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Expression of chloramphenicol acetyltransferase in *Bacillus subtilis* under the control of a phytoplasma promoter

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Abstract

A cloned putative promoter region upstream of the 16S rRNA gene of the western X-disease phytoplasma was inserted behind the promoterless chloramphenicol acetyltransferase gene of plasmid pPL603. The DNA construct was used to transform *Bacillus subtilis* cells. The transformants were assayed for chloramphenicol acetyltransferase activity, showing that the phytoplasma promoter is efficiently expressed in a *B. subtilis* background. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Phytoplasma; *Bacillus subtilis*; 16S rRNA gene; Promoter

1. Introduction

The phytoplasmas are a monophyletic group of mollicutes associated with numerous diseases which affect hundreds of plant species [1]. The inability to culture these microorganisms in vitro has severely hampered the determination of their genetic, physiological and biochemical characters. In a previous work Kuske and Kirkpatrick [2] reported the sequence analysis of a cloned fragment of the severe strain of the western aster yellows phytoplasma. It consisted of the 16S rRNA gene and a 554 bp long upstream sequence, possibly containing the promoter sequence. In this paper we report the sequence upstream of the 16S rRNA gene of another phytoplasma, the western X-disease phytoplasma, and present evidence of the functional activity of the alleged promoter sequence in a phylogenetically related organism, *Bacillus subtilis*.

2. Materials and methods

Plasmid pPY6, a derivative of the *Escherichia coli* plasmid pUC19, was obtained by screening a plasmid library made from the DNA of the western X-disease phytoplasma with a probe for the 16S rRNA gene [3], following essentially the method used by Kuske and Kirkpatrick [2] for the aster yellows phytoplasma. *E. coli* exonuclease III nested deletions of the cloned insert were subcloned and sequenced using standard methodologies [4].

The *B. subtilis* plasmid pPL603 specifies neomycin resistance and contains a structural gene for chloramphenicol acetyltransferase (*cat*) which could not be transcribed and expressed because the promoter had been deleted [5].

In the cloning experiments, the recipient strain *E. coli* DH5α was transformed by the $CaCl_2$ method [4] and *B. subtilis* BR151 according to Bott and Wilson [6].

The chloramphenicol acetyltransferase enzyme activity was assayed using the Fast Cat Yellow Chloramphenicol Acetyltransferase Assay kit (Molecular Probes). The reaction products were separated by thin layer chromatography on silica gel plates and photographed under UV light.

3. Results

A plasmid clone, named pPY6, reacted positively when hybridized to a DNA probe consisting of the 16S rRNA

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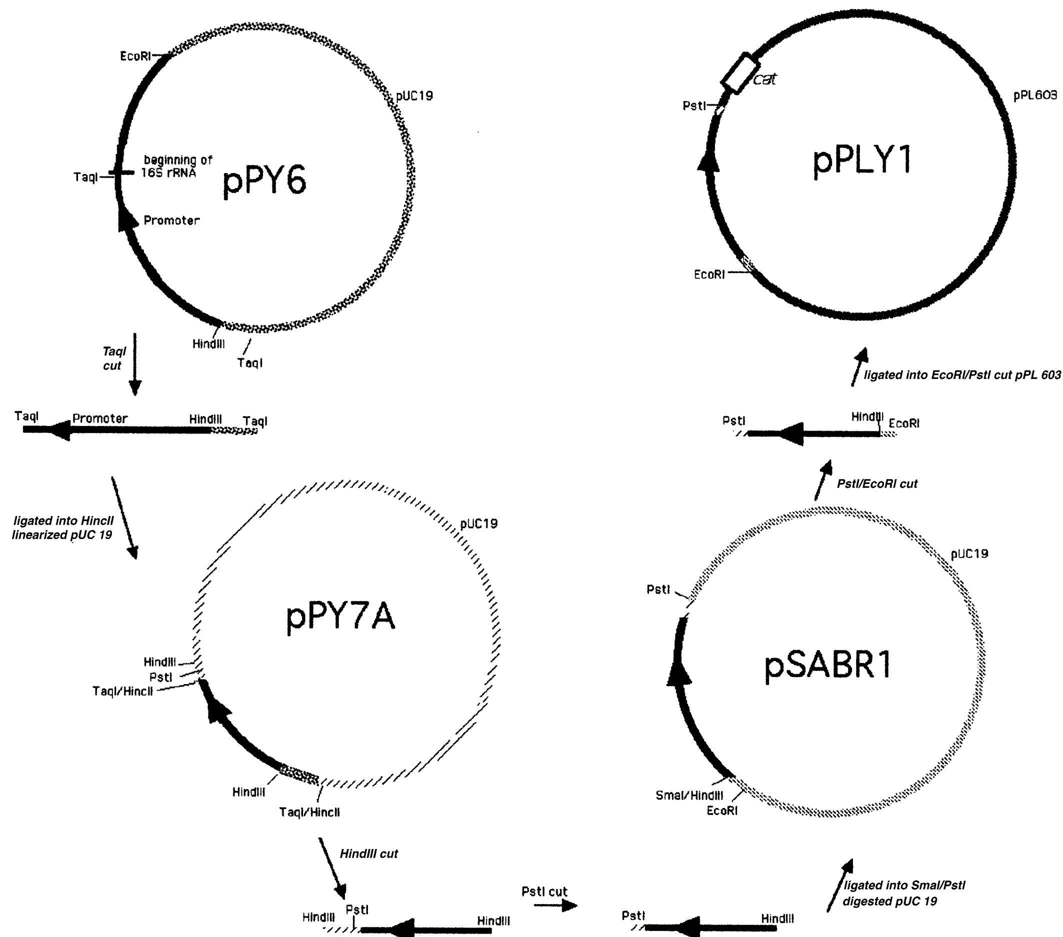

Fig. 1. Outline of the DNA manipulations carried out to obtain plasmid pPLY1. Plasmid pPY6 was digested with *Taq*I and the DNA fragment containing the sequence upstream of the 16S rRNA gene was purified and ligated to pUC19 linearized with *Hin*cII. The plasmid DNA of one of the clones obtained after transformation (pPY7A) was digested with *Hin*dIII and the recessed ends created were filled with the Klenow fragment of *E. coli* DNA polymerase I. The linear plasmid was purified and then digested with *Pst*I, purified again, ligated to a *Sma*I and *Pst*I digested pUC19 and used to transform competent *E. coli* DH5α cells. A selected recombinant plasmid (pSABR1) was digested with *Eco*RI and *Pst*I restriction enzymes to obtain a fragment which was ligated into the unique *Eco*RI and *Pst*I sites of the pPL603 plasmid; the ligation mixture was then used to transform *B. subtilis* cells, which were plated on agar containing neomycin to obtain pPLY1.

gene of an aster yellows phytoplasma. The cloned insert of pPY6 was found to contain about 605 bp corresponding to the 5′ region of the 16S rRNA and 1250 bp corresponding to the upstream sequence [7]. The latter (accession number AJ296279) did not contain a tRNA gene and showed limited similarity to the orthologous sequence of the aster yellows phytoplasma [2]. The only remarkable similarity was found around the 34-bp sequence (nucleotides 1073–1107) which forms a stem–loop structure by base pairing the inverted repeat in the 16S/23S spacer sequence. Despite their limited overall similarity, the base composition of the two sequences was very similar, as the trinucleotides TTT and AAA were about two to three times more strongly represented than TAT, TTA, TAA, AAT, ATA, ATT.

A very conserved promoter consensus sequence (TTGAAAN_{17}TATAAT) was found between nucleotides 982 and 942. The putative −10 sequence (TATAAT) was identical to that determined for one of the two possible

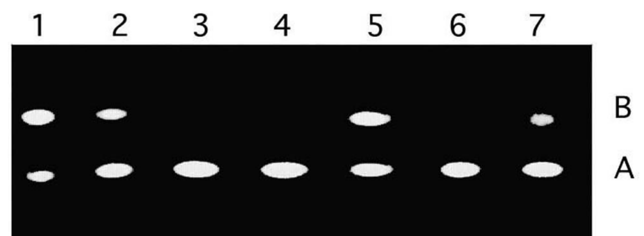

Fig. 2. Photograph under UV light of the results of thin layer chromatography of the chloramphenicol acetyltransferase assay. Lane 1: control reaction. Lane 2: extract from *B. subtilis* BR151 (pPLY1) incubated for 15 min. Lane 3: extract from *B. subtilis* BR151 incubated for 15 min. Lane 4: extract from *B. subtilis* BR151 (pPL603) incubated for 15 min. Lane 5: Extract from *B. subtilis* BR151 (pPLY1) incubated for 60 min. Lane 6: extract from *B. subtilis* BR151 incubated for 60 min. Lane 7: extract from *B. subtilis* BR151 (pPL603) incubated for 60 min. The fluorescent spots correspond to substrate (A) and product (B) of the chloramphenicol acetyltransferase assay.

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promoter sequences in the aster yellows phytoplasma orthologous sequence.

The sequence upstream of the phytoplasmal 16S rRNA gene cloned in pPY6 was placed behind the promoterless *cat* gene cloned in pPL603 (Fig. 1). This was accomplished thanks to the presence of a *Taq*I site a few nucleotides before the beginning of the 16S rRNA gene in pPY6 and the unique *Eco*RI and *Pst*I sites in pPL603. Details on the manipulations performed to obtain a recombinant plasmid with the phytoplasma promoter controlling the expression of the *cat* gene are reported in the legend of Fig. 1.

Three clones grown after plating transformed *B. subtilis* cells on neomycin containing plates (Fig. 1) were evaluated for *cat* gene expression by performing a semiquantitative analysis. For the cell extract of a typical recombinant clone (named pPLY1) (Fig. 2), about 50% of the substrate was acetylated after 15 min incubation. Under the conditions of the assay, this rate corresponded to a high level of expression of the *cat* gene.

4. Discussion

Due to the inability to culture phytoplasmas in vitro, information on phytoplasma genetics is still limited. Most available information has been restricted to the sequence of structural genes, mainly ribosomal. In this context, functional analysis of phytoplasma gene products can be accomplished only through the expression in heterologous hosts. The expression of phytoplasma membrane proteins in *E. coli* has recently been achieved ([8,9], C. Blomquist and B.C. Kirkpatrick, in preparation). The use of *E. coli* as a host, however, might not be appropriate for the study of genetic control of gene expression which is of primary interest as the phytoplasmas may infect both plants and insects. We therefore carried out a preliminary evaluation of the suitability of the more closely related *B. subtilis* as a host for expression studies.

The results presented in this paper show that the promoter region of a phytoplasma ribosomal RNA operon permitted the transcription of the *cat* gene in *B. subtilis*. This suggests that other phytoplasma genes may also be efficiently expressed in a *B. subtilis* background. In addition our results suggest that the promoter probe/host system pPL603/*B. subtilis* may be used to study at least some phytoplasma promoter sequences, as has been done for other Gram-positive bacteria such as *Bacillus* and *Streptococcus* spp. [5,10].

The identification of a sequence containing a promoter may also be of value for the construction of a transformation vector for phytoplasmas; constructs containing selectable markers positioned downstream of this sequence may be tested for functionality in an alternative, in vitro cultivable host, before the transformation into phytoplasma cells is attempted.

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