Cloning and Characterization of the Tetracycline Resistance Determinant of and Several Promoters from within the Conjugative Transposon Tn919

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Tn919 is a 15- to 16-kilobase (kb) tetracycline resistance conjugative transposon that was originally isolated from Streptococcus sanguis FC1. The tetracycline resistance determinant (tet) was found on a 4.2-kb HindII fragment by in vitro deletion analysis. This fragment was subcloned to a pWV01 origin capable of redirecting replication in Escherichia coli, Bacillus subtilis, and Streptococcus lactis, and expression was observed in all three genera. In all cases, expression was weaker when only the 4.2-kb cloned fragment rather than the full transposon was present. The resistance gene is of the streptococcal tetM class and codes for a protein of approximately 70 kilodaltons. The restriction map resembles that of the tetM gene of Tn545 (P. Martin, P. Trieu-Cuot, and P. Courvalin, Nucleic Acids Res. 14:7047-7058, 1986), which codes for a protein of 72.5 kilodaltons. A number of transposon-derived promoter-bearing fragments were also cloned and sequenced. These closely resemble the consensus sequence of E. coli and B. subtilis promoters. Fusion experiments with a truncated lacZ gene indicate the possibility of an open reading frame for one of the promoters.

MATERIALS AND METHODS

Bacteria, media, and chemical reagents. Bacterial strains and their plasmid content, where relevant, are described in Table 1. S. lactis strains were grown in M17 medium (32) supplemented with 0.5% glucose (GM17). B. subtilis and E. coli were routinely grown in LB medium (10). Solid media contained 1.5% agar (no. 3; Oxoid Ltd., London, England). Antibiotics present in selective media were added at the following concentrations: for streptococci, tetracycline, 10 μg/ml; for bacilli, tetracycline, 10 μg/ml; chloramphenicol, 5 μg/ml; for E. coli, ampicillin, 100 μg/ml; tetracycline, 5 μg/ml; kanamycin, 50 μg/ml. Restriction enzymes and the Klenow fragment of DNA polymerase I were obtained from Boehringer Corp., Dublin, Ireland, and used as specified by the manufacturer.

Molecular cloning and transformation. Plasmids were isolated as previously described (19). General procedures for DNA manipulations and cloning were essentially as described by Maniatis et al. (23). DNA fragments were recovered from agarose gels by the method of Tautz and Renz (30). Transformation of competent E. coli was by the method of Mandel and Higa (22). Protoplasts of B. subtilis PSL-1...
TABLE 1. Bacterial strains and genotypes

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
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<tr>
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<td></td>
<td>pGV1</td>
<td>kan tet</td>
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<td>pCI172</td>
<td>kan tet</td>
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<td>38</td>
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<td>CH919</td>
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<tr>
<td>IL5919</td>
<td>pCI172</td>
<td>tet kan</td>
<td>This study</td>
</tr>
</tbody>
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were transformed as described by Chang and Cohen (6). Transformation of S. lactis MG1363 and IL1403 protoplasts was achieved by following the protocol of van der Vossen et al. (36).

Determining MICs. Cultures were inoculated (0.1%) into an appropriate medium containing increasing concentrations of tetracycline (from 0 to 100 μg/ml) and grown for 16 h at optimum growth temperatures (30°C for S. lactis and 37°C for B. subtilis and E. coli), after which the optical density at 550 nm (OD550) was read. The tetracycline concentration at which the OD550 equaled half the OD550 in the absence of tetracycline was taken as the MIC for each strain. MICs of chloramphenicol were essentially as described above, except that the OD550 was read after 6 h to rule out secondary growth after chloramphenicol breakdown.

In vitro transcription and translation. The Amersham procaryotic DNA-directed cell-free coupled transcription-translation system derived from E. coli was used as specified in the protocol supplied by the manufacturer (Amersham International, Little Chalfont, Buckinghamshire, England). CsCl-purified DNA (0.5 to 1.0 μg) was used in the reaction in which proteins were labeled with L-[35S]metionine. One-third of the reaction mixture was analyzed on 15% polyacrylamide gels (20). After electrophoresis, the gels were processed by fluorography as described by Skinner and Griswold (28) and exposed for at least 16 h to RXNIF 100 films (Fuji; J. J. Silber, Dublin, Ireland). DNA sequence analysis. The promoter-bearing fragments of Tn919 were cloned in M13mp18 and M13mp19 (38) and sequenced by the dideoxynucleotide method with [α-35S]dATP (26) essentially as described in the Amersham Cloning and Sequencing Handbook (Amersham International). M13 and its derivatives were propagated in E. coli JM109 (38).

RESULTS

Cloning of the tetracycline resistance determinant of Tn919. Tn919 was cloned in the pBR322-derived vector pGL101 on a small EcoRI fragment of pAD1, a Streptococcus faecalis plasmid, resulting in pAM554 (11) (Fig. 1). A spontaneous deletion derivative of pAM554 was observed in E. coli and was designated pCI160 (Fig. 1). Unlike the original plasmid, pAM554, this molecule is extremely stable in E. coli and shows no tendency to lose Tc', even in the absence of selective pressure. For these reasons pCI160 was chosen as a starting point for generating in vitro deletions of Tn919 to establish the location of the Tc' determinant (tet). HindIII was chosen for deletion construction, since the pGL101 origin is completely contained within one of the eight fragments generated by this enzyme (Fig. 1, A to H). On restriction of pCI160 with HindIII and religation, a large number of transformants of E. coli DH1 were obtained on plates containing 5 μg of tetracycline per ml. Two recombinant plasmid types were found among the transformants, the smaller of which was the result of recombination between fragments B and C. A representative of this class, designated pCI182, is shown in Fig. 2, line a. The second plasmid type contained fragments B, C, and F (data not shown). Fragment B (4.2 kb) must contain the tet gene, since this is the only transposon-derived fragment.

Insertional inactivation experiments were used to delineate the tet gene within this 4.2-kb HindIII fragment. When the kan gene of pKM1 (18) was cloned as a 1.4-kb EcoRI fragment into the unique EcoRI site of pCI182 (pCI188; Fig. 2, line b), there was no effect on Tc', since the EcoRI site is outside the 4.2-kb fragment. However, when the kan gene was introduced into the single HindIII site of pCI182 (the 1.4-kb kan fragment was recovered from an agarose gel after HindIII digestion of pKM1 and ligated to pCI182 which had been digested with HindIII and blunt ended with Klenow fragment) to create pCI186 (Fig. 2, line c), the tet gene was completely inactivated.

In a further experiment, pCI188 (Fig. 2, line b) was digested with ScaI, and the resultant sticky ends were filled in with Klenow fragment. After treatment with an excess of ScaI, the mixture was transformed to E. coli and selected on kanamycin. A total of 97% of the resultant transformants were Tc'. These data suggest that both the HindIII and SacI restriction sites, separated by 1.5 kb, lie within the resistance gene.

These results, and comparison of restriction sites mapped on the 4.2-kb fragment (Fig. 3) with the published restriction maps of DNA fragments containing tetM genes (3, 24, 31, 34), allowed us to align the putative location and direction of the Tn919 tet gene (Fig. 3).
Subcloning the 4.2-kb HindII fragment on a pWVO1 origin.
The expression of the Tc+ determinant (tet) in S. lactis and B.
subtilis was examined by subcloning the HindII B fragment
from pCI160 to a plasmid containing an origin capable of
directing replication in those genera. A number of plasmids
have been constructed based on the streptococcal origin of
the small cryptic plasmid pWVO1 which can replicate in
those hosts in addition to E. coli (19, 35, 37). Attempts to
clone the 4.2-kb fragment in one such plasmid, pGBK210
(35), proved unsuccessful in E. coli, selecting on tetracycline
at 5 μg/ml. No transformants were obtained even when the
fragment was inserted next to strong promoters previously
cloned in pGBK210 (data not shown). All efforts to clone
larger fragments of both pCI160 and pCI182 also failed.
However, pCI160 fragments A and B could be cloned in
pGV1, a vector constructed by Vosman et al. (37) which
contains the multiple cloning site and the alpha-lacZ region
of M13mp10 in addition to a Kan' gene (originally from
pJH1) on a pWVO1 origin (37). This plasmid allows for the
selection of recombinants in E. coli JM83 on plates contain-
ing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
(X-Gal). Fragments A and B were extracted from a gel and
cloned in the unique SmaI site of pGV1. Blue and white
(insertionally inactivated) colonies arose on plates contain-
ing kanamycin, but not on plates containing tetracycline,
with or without kanamycin. A total of 20 white colonies were
lysed, and 18 contained plasmids with inserts, equally di-
vided between fragments A and B. On being restreaked onto
plates containing tetracycline, only those with fragment B
showed growth. A representative plasmid, pCI172, was
mapped and found to contain the entire 4.2-kb insert (Fig. 2,
line d). When pCI172 was retransformed to both E. coli
JM83 and DH1, transformants could be obtained only by
selecting on kanamycin, not on tetracycline. The Kan' colonies
could again grow on tetracycline on subsequent
restreaking.

Cell-free transcription-translation. Cell-free transcrip-
tion-translation studies provide an alternative to minicell exper-
iments in visualizing proteins produced by DNA inserts. The
proteins produced by some of the plasmids described above
are shown in Fig. 4. The proteins derived from pGL101 (21)
are shown in lane 1. The three heavy bands are products of
the ampicillin gene and represent pre-β-lactamase, mature
β-lactamase, and a specific breakdown product. Other minor
bands are probably nonspecific degradation products due to
enzymes present in the cell extract. The gene products of

FIG. 1. Restriction endonuclease site map of pAM554 (21.0 kb) and pCI160 (18.0 kb). Symbols: ———, region corresponding to Tn919;
dna of pAD1 origin; ———, pGL101 DNA; ———, extent of the in vivo deletion in pAM554. The inner circle in pCI160 depicts the eight
Hind II fragments (A to H). Restriction endonucleases: V, HindII; E, EcoRI; H, HindIII; P, PstI; Sa, SacI; Sc, ScaI.

FIG. 2. Restriction endonuclease site maps of pCI182 (line a), pCI188 (line b), pCI186 (line c), and pCI172 (line d). Symbols: ———,
Tn919; ———, pGL101; ———, pAD1; ———, pGV1. Restriction endonucleases: V, HindII; E, EcoRI; H, HindIII; P, PstI; Sa, SacI; Sc, ScaI;
V, HindII-SmaI fusion; V, HindII- HindIII fusion.
pCI160 are shown in lane 2. Since it is difficult to distinguish between original gene products and degraded derivatives, only the uppermost band, representing a protein of approximately 70 kDa, can be designated an original product. The amp gene products are also visible in this lane. The proteins in lane 3 were produced by the in vitro deletion plasmid pCI182. Most, although not all, of the bands present in lane 2 are repeated here with the exception of the amp gene products (pCI182 is pCI160 is Ap<sup>a</sup>). This indicated that bands present in both lanes arose from a small number of proteins, the gene products for which are contained within the 4.2-kb HindII B fragment. Two small extra bands present in lane 3 disappeared when pCI182 was cut with HindII (data not shown), which suggests that novel ORFs were created by the in vitro rearrangement of fragments B and C of pCI160. The gene products of pCI188, i.e., pCI182 with the kan gene inserted in the EcoRI site (and therefore outside the 4.2-kb fragment), are shown in lane 4 and are identical to those in lane 3, except that the kan gene product is clearly visible. In pCI186 (lane 5), the kan gene was introduced into the unique HindIII site, resulting in insertional inactivation of tet. In this lane the largest 70-kDa band is missing, as are a number of smaller, presumably breakdown, products.

In additional experiments (data not shown), it was shown that the 70-kDa protein (also synthesized by pCI172) was no longer present in HindIII-digested pCI182, although it was unaffected by HindII digestion. These results suggest that the tet gene codes for a 70-kDa protein.

Expression of the tetracycline resistance determinant of Tn919. We examined tet expression either by different plasmids containing the cloned tet gene or by the original Tn919 in different hosts which were obtained as follows. pCI172 was transformed to S. lactis MG1363 and IL1403 and to B. subtilis PSL-1. In all cases, transfectants could be readily selected on tetracycline at 10 μg/ml. S. lactis CH919, constructed in an earlier study (16), is a derivative of MG1363 which contains a single copy of Tn919 in a chromosomal location. S. lactis IL5919 is a derivative of S. lactis IL1403 (7) which contains a single copy of Tn919 located on the chromosome. Tn919 was introduced to B. subtilis PSL-1 in filter matings by using an S. faecalis GF590 donor (11) to create strain PSL-919.

MICs of tetracycline are presented for all strains in Table 2. Strains containing the entire transposon (or, for E. coli, a slightly deleted form in pCI160) showed elevated resistance levels compared with strains possessing the 4.2-kb cloned fragment as pCI172 or pCI182. The reduced MIC of pCI172 compared with pCI182 probably accounts for the fact that pCI172 cannot be selected for directly on tetracycline plates after transformation. No induction of tet in E. coli was observed after pregrowth in the presence of 5 μg of tetracycline per ml.

The small fragment of the S. faecalis plasmid pAD1 present in pCI182 (Fig. 1) was found in subsequent experiments to contain a number of promoters (data not shown). This fragment could be removed from pCI182 by digestion with HindII and subsequent religation. Of the four fragments generated by HindII, only two were necessary for elevated tet expression (the 4.2-kb tet fragment and the 2.2-kb ori fragment). This suggests that the pAD1 promoter-rich region does not play a part in the elevated expression of pCI182 over pCI172.
quence. pGKV210 is a promoter-screening vector which replicates in B. subtilis, S. lactis, and E. coli (35). This plasmid was used to select for promoterlike regions of Tn919 by cloning small fragments into the multiple cloning site of the vector and selecting for expression of the promoterless chloramphenicol acetyltransferase (CAT) gene in B. subtilis. Using both HindII and Sau3A to generate fragments of pCI160, we isolated a large number of promoter-bearing recombinants. At least seven different promoter-bearing Sau3A fragments were found by Southern hybridization to have originated from the HindIII C fragment (Fig. 1), originating in part from pGL101 and in part from pAD1. Because of the profusion of promoters in this region, the HindIII C fragment was removed from a gel and the remaining seven fragments of pCI160 were eluted and subsequently redigested with Rsal. This generated a large number of fragments (>10) which were less than 1.0 kb in size. These were shotgun cloned in the single Smal site of pGKV210 and transformed to B. subtilis PSL-1, selecting for erythromycin (5 μg/ml) and chloramphenicol (5 μg/ml). Transformants were divided into different classes based on the location of the inserted DNA. The three smaller inserts (from pCI139, pCI140, and pCI144) were cloned in M13mp18 and M13mp19 and sequenced by the dideoxy method (the fragment contained in pCI144 could not be subcloned in M13mp19, a problem often encountered with promoter-bearing fragments, and the sequence obtained from repeated sequencing in one direction). Only one putative promoter sequence was identified in each fragment. The sequences of interest are shown in Table 3, along with the MICs obtained after growth for 6 h in broth containing various levels of chloramphenicol. The putative promoter sequences closely resemble the consensus sequences of E. coli, B. subtilis, and S. cremoris (36). This is to be expected, since fragments were screened in B. subtilis and also since the transposon functions in all three genera. However, we have as yet no evidence that these sequences promote transcription of Tn919 genes.

Only promoter 144 has an ORF following immediately downstream. This ORF also contains a putative ribosomal binding site (RBS; free energy, −13.3 kcal [−55.6 kJ]) in a suitable location (Fig. 5). However, the ORF is only 7 amino acids in length. Although this may be the gene product for which the promoter is meant, there is a second possibility. Soon after this ORF there is a second, stronger, RBS (free energy −16 kcal [−66.9 kJ]), and within 10 base pairs there is a possible start codon TTG. It is impossible to determine whether this represents a second ORF, since the limit of the inserted DNA does not extend beyond this point. It is possible, however, by conducting fusion experiments with a lacZ gene missing its first nine codons and expression signals, to determine whether the second RBS and start codon can act as translational start signals. Consequently, promoter 144 was cut with EcoRI and HindII and ligated to the EcoRI-Smal-digested vectors pMLB1034, pMG4, and pMG7, which contain the Smal site in three different frames relative to the truncated lacZ gene (Fig. 5). Only fusions with pMLB1034 showed any expression of lacZ, this being the only instance in which the TTG of the putative second ORF is in frame. Expression was weak (light-blue colonies on X-Gal) compared with that in a control experiment in which a fragment containing promoter 144 plus the first codons of the CAT gene was ligated in frame to pMLB1034 (intensely blue colonies; data not shown). These data confirm that promoter 144 can direct, if only weakly, transcription and expression.

### Table 3. Tn919 promoter sequences and E. coli consensus

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<th>Promoter</th>
<th>Nucleotide sequence</th>
<th>Chloramphenicol MIC (μg/ml)</th>
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<td>144</td>
<td>TTGACA AATATCTTTAAGCTGC (17 nt)</td>
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<td>TTGACA TACGTCAGAAATTTTG (15 nt)</td>
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<tr>
<td>139</td>
<td>CTGACA AACAAGTTACCAGTAC (17 nt)</td>
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</tr>
<tr>
<td>Consensus</td>
<td>TTGACA 16 of 18 nt</td>
<td>TATAAT</td>
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</table>

* Nucleotide sequence of three Tn919 putative promoters. The sequences shown were the only ones within each fragment which resembled the consensus promoter sequence as determined for E. coli and B. subtilis. The consensus sequence is included for comparison.

* nt, Nucleotide.
translation of a second ORF, assuming that one exists, starting from the TTG at the end of the sequenced insert.

**DISCUSSION**

Tn919 and related elements show great potential as genetic tools for the analysis of lactic streptococci (9, 17). However, little information is available regarding the characterization of Tn919. In this study we describe the cloning of both the Tc' determinant (tet) and promoter-bearing fragments of Tn919. Although more than 90% of Tn919 has been subcloned in our laboratory (unpublished data), the work presented here focuses on an internal 4.2-kb HindII fragment which was found by in vitro deletion analysis to contain the resistance gene. Initial attempts to subclone this fragment in *E. coli* on an origin which can also function in *S. lactis* and *B. subtilis*, selecting on tetracycline at 5 µg/ml, proved unsuccessful, even though the original in vitro deletion derivative, pCI182, could be readily selected after transformation under similar selection conditions. However, the fragment was cloned by using the vector pGV1, in which direct selection for Tc' resistance is not necessary to select recombinants in *E. coli*. The resultant plasmid, pCI172, showed limited Tc’ in *E. coli* JMB3, but was still unable to transform this strain with selection for tetracycline. To rule out any possible strain influence, pCI172 was introduced to *E. coli* DH1, but again no Tc' transformants were obtained, even though Kan' colonies did display Tc' upon further restreaking. The possibility that the difference between pCI172 and pCI182 was due to a strong promoter region present in pCI182 and originating from pAD1 was ruled out, since this promoter-rich region could be removed from pCI182 without preventing transformation and selection on tetracycline at 5 µg/ml. The most obvious remaining difference between pCI172 and pCI182 was their relative copy number, which was significantly higher (as determined visually from agarose gels) for the latter plasmid.

The tet determinant was expressed in *S. lactis* and *B. subtilis* and pCI172 could be readily selected in these strains after transformation, with tetracycline at 10 µg/ml. This result, together with the higher tetracycline MICs for pCI172 in gram-positive than gram-negative strains, suggests that the gene product either functions, or is expressed, more efficiently in those genera. It does appear that the tet gene is more efficiently expressed by the full transposon than when subcloned on the 4.2-kb fragment. Strains containing a single chromosomal copy of Tn919 were resistant to higher levels of tetracycline than were those containing more than one copy of pCI172. For *E. coli* also, a higher level of resistance was observed in strains harboring pCI160 (which contains almost the entire transposon) than in those containing the deletion derivative pCI182. This suggests that DNA from outside the 4.2-kb fragment has a role in determining the expression of the tet gene. Tobian et al. (33) also found that a tetM gene cloned from the chromosome of *Streptococcus mutans* V825 gave higher levels of resistance in *E. coli* (tetracycline MIC > 20 µg/ml) than in *Streptococcus sanguis* (tetracycline MIC, ca. 5 µg/ml). The levels of resistance in *S. sanguis* were comparable to those in *S. mutans* V825. However, the cloned and chromosomal determinants were not directly compared in an isogenic background.

Insertional inactivation of tet at the HindIII site of pCI182 results in the loss of a 70-kDa protein, suggesting that this is the tet gene product. Similar results have been reported for the tetM gene products of Tn1545 (24) and the *Campylobacter jejuni* plasmid pUA466 (34). In contrast, the product of a tetM gene isolated from the chromosome of *S. mutans* codes for at least one of two proteins of 33 and 35 kDa (33). At a restriction site level, similarities are also apparent between different tetM genes (e.g. six of seven enzyme sites mapped within the Tn919 gene can be located on the sequence of the Tn1545 tetM gene in similar positions). However, the sequences are not identical, since no Clal, Sau3A, or KpnI site could be identified, although all appear in Tn1545. It may be concluded that although the tetM determinants encoded by these elements are similar, there has been some divergence at a sequence level, resulting in altered restriction patterns.

Since Tn919 can function in a number of genera, it was of interest to clone and sequence transposon promoter regions. The information generated could be useful in at least two respects: first, in constructing expression vectors capable of functioning in different genera, and second, in helping to locate different ORFs on the transposon. Initial attempts were unsuccessful, in that promoter-bearing fragments were isolated at a high frequency from one particular 3.3-kb fragment of pCI160 which contains DNA of pG1101 and pAD1 origin. When this fragment was removed prior to cloning, a large number of Cm’ transformants were isolated. Four recombinant plasmids were chosen and probed against pCI160. All four showed homology with the 4.2-kb HindII fragment. The three smaller inserts were cloned in M13 and sequenced. One promoterlike sequence was found in each insert, all of which closely resemble the canonical consensus sequences reported for *E. coli*, *B. subtilis*, and, most recently, *S. cremoris* (36). It was found that the promoter most closely resembling the consensus sequence was strongest. A similar finding was reported by van der Vossen et al. (36) for promoters from *S. cremoris*. The same authors suggested a possible involvement of a TG sequence separated from the −10 region by a single nucleotide in strong promoters. This TG sequence was also found in promoter 144.

It is difficult to determine whether the promoters isolated in this study are actually involved in the expression of transposon functions, since in only one case was an ORF found on the same fragment. This probably reflects the small sizes of the inserts which were sequenced. It is notable in this respect that no obvious promoter was found for the tetM gene of Tn1545 in the 130 base pairs preceding the ORF. One promoter in this study, cloned in pCI144, does have an extremely small ORF and a RBS downstream, but this would code for a peptide of only 7 amino acids. A second RBS and start codon were detected downstream of the ORF, just within the limits of the insert. The start codon in this instance is TTG, which is less common than ATG, but is certainly capable of functioning in this role (the promoterless CAT gene used in this study begins at TTG). Fusion experiments demonstrated that this second RBS and start codon allow expression of lacZ in *E. coli*, although weakly when compared with the expression of a fused CAT-lacZ under the same promoter. It has been found that a small ORF preceding the ermC gene in *B. subtilis* (1) is most probably a regulatory device, but the kind of hairpin structures described in this study were not found in this instance.

The combined results of transcription-translation experiments and cloning of the promoter-bearing fragments may suggest a concentration of expression signals and gene products on the 4.2-kb HindII fragment. An alternative explanation may be that only expression signals recognized by *E. coli* and *B. subtilis* lie in this region; it was expected that the resistance gene and promoters isolated in this study, which function in three unrelated genera, will find a wide
application in the design of shuttle vectors, a number of which are currently being constructed in our laboratory.

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LITERATURE CITED