Construction of Cloning, Promoter-Screening, and Terminator-Screening Shuttle Vectors for Bacillus subtilis and Streptococcus lactis

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Shuttle vectors have been constructed which are suitable both for the selection of regulatory sequences and for gene cloning in Bacillus subtilis and Streptococcus lactis. The promoter screening vectors contain a promotorless chloramphenicol acetyltransferase gene; the insertion of suitable DNA fragments upstream of the gene restored the enzyme activity. With a related set of vectors, transcription termination signals can be selected.

Recently, we have described the construction of a set of cloning vectors for lactic streptococci (9). The vectors used in this system also replicate in Bacillus subtilis and Escherichia coli. However, screening of regulatory sequences for gene expression with these vectors was not possible. In view of the development of plasmid vectors which would allow the proper expression of heterologous genes in lactic streptococci and to obtain information concerning the structure of lactic streptococcal promoters, the availability of vectors for screening promoter and transcription termination sequences is desirable.

In this paper we describe the construction of a shuttle vector family which allows the isolation of promoter and transcription termination signals in lactic streptococci. The method of detecting promoter activity in this system is analogous to that described for screening promoter activity in B. subtilis by means of pPL603 (23).

The starting material for the construction of the screening vectors were (i) pGK3, which contains the largest ClaI fragment of the cryptic Streptococcus cremoris Wg2 plasmid pWVO1 and the erythromycin resistance (Em') gene on a ClaI-HpaII fragment of pE194 cop-6 (22) and (ii) the B. subtilis vector pPL608 (23) carrying the chloramphenicol acetyltransferase (CAT) gene from B. pumilus (Fig. 1). pGK3 and pPL608 were isolated from B. subtilis 8G-5 (2) after the cells were grown in TY broth (18) with 5 μg of kanamycin per ml by the method of Ish-Horowicz and Burke (8) with some modifications (9). Restriction enzymes were used as recommended by the manufacturer. Digested DNA was analyzed in 0.8% agarose gels (11). pPL608 was completely digested with PvuII and partially with EcoRI restriction endonucleases. The largest PvuII-EcoRI fragment, containing the SPO2 promoter and the CAT gene, was isolated by electrophoresis from the agarose gel. After filling in the ClaI-linearized pGK3 with the Klenow fragment of E. coli DNA polymerase I and the EcoRI cohesive end of the PvuII-EcoRI fragment mentioned above, these molecules were ligated. Subsequently, protoplasts of B. subtilis PSL1 (16) were exposed to the ligation mixture by the method of Chang and Cohen (3), and Em' chloramphenicol-resistant (Cm') transformants were selected. Two types of transformants were obtained, one carrying plasmid pGKV1 (Fig. 1) and the other carrying pGKV2 (data not shown). The difference between pGKV1 and pGKV2 concerns the orientation of the CAT gene. Both vectors transformed protoplasts of S. lactis to Em' (5 μg/ml) and Cm' (4 μg/ml) at a frequency of approximately 10^8 transformants per μg of DNA. The S. lactis protoplasts were prepared as described by Okamoto et al. (15), except that protoplasts were made by incubating the cells for 1 h in 30 mM Tris-hydrochloride buffer (pH 8.0)–3 mM MgCl_2–25% sucrose–30 μg of lysozyme per ml. Transformation was performed by the method of Kondo and McKay (10) with some modifications (9).

Since fusion of the filled in recessed ends of the ClaI and EcoRI sites restored the EcoRI site, the SPO2 promoter was deleted in vitro by EcoRI digestion, resulting in pGKV10 (Fig. 1) and pGKV20 with opposite orientations of the CAT gene (data not shown). Both plasmids were transformed to B. subtilis PSL1 and gave rise to Em' Cm' colonies. The CAT gene was not expressed in either pGKV10 or pGKV20, indicating that no sequence on the pWVO1 part of the vector promoted the expression of the gene, or that the CAT gene was expressed along with the Em' gene under the Em' promoter. This is in accord with the presence of a termination signal downstream of the Em' gene (7).

Although the two vectors pGKV10 and pGKV20 can be used to select for promoter activity on DNA fragments generated by EcoRI, more versatile derivatives were made by replacing the EcoRI-PstI fragment by a multiple cloning site of double-stranded MI3mp1 (13), resulting in pGKV110 (Fig. 1) and pGKV210 with opposite orientations of the CAT gene (data not shown), which carries unique EcoRI, Smal, Xmal, BamHI, Sall, and PstI sites upstream of and adjacent to the CAT gene. Transformation of S. lactis protoplasts with pGKV110 and pGKV210 gave rise to Em' Cm' transformants. The observations that pGKV1 and pGKV2 transformed B. subtilis and S. lactis cells to Cm' and that pGKV110 and pGKV210 failed to do so indicate that the CAT gene is expressed in S. lactis under the control of the B. subtilis phage promoter. To examine whether promoter sequences could be selected with the aid of the vector pGKV110, MboI fragments of B. subtilis OG-1 (2) and S. cremoris Wg2 (17) DNA were cloned into the unique BamHI site of pGKV110. Protoplasts of B. subtilis PSL1 were exposed to the ligation mixture, and transformants were selected on DM3 plates (3) with 5 μg of erythromycin and 5 μg of chloramphenicol per ml. Promoter selection efficiency in B. subtilis was 500 to 1,000 Cm' transformants per μg of DNA.

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DNA, irrespective of the source of the DNA. Direct transformation of the ligation mixture to S. lactis MG1363 (5) proved to be unsuccessful. However, it was possible to transform S. lactis protoplasts to Em<sup>+</sup> with plasmids derived from the Em<sup>+</sup> Cmr<sup>+</sup> B. subtilis transformants. The results in Table 1 show that none of the B. subtilis promoters were active in S. lactis, with the exception of the SPO2 promoter. Table 1 also shows that S. cremoris promoters were active in B. subtilis and that the majority of these promoters also functioned in S. lactis, although at a substantially reduced level. Whether the difference in promoter activity between the two bacterial species is caused by intrinsic differences in the structure of the promoters or is attributable to different stability of the mRNA or the gene product remains to be established.

Reinserting the SPO2 promoter on a 0.18-megadalton (Md) EcoRI fragment into the EcoRI site of pGKV110 and pGKV210 restored the CAT activity; B. subtilis cells harboring both plasmids pGKV11 and pGKV21 with opposite orientations of the CAT gene (data not shown) were Cmr<sup>+</sup>. Since the BamHI site of the multiple cloning site on plasmid pGKV11 is located between the promoter and the ribosomal binding site of the CAT gene (4, 6, 19), it may be expected that the insertion of DNA fragments carrying a transcription termination sequence in this site will abolish CAT expression and therefore that pGKV11 may be used as a transcription termination selection vector. To test this, MboI fragments of pPLP1 were inserted in the BamHI site of plasmid pGKV11. pPLP1 is a recombinant plasmid, constructed in our laboratory (J. van Randen, unpublished results), carrying the penicillinase gene of B. licheniformis on a AhaIII fragment (14) inserted into the HindIII site of pPL608. The transcription termination signal of the penicillinase gene on this plasmid is located on a 0.4-Md MboI fragment. Among the Em<sup>+</sup> B. subtilis transformants, Cmr<sup>+</sup> colonies were isolated with a frequency of 5%. These colonies either contained a plasmid consisting of pGKV11 with a 0.4-Md insert (pGKV12) or a plasmid consisting of pGKV11 carrying a 0.7-Md insert (pGKV13). Restriction enzyme analysis and Southern hybridization (data not shown) indicate that pGKV12 contained the 0.4-Md MboI fragment harboring the transcription termination signal of the penicillinase gene, which is a complementary repeat followed by oligodeoxythymidylicate (14). Transformation of S. lactis protoplasts with the recombinant plasmid pGKV12 gave rise to Em<sup>+</sup> Cmr<sup>+</sup> transformants, indicating that a Bacillus transcription termination signal also functioned in S. lactis. The sequence of the 0.7-Md insert in pGKV13 is only partially known (12). The lack of CAT expression suggests the presence of a transcription terminator in the unknown sequence.

Some of the vectors constructed can also be used as regular cloning vehicles. This applies to the vectors pGKV1 and pGKV2, carrying unique HindIII and HpaII sites in the CAT gene, and to pGKV11 and pGKV21, carrying a unique HindIII site in the CAT gene.

### Table 1. Cmr properties of pGKV1 and derivative plasmids<sup>a</sup>

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source of insert</th>
<th>CAT activity (µg/ml for B. subtilis)</th>
<th>Cmr&lt;sup&gt;+&lt;/sup&gt; (µg/ml for S. lactic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGKV110</td>
<td>None</td>
<td>0.1</td>
<td>&lt;5</td>
</tr>
<tr>
<td>pGKV1</td>
<td>EcoRI of SPO2</td>
<td>2.5</td>
<td>30</td>
</tr>
<tr>
<td>pGKV11</td>
<td>MboI of B. subtilis</td>
<td>0.5</td>
<td>30</td>
</tr>
<tr>
<td>pGKV112</td>
<td>MboI of B. subtilis</td>
<td>2.1</td>
<td>30</td>
</tr>
<tr>
<td>pGKV113</td>
<td>MboI of B. subtilis</td>
<td>2.0</td>
<td>30</td>
</tr>
<tr>
<td>pGKV114</td>
<td>MboI of B. subtilis</td>
<td>0.6</td>
<td>10</td>
</tr>
<tr>
<td>pGKV143</td>
<td>MboI of S. cremoris</td>
<td>0.8</td>
<td>30</td>
</tr>
<tr>
<td>pGKV144</td>
<td>MboI of S. cremoris</td>
<td>4.0</td>
<td>30</td>
</tr>
<tr>
<td>pGKV145</td>
<td>MboI of S. cremoris</td>
<td>3.6</td>
<td>30</td>
</tr>
<tr>
<td>pGKV146</td>
<td>MboI of S. cremoris</td>
<td>0.9</td>
<td>30</td>
</tr>
</tbody>
</table>

<sup>a</sup> CAT activity was assayed in cell extracts grown in TY (B. subtilis) or M17 (S. lactic) after induction of the CAT gene with 4 µg of chloramphenicol per ml when the cells were in the mid-logarithmic phase. Protein was measured by the method of Bradford (1). The maximum Cmr<sup>+</sup> is given in micrograms per milliliter for B. subtilis and was determined as described by Williams et al. (25) in TY containing 0.5, 10, 30, and 100 µg of chloramphenicol per ml. Resistance in S. lactic was determined by plating a 10<sup>5</sup>-diluted culture on M17 plates with 0.1, 1, 2, 3, and 4 µg of chloramphenicol per ml, respectively.

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**FIG. 1.** Vector construction strategy and restriction endonuclease maps of PGK3, pPL608, pGKV1, pGKV10, pGKV110, and pGKV11. The origin of the plasmid segments is indicated in the key. Only relevant restriction sites for construction and use of the recombinant vectors are given. Unique restriction sites of the multiple cloning site derived from double-stranded M13mp11 are EcoRI, Smal, Xmal, BamHI, SalI, and PstI, in the order given. For details, see text.
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LITERATURE CITED