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van der Vossen, Josephus; Kok, Jan; Venema, Gerhardus

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

JOS M. B. M. VAN DER VOSSEN,* JAN KOK, AND GERARD VENEMA
*Department of Genetics, University of Groningen, 9751 NN Haren, The Netherlands*

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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 promoterless 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 *Cla*I fragment of the cryptic *Streptococcus cremoris* Wg2 plasmid pWVO1 and the erythromycin resistance ($Em^r$) gene on a *Cla*I-*Hpa*II 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 *Pvu*II and partially with *Eco*RI restriction endonucleases. The largest *Pvu*II-*Eco*RI fragment, containing the SPO2 promoter and the CAT gene, was isolated by electroelution from the agarose gel. After filling in the *Cla*I-linearized pGK3 with the Klenow fragment of *E. coli* DNA polymerase I and the *Eco*RI cohesive end of the *Pvu*II-*Eco*RI 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 $Em^r$ chloramphenicol-resistant ($Cm^r$) 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 $Em^r$ (5 μg/ml) and $Cm^r$ (4 μg/ml) at a frequency of approximately $10^3$ 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 *Cla*I and *Eco*RI sites restored the *Eco*RI site, the SPO2 promoter was deleted in vitro by *Eco*RI 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 $Em^r$ $Cm^s$ 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 $Em^r$ gene under the $Em^r$ promoter. This is in accord with the presence of a termination signal downstream of the $Em^r$ gene (7).

Although the two vectors pGKV10 and pGKV20 can be used to select for promoter activity on DNA fragments generated by *Eco*RI, more versatile derivates were made by replacing the *Eco*RI-*Pst*I fragment by a multiple cloning site of double-stranded M13mp11 (13), resulting in pGKV110 (Fig. 1) and pGKV210 with opposite orientations of the CAT gene (data not shown), which carries unique *Eco*RI, *Sma*I, *Xma*I, *Bam*HI, *Sal*I, and *Pst*I sites upstream of and adjacent to the CAT gene. Transformation of *S. lactis* protoplasts with pGKV110 and pGKV210 gave rise to $Em^r$ $Cm^s$ transformants. The observations that pGKV1 and pGKV2 transformed *B. subtilis* and *S. lactis* cells to $Cm^r$ 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, *Mbo*I fragments of *B. subtilis* 0G-1 (2) and *S. cremoris* Wg2 (17) DNA were cloned into the unique *Bam*HI 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 $Cm^r$ transformants per μg of

* Corresponding author.

TABLE 1. Cm$^r$ properties of pGKV1 and derivative plasmids[a]

| Plasmid | Source of insert | CAT activity (mU/mg of protein) for *B. subtilis* | Cm$^r$ (μg/ml) for *B. subtilis* | CAT activity (mU/mg of protein) for *S. lactis* | Cm$^r$ (μg/ml) for *S. lactis* |
|---|---|---|---|---|---|
| pGKV110 | None | 0.1 | <5 | 0 | <3 |
| pGKV1 | *Eco*RI of SPO2 | 2.5 | 30 | 0.1 | 4 |
| pGKV111 | *Mbo*I of *B. subtilis* | 0.5 | 30 | 0 | <3 |
| pGKV112 | *Mbo*I of *B. subtilis* | 2.1 | 30 | 0 | <3 |
| pGKV113 | *Mbo*I of *B. subtilis* | 2.0 | 30 | 0 | <3 |
| pGKV114 | *Mbo*I of *B. subtilis* | 0.6 | 10 | 0 | <3 |
| pGKV143 | *Mbo*I of *S. cremoris* | 0.8 | 30 | 0 | <3 |
| pGKV144 | *Mbo*I of *S. cremoris* | 4.0 | 30 | 0.07 | 4 |
| pGKV145 | *Mbo*I of *S. cremoris* | 3.6 | 30 | 0.003 | 4 |
| pGKV146 | *Mbo*I of *S. cremoris* | 0.9 | 30 | 0.002 | 4 |

[a] CAT activity was assayed in cell extracts grown in TY (*B. subtilis*) or M17 (*S. lactis*) 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 Cm$^r$ is given in micrograms per milliliter for *B. subtilis* and was determined as described by Williams et al. (23) in TY containing 0, 5, 10, 30, and 100 μg of chloramphenicol per ml. Resistance in *S. lactis* was determined by plating a $10^5$-diluted culture on M17 plates with 0, 1, 2, 3, and 4 μg of chloramphenicol per ml, respectively.

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$^r$ with plasmids derived from the Em$^r$ Cm$^r$ *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) *Eco*RI fragment into the *Eco*RI site of pGKV110 and pGKV210 restored the CAT activity; *B. subtilis* cells harboring both plasmids pGKV11 (Fig. 1) and pGKV21 with opposite orientations of the CAT gene (data not shown) were Cm$^r$. Since the *Bam*HI 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, *Mbo*I fragments of pPLP1 were inserted in the *Bam*HI 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 *Aha*III fragment (14) inserted into the *Hin*dIII site of pPL608. The transcription termination signal of the penicillinase gene on this plasmid is located on a 0.4-Md *Mbo*I fragment. Among the Em$^r$ *B. subtilis* transformants, Cm$^s$ 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 *Mbo*I fragment harboring the transcription termination signal of the penicillinase gene, which is a complementary repeat followed by oligodeoxythymidylate (14). Transformation of *S. lactis* protoplasts with the recombinant plasmid pGKV12 gave rise to Em$^r$ Cm$^s$ 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 *Hin*dIII and *Hpa*II sites in the CAT gene, and to pGKV11 and pGKV21, carrying a unique *Hin*dIII site in the CAT gene.

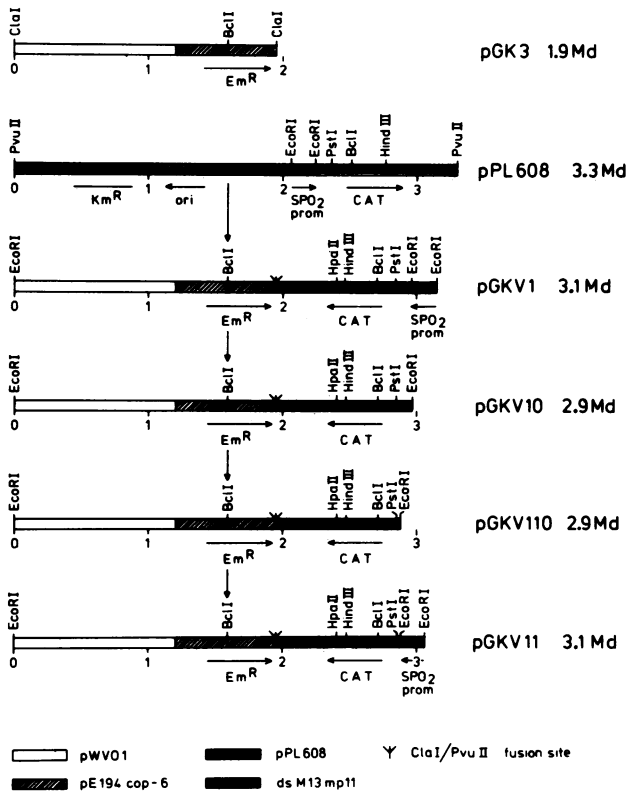


FIG. 1. Vector construction strategy and restriction endonuclease maps of PGK3, pPL608, pGKV1, pGKV10, pGKV110, and pGKV11. The origin of the plasmid segments is indicated in the key. Only relevant restriction sites for construction and use of the recombinant vectors are given. Unique restriction sites of the multiple cloning site derived from double-stranded M13mp11 are *Eco*RI, *Sma*I, *Xma*I, *Bam*HI, *Sal*I, and *Pst*I, in the order given. For details, see text.

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