^R transformant.](Image)
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 GM4855 were cloned using the pTV21A2-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 ΔlacZ 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 PBSI transducing lysate was prepared from strain GM485 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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