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Analysis of *Streptococcus pyogenes* Promoters by Using Novel Tn*916*-Based Shuttle Vectors for the Construction of Transcriptional Fusions to Chloramphenicol Acetyltransferase

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We have developed a series of shuttle vectors based on the conjugative transposon Tn*916* that have been designed for the analysis of transcriptional regulation in *Streptococcus pyogenes* and other gram-positive bacteria. Designated the pVIT vectors (vectors for integration into Tn*916*), the vectors are small, stable plasmids in *Escherichia coli* to facilitate the fusion of promoters from cloned *S. pyogenes* genes to a promoterless gene which encodes chloramphenicol acetyltransferase. The vectors each contain one or more small regions of Tn*916* to direct the integration of the transcriptional fusion into the transposon via homologous recombination following transformation of *S. pyogenes* or other suitable gram-positive hosts. Integration can be monitored by the inactivation or replacement of an antibiotic resistance determinant in modified derivatives of Tn*916*. Promoter activity can then be quantitated by the determination of chloramphenicol acetyltransferase-specific activity. In addition, since integration is into loci that do not disrupt the conjugative transpositional functions of Tn*916*, the vectors are useful for analysis of regulation in strains that are difficult or impossible to transform and can be introduced into these strains by conjugation following transformation of an intermediate host. The promoters for the genes which encode both the M protein and protein F of *S. pyogenes* were active in pVIT vectors, as was the region which controls transcription of *mry*, a *trans*-acting positive regulator of M protein expression. However, neither of the two characterized promoters for *mry* demonstrated activity when independently analyzed in pVIT-generated partial diploid strains, suggesting that regulation of *mry* is more complex than predicted by current models. The broad host range of Tn*916* should make the pVIT vectors useful for analysis of regulation in numerous other bacterial species.

The gram-positive bacterium *Streptococcus pyogenes* (group A streptococcus) is an important human pathogen and the causative agent of numerous suppurative infections of the throat and skin, including pharyngitis (strep throat), impetigo, cellulitis, and necrotizing fasciitis. Of particular concern, is that the incidence of severe streptococcal diseases, including a toxic shock-like syndrome, several types of invasive infections and rheumatic fever, a nonsuppurative sequela of infection, appears to be increasing in recent years (37). The importance of *S. pyogenes* as an agent of human infection has stimulated considerable investigation into the pathogenesis of the different streptococcal diseases.

Significant progress in understanding the molecular mechanisms by which *S. pyogenes* causes disease has been made possible by the development of technology for the genetic manipulation of this organism (reviewed by Caparon and Scott [5]). For example, allelic replacement techniques have been used to confirm the important role of the M protein in protecting the organism from being killed by phagocytic cells during infection (25). Fusion between the reporter gene which encodes chloramphenicol acetyltransferase (CAT) and the promoter for the gene which encodes the M protein (*emm*) has been used to demonstrate that transcription of *emm* is environmentally regulated in response to alterations in atmosphere (2, 27) and is stimulated by elevated tensions of carbon dioxide (2). Reporter gene fusions have also been used to demonstrate that environmental regulation requires the participation of *mry* (also known as *virR*) (23, 27), a *trans*-acting positive regulator of both *emm* (3, 24, 27) and the genes which encode several other streptococcal surface proteins (27).

Detailed analyses of the regulation of *mry* has shown that transcription of *mry* itself is regulated in response to the level of CO_2 (23). In addition, *mry* is positively autoregulated, and regulation requires the participation of extensive DNA sequences which extend at least 473 bp upstream of the *mry* coding region (23). Nuclease protection analysis of RNA has indicated that transcription of *mry* is initiated from two separate promoters located within this extended regulatory region (23). These data, in combination with other transposon mutagenesis, complementation, and DNA sequence analysis studies (3, 23, 24, 27) have suggested a model of *mry* activation involving differential initiation of transcription from the two promoters (23). In this model, the distal promoter (P_1) is responsible for low-level transcription of *mry* under noninducing environmental conditions while the proximal promoter (P_2) is not active. Following an encounter with a stimulating environment, Mry is modified, possibly through phosphorylation by a membrane-spanning sensor protein, and then stimulates its own transcription via a feedback loop through activation of P_2. The resulting high levels of Mry now activate promoters for the genes which encode Mry-regulated surface proteins.

To test this model, we have developed a novel vector for the high-resolution analyses of cloned *S. pyogenes* promoters that is based on the conjugative transposon Tn*916*. This transposable element endows the gram-positive hosts within which it resides with the ability to act as a conjugal donor for transmission of the element to a new host (reviewed by Clewell and Gawron-Burke [6] and Scott [30]). The observation that for-

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TABLE 1. Bacterial strains used in this study

Strain	Relevant characteristic(s)[a]	Comment	Reference or source
E. coli DH5α	*recA1 endA1 hsdR17*		BRL[b]
B. subtilis			
MEN207	xxx::Tn*916*-5K3	Derivative of Marburg 168	21
JJA100	xxx::Tn*916*-J4	Derivative of MEN207[c]	This work
E. faecalis OG1RF	Rif^r		7
S. pyogenes			
JRS4	*emm*6.1 Sm^r	Spontaneous Sm^r derivative of D471	31
JRS15	zzz::Tn*916*	Derivative of JRS4	3
RTG229	yyy::Tn*916*-J4	Derivative of JRS4[d]	This work

[a] Rif^r, rifampin resistant; Sm^r, streptomycin resistant.
[b] BRL, GIBCO-Bethesda Research Laboratories, Inc.
[c] Transformation of MEN207 by pEU3070. Recombination between pEU3070 and Tn*916*-5K3 produced Tn*916*-J4.
[d] Transconjugant derived from mating JJA100 and JRS4.

eign DNA can be inserted into a specific locus of Tn*916* without affecting the ability of the resulting chimeric element to undergo conjugative transposition (21) coupled with reports of other shuttle vectors which have exploited various properties of conjugative transposons (28, 38) has suggested that these elements can be developed as vectors for the analysis of gene regulation.

For analysis of *mry*, we have developed Tn*916*-based vectors with the following features. When present in *Escherichia coli*, the vectors are small and stable plasmids that facilitate the manipulation and fusion of streptococcal promoters to a promoterless CAT allele. A salient feature is the inclusion of one or more DNA fragments of Tn*916* which target the integration of the vectors into a resident chromosomal copy of Tn*916* following transformation of *S. pyogenes* or other gram-positive hosts. Thus, these plasmids have been designated the pVIT vectors (for vectors for integration into Tn*916*). Since integration is into loci of Tn*916* that do not affect the ability of the element to undergo conjugative transposition, the pVIT vectors can be used for analysis of promoters in strains of *S. pyogenes* that have proven difficult or impossible to transform. Additional advantages include the ability to analyze promoters in a chromosomal environment and at low copy number, as well as the capacity to construct stable partial diploid strains for analysis of autoregulated genes. The usefulness of these vectors was demonstrated in an analysis of the regulation of *mry*, as evidence was obtained to suggest that transcriptional regulation of *mry* is more complex than that proposed in our previous model.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. The construction of additional strains is described in Results. *E. coli* and *Bacillus subtilis* were cultured in Luria-Bertani broth (29), and *S. pyogenes* strains were cultured in Todd-Hewitt medium (Difco) supplemented with 0.2% yeast extract (THY medium). Where appropriate, the following antibiotics were added to media at the indicated concentrations: ampicillin at 50 μg/ml for *E. coli*, kanamycin at 25 μg/ml for *E. coli* and *B. subtilis* and 500 μg/ml for *S. pyogenes*, tetracycline at 10 μg/ml for *B. subtilis* and 5 μg/ml for *S. pyogenes*, erythromycin at 1 μg/ml for *B. subtilis* and *S. pyogenes*, and rifampin at 25 μg/ml for *S. pyogenes* and *Enterococcus faecalis*.

Transformation of bacteria. *E. coli* was transformed by plasmid DNA by the method of Kushner (16). DNA was introduced into *B. subtilis* by transformation of competent cells (11). Electroporation was used to transform *S. pyogenes* (5). Since resistance to penicillin has not been described for any natural isolate of *S. pyogenes*, no pVIT vector contains an intact β-lactamase gene. Integration of DNA into the correct locus was confirmed for all *S. pyogenes* transformants by Southern blot analysis (36) or by polymerase chain reaction (PCR) (15) with ^{32}P-labeled probes (8) or oligonucleotide primers of the appropriate sequences.

Bacterial matings. Transfer of Tn*916* by conjugation from *S. pyogenes* to *E. faecalis* was performed as described elsewhere (5). For conjugal transfer of Tn*916* from *B. subtilis* to *S. pyogenes*, the method was modified as follows: the *B. subtilis* donor was cultured overnight in THY broth (THYB) at 30°C, and the *S. pyogenes* recipient was cultured overnight in THYB at 37°C. Aliquots of the overnight cultures (10 ml each) were harvested by centrifugation (7,500 × *g*, 14°C, 10 min), washed once in 10 ml of THYB, and resuspended in THYB to a final volume of 1 ml. Equal volumes (200 μl each) of donor and recipient were added to a microcentrifuge tube. This represents approximately a 10-to-1 ratio of donors to recipients based on CFU. The cells in the mixture were pelleted by brief centrifugation (3 min), and the supernatant fluids were carefully removed. The mating mixture was resuspended in 100 μl of a buffer which contains 0.5 M sucrose, 0.02 M malic acid, 0.02 M $MgCl_2$, and 40% polyethylene glycol (average molecular weight, 8,000) at pH 7.1, and the entire mixture was spotted on the center of a THY plate. Following an overnight incubation at room temperature, transconjugants are harvested and enumerated as described previously (5). Conjugation frequency is reported as the ratio of CFU of transconjugants per surviving recipient.

Oligonucleotides. When synthetic oligonucleotides TER-1 (GATCCCGGGTGATTGATTGA) and TER-2 (GATCTCA ATCAATCACCCGG) are mixed together at equimolar ratios, heated to 100°C, and then allowed to slowly cool to room temperature, they will anneal to form a short double-stranded molecule (designated the TER fragment) that can be ligated into a *Bam*HI site. The sequence of the TER fragment was designed to regenerate the *Bam*HI site at only one side of inserted linker. A *Sma*I site is introduced adjacent to the regenerated *Bam*HI site, and adjacent to the *Sma*I site are TGA stop codons for all three reading frames that proceed toward the inactivated *Bam*HI site at the opposite end of the inserted linker. Oligonucleotides CAT86-4 (GTGAAAGTGC TCTTTTCG) and Ω-1 (CGGTTTACAAGCATAAAG) anneal at the amino-terminal end of *cat*86 (1) and the inverted repeat of ΩKm-2 (24), respectively. When oligonucleotides

MRY-B1 (GGATCCCTTAAAAACTGAAATCAGAC) and MRY-S2 (GAGCTCCTTGTAAACAACTTACTTAC) are used as amplimers in PCR, they produce a 386-bp product that contains the region between nucleotides −366 and +7 of the *mry* sequence (23) that is flanked by *Bam*HI and *Sst*I sites to assist cloning (see below).

Plasmid constructions. Insertion of a 660-bp *Dra*I fragment of pMGC2 (2) which contains the promoter for *emm*6.1 (13) into the *Sma*I site of pVIT162.1 (Fig. 1) generated pMGC21. Insertion of a 604-bp *Hpa*I fragment of pMGC8.1 (23) which contains the entire *mry* regulatory region (23) into the *Sma*I site of pVIT162.1 produced pRTG3. The chimeric plasmid pRTG4 was constructed by the insertion of a 526-bp *Dra*I fragment of pJRS1020 (4) into the *Sma*I site of pVIT162.1. Insertion of a 3,040-bp *Dra*I fragment of pMGC8.0 (23) into the *Sma*I site of pUC18 (39) generated pMGC15, and insertion of a 245-bp *Hpa*I-*Sst*I fragment of pMGC15 between the *Sma*I and *Sst*I sites of pVIT164 (Fig. 1) produced pRTG7. Insertion of the 386-bp PCR product of MRY-B1 and MRY-S2 between the *Bam*HI and *Sst*I sites of pVIT164 generated pRTG13. The relevant *mry* regions contained by pRTG3, pRTG4, pRTG7, and pRTG13 are summarized in Fig. 2. The chimeric plasmids pCIV8 and pCIV9 were constructed by insertion of 526-bp *Dra*I and 564-bp *Pst*I-*Hpa*I fragments of pMGC9 (23), respectively, into pVIT201 (Fig. 3). The relevant *mry* regions contained on pCIV8 and pCIV9 are summarized in Table 3. Insertion of a 560-bp *Eco*RI-*Sph*I fragment of the chimeric plasmid pPTF5, which contains the promoter for protein F (12), into pVIT201 resulted in pCIV6. All restriction nucleases, ligases, and polymerases were used according to the recommendations of the manufacturers. When required, incompatible restriction fragment ends were ligated following treatment with T4 DNA polymerase to produce blunt fragment ends. Correct insertion of all fragments was confirmed by sequence analyses of plasmid DNA (40) by using primers of the appropriate sequences.

Construction of Tn*916*-J4. A derivative of Tn*916* (Tn*916*-J4) was constructed to contain an erythromycin resistance determinant (*erm*) at the *Bst*XI site of Tn*916* as follows. A 2.3-kb *Ava*I-*Sal*I fragment containing the *erm* gene of pVA838 (19) was inserted into the *Sal*I site of pEU3025 (21) to generate pEU3025E. A 1.5-kb *Hin*dIII fragment containing *erm* from pEU3025E was inserted into the *Bam*HI site of pVIT130 (5), which contains regions which flank the *Bst*XI site of Tn*916* (Fig. 1) to construct pEU3070. Competent *B. subtilis* MEN207 was transformed with pEU3070 that had been linearized by digestion with *Pvu*I. Transformants resistant to erythromycin were generated by homologous recombination between the Tn*916* regions that flank *erm* in pEU3070 and the resident derivative of Tn*916* present in MEN207. One transformant (JJA100) was analyzed in detail and was shown to contain the expected derivative of Tn*916* (designated Tn*916*-J4) (Fig. 1C) and was found to transfer resistance to erythromycin and tetracycline to *S. pyogenes* at a frequency of approximately 10^{-8}.

Determination of CAT-specific activities. The preparation of cell extracts and the determination of CAT-specific activities were performed as described previously for streptococci cultured in liquid THY media (2) with [^{14}C]chloramphenicol (Amersham) by using the chromatographic assay of Shaw (33). For constructs derived from transformation of *S. pyogenes*, the data presented represent the means and standard errors of the means derived from two different extracts prepared from each of two independent transformants. For constructs introduced into *S. pyogenes* by conjugation, the data represent the means and standard errors of the means derived from two different extracts prepared from an individual transconjugant.

RESULTS

Construction of pVIT vectors for the construction and analysis of transcriptional fusions to CAT. Previous work has shown that foreign DNA can be inserted into the *Bst*XI site of Tn*916* without affecting the element's transpositional or conjugative abilities (21). We have exploited this observation to construct pVIT shuttle vectors that can be used to construct transcriptional fusions of cloned streptococcal promoters to a promoterless CAT reporter gene (Fig. 1). To maximize expression of CAT in the gram-positive bacterium *S. pyogenes*, a CAT allele (*cat*86C2-L22) from the gram-positive bacterium *Bacillus pumilis* that has been further modified by the deletion of a translational attenuator (C2 mutation) (17) and the substitution of an ATG start codon for the wild-type gene's TTG start codon (L22 mutation) was utilized (1). Additional important features of the pVIT vectors include (i) a number of different restriction enzyme sites for cloning promoters adjacent to *cat86* (the pVIT vectors pVIT162.1, pVIT163, and pVIT164 differ only by the enzyme sites available for cloning [Fig. 1A and B]), (ii) the introduction of translational stop codons in all three reading frames between the promoter cloning sites and *cat86* (Fig. 1A) (this feature will prevent the formation of undesired translational fusion proteins that may interfere with the enzymatic activity of *cat*86 and allow the direct comparison of promoter activity from different constructs), (iii) the introduction of a strong transcriptional terminator (present on the ΩKm-2 element [Fig. 1A]) (24) upstream of the promoter cloning sites (this will insure that all transcription of *cat*86 is initiated from the cloned promoter and is not the result of read-through transcription from any vector, transposon, or other chromosomal sequences), (iv) the *aphA3* kanamycin resistance determinant (present on ΩKm-2) that can be selected for in both gram-positive and gram-negative hosts, and (v) inclusion of the two regions of Tn*916* which immediately flank the *Bst*XI site (designated "L" and "R" [Fig. 1A and B]) so that they bracket ΩKm-2 and *cat*86. Details of the construction of these pVIT vectors is provided in the legend to Fig. 1.

The strategy for using the pVIT vectors is as follows. The cloning and manipulation of streptococcal promoters is facilitated by the ability of the vectors to replicate autonomously as stable and relatively small plasmids in *E. coli*. Once the transcriptional fusion has been constructed, plasmid DNA is purified from *E. coli* and is converted to a linear molecule by digestion with a restriction enzyme which recognizes a site in the pUC-derived region of the vector (e.g., *Pvu*II [Fig. 1C]). The linearized molecule is then used to transform a host that contains a resident chromosomal copy of a derivative of Tn*916* that has been modified to contain *erm* at the *Bst*XI site of the transposon (Tn*916*-J4) (Fig. 1C) (see also Materials and Methods), which can be either a competent intermediate host (e.g., *B. subtilis*) or a transformable *S. pyogenes* strain (see below). Homologous recombination between the L and R regions contained on both the vector and Tn*916*-J4 will result in the replacement of *erm* with both ΩKm-2 and the transcriptional fusion (Fig. 1C) and generate stable transformants that are resistant to kanamycin and sensitive to erythromycin.

The *emm*6.1 promoter is active when expressed in a pVIT vector. In order to test the usefulness of the pVIT vectors for the analysis of promoter function, we examined the activity of the well-characterized promoter for *emm*6.1 ($P_{emm6.1}$), the gene which encodes the type 6 M protein of *S. pyogenes* JRS4 (13). A fragment which contains $P_{emm6.1}$ and 122 bp of the 3′

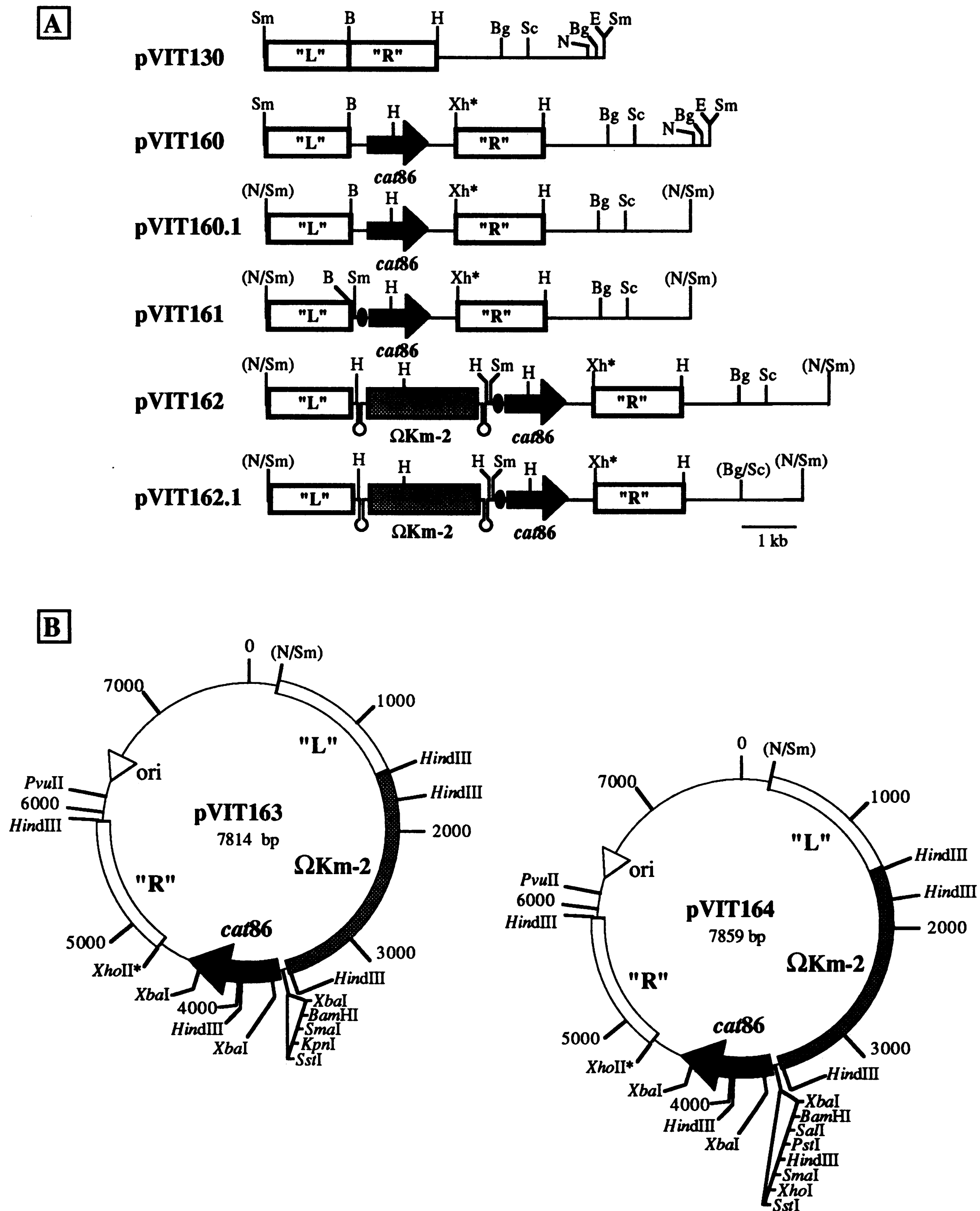
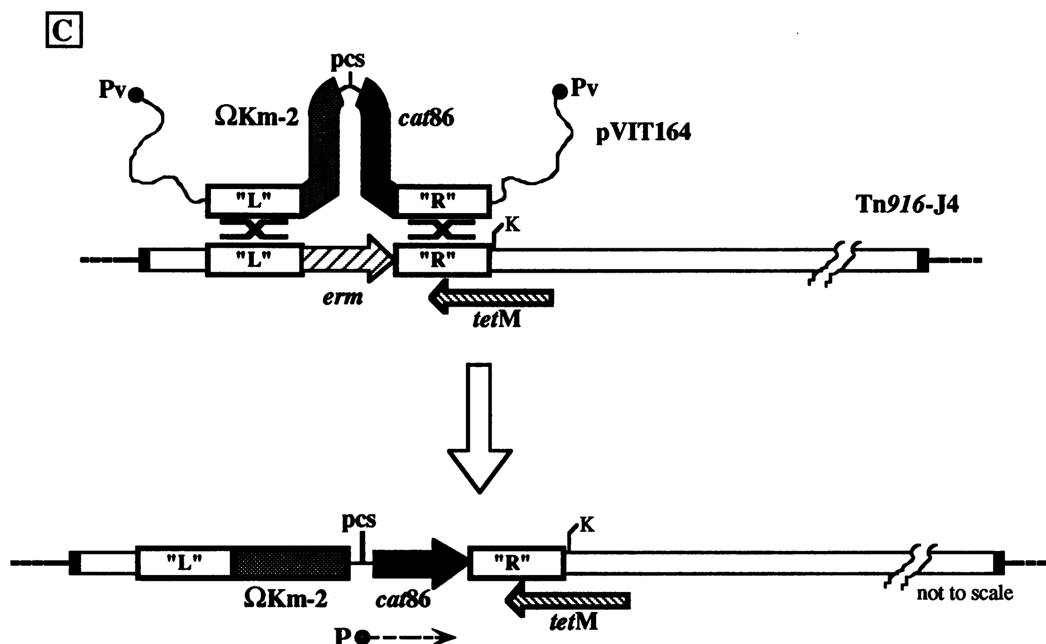


FIG. 1. Construction and use of pVIT vectors for analysis of gene regulation. (A) The chimeric plasmid pVIT130 (shown linearized at a *Sma*I site) contains approximately 1.2 kb of DNA located immediately to the left (L) and to the right (R) of the *Bst*XI site of Tn*916* that has been cloned into pUC9, and the *Bst*XI site was converted to a *Bam*HI site (5). Insertion of a 1.1-kb *Xho*II fragment from pBR322-L22 (1, 17) into the *Bam*HI site of pVIT130 introduced the promoterless *cat*86C2-L22 allele and regenerated a *Bam*HI site upstream of *cat*86. The location and direction of transcription of *cat*86 in the resulting plasmid (pVIT160) are indicated by the closed bar and arrow. The plasmid pVIT160.1 was generated by digestion of pVIT160 with *Sma*I and *Nar*I followed by religation to inactivate the *Sma*I and *Nar*I sites and to delete an *Eco*RI and *Bgl*I site. Insertion of the TER fragment (see Materials and Methods) into the *Bam*HI site of pVIT160.1 regenerates a *Bam*HI site and introduces a *Sma*I site and translational terminators for all three reading frames (●) (see pVIT161). Insertion of a blunted 2.2-kb *Bam*HI fragment containing



ΩKm-2 (24) into the blunted *Bam*HI site of pVIT161 generates pVIT162, inactivates all *Bam*HI sites, and introduces the *aph*A3 kanamycin-resistance determinant and strong transcriptional terminators (indicated by the stem-loop structures) upstream of the *Sma*I site. Digestion of pVIT162 with *Bgl*I and *Sca*I followed by religation inactivates a β-lactamase gene and produces pVIT162.1. (B) Additional restriction sites for cloning promoters were introduced into pVIT162.1 to generate pVIT163 and pVIT164 as follows. An *Eco*RI-*Hin*cII fragment of pUC18 was inserted into the *Sma*I site of pVIT162.1 to produce pVIT163. Insertion of a *Bam*HI-*Sst*I fragment from pTL41 (18) between the *Bam*HI and *Sst*I sites of pVIT163 generates pVIT164. The numbers located along the circumference of the circular molecules indicate distance in base pairs. (C) The strategy for using pVIT vectors involves first cloning a promoter into a vector by using the restriction sites upstream of *cat*86 (promoter cloning sites, shown as pcs). The chimeric plasmid is purified from *E. coli*, converted to a linear molecule by digestion with an enzyme which cuts only in the pUC9 vector (e.g., *Pvu*II), and used to transform a host which contains a chromosomal copy of a derivative of Tn*916* (Tn*916*-J4; see Materials and Methods) that has been modified to contain an erythromycin-resistance gene (*erm*; the direction of transcription of *erm* is indicated by the striped bar and arrow) at the *Bst*XI site of Tn*916*. Homologous recombination (each recombination event is indicated as an X between the incoming DNA and transposon) results in the replacement of *erm* by ΩKm-2 and the promoter-*cat*86 fusion and the generation of kanamycin-resistant and erythromycin-sensitive transformants. If an intermediate host was transformed (e.g., *B. subtilis*) the chimeric transposon can be transferred by conjugation to an *S. pyogenes* host for analysis of the cloned promoter (shown as the dashed line and arrow labeled P). When indicated, incompatible restriction fragment ends were ligated following treatment with T4 DNA polymerase to produce blunt fragment ends. Restriction enzyme sites: B, *Bam*HI; Bg, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nar*I; Pv, *Pvu*II; Sc, *Sca*I; Sm, *Sma*I; Xh, *Xho*II. Xh* indicates that the plasmids contain other *Xho*II sites in addition to the site shown. Restriction enzyme sites within parentheses are inactive. The thin solid bar of each plasmid represents the pUC9 vector sequences.

end of *mry*, which is located upstream of *emm*6.1 (the structure of the *mry*-*emm*6.1 locus is shown in Fig. 2), was cloned into pVIT162.1 in the correct orientation to transcribe *cat*86 (see Materials and Methods). The resulting plasmid (pMGC21), as well as the pVIT162.1 vector, was purified from *E. coli* and digested with *Pvu*II, and approximately 1 μg of the digests was used to transform *B. subtilis* JJA100 to kanamycin resistance (Table 2). All transformants analyzed were found to be sensitive to erythromycin, which indicates that the expected homologous recombination events occurred. Single-colony PCR of several individual transformants derived from pMGC21 was performed with oligonucleotides CAT86-4 and Ω-1, which anneal to sites which flank the promoter cloning sites of pVIT162.1 (see Materials and Methods). For each transformant analyzed, a single fragment of the expected size was amplified to confirm insertion of the $P_{emm6.1}$-containing construct into the *B. subtilis* chromosome (data not shown).

In several independent matings between a single transformant derived from pMGC21 (RTG104) or pVIT162.1 (RTG103) and *S. pyogenes* JRS4, the chimeric transposons transferred at frequencies which ranged from approximately 1×10^{-8} to 4×10^{-8} (Table 2). This frequency was similar to the frequency of transfer of Tn*916*-J4 from JJA100 (Table 2) and that reported for transfer of Tn*916*-E between *B. subtilis* and *S. pyogenes* (22). The activity of $P_{emm6.1}$ following transfer into *S. pyogenes* was then determined by analysis of CAT-specific activities. While an *S. pyogenes* transconjugant (RTG238) containing the chimeric transposon derived from vector pVIT162.1 alone demonstrated no detectable CAT activity, high levels of activity were detected in extracts prepared from three independent transconjugants that contained the $P_{emm6.1}$-*cat*86 fusion (Table 2). One of the three transconjugants (RTG239) produced approximately threefold lower activity than the other two (RTG421 and RTG422) (Table 2); this finding likely reflects the fact that a Southern blot analysis indicated that the latter two transconjugants had acquired at least two copies of the chimeric transposon (data not shown).

Analysis of the *mry* regulatory region. Previous analyses with integrational plasmids in combination with reporter gene fusions have revealed that the region involved in transcriptional control of *mry* extends 473 bp upstream of the Mry-coding region (23). Nuclease protection analysis of RNA has indicated that transcription of *mry* is initiated from two separate promoters (designated P_1 and P_2) located within this

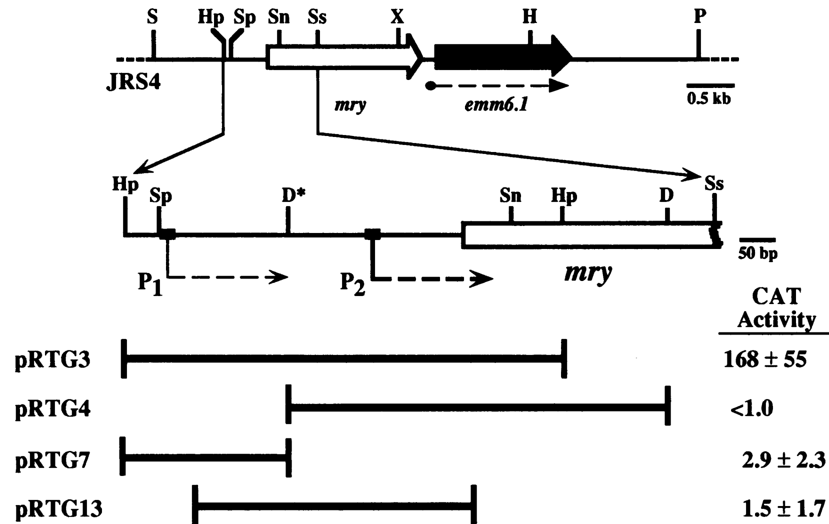


FIG. 2. Analysis of *mry* regulatory region. A partial restriction map of the *mry* and *emm* locus of *S. pyogenes* JRS4 is illustrated. The location and direction of transcription of *mry* (open bar) and *emm*6.1 (closed bar) are shown by arrows. The location of the promoter for *emm*6.1 is indicated by the broken line and arrow below the restriction map. Details of the *mry* regulatory region are provided in the expanded section, including the location of promoter P_1 (shown by the broken line and arrow under the restriction map) and stronger promoter P_2 (shown by the heavier broken line and arrow). The different DNA fragments from the *mry* regulatory region that were introduced into pVIT vectors to construct the indicated plasmids (see Materials and Methods for details) are indicated by the bars shown below the expanded section. Each indicated plasmid was converted to a linear molecule by *Pvu*II digestion and used to transform *S. pyogenes* RTG229, which contains Tn*916*-J4 at an unrelated locus, to kanamycin resistance. CAT-specific activities (reported as micromoles of chloramphenicol acylated min^{-1} mg of $protein^{-1}$) were then determined for the resulting erythromycin-sensitive transformants. Each value reported represents the mean (± standard error of the mean) obtained from duplicate determinations from at least two independent transformants. Restriction sites: D, *Dra*I; Hp, *Hpa*I; S, *Sal*I; Sn, *Sna*BI; Sp, *Ssp*I; Ss, *Sst*I; P, *Pst*I; X, *Xba*I. Other restriction sites are as indicated in the legend to Fig. 1. D* indicates the location of a Tn*916* insertion into a *Dra*I site that inactivates the function of both P_1 and P_2 (23).

extended regulatory region (23) (Fig. 2). Previous experiments have also demonstrated that *mry* is positively autoregulated; however, it was not possible to determine whether both or just one of the promoters required Mry for activation (23). The ability of pVIT vectors to generate stable partial diploid strains was expected to allow the independent analysis of P_1 and P_2 in a background in which the native *mry* locus is unaltered and provides a source of Mry. To test this, a series of plasmids was then constructed in which the entire regulatory region or just regions which contain only P_1 or P_2 were cloned into pVIT vectors in the appropriate orientation to transcribe *cat*86 (see Materials and Methods) (Fig. 2).

Since it is possible to conduct allele replacement in *S. pyogenes* JRS4 by electroporation of linear DNA (2, 12, 25), each construct was linearized by *Pvu*II digestion and used to transform to kanamycin resistance a derivative of JRS4 (RTG229) which contains a single copy of Tn*916*-J4 at a locus unlinked to *mry*. Transformants were obtained at frequencies which ranged from 10 to 100/μg of DNA, and the majority were sensitive to erythromycin. PCR analyses of selected erythromycin-sensitive transformants confirmed integration of the appropriate DNA segment into the RTG229 chromosome (data not shown). When CAT-specific activities were determined for the resulting strains, considerable activity was associated with strains derived from transformation of a plasmid which contained the entire regulatory region (pRTG3) (Fig. 2). However, no CAT activity was generated by strains transformed by a construct which contained only P_2 (pRTG4) (Fig. 2) at any levels greater than that obtained from transformation of the pVIT162.1 vector with no insert. Similarly, activities from strains transformed by a construct which contained solely P_1 were only slightly greater than background activity (pRGT7) (Fig. 2). A region which included P_2, as well as all the DNA which extends up to, but did not include P_1, also demonstrated only minimal activity (pRTG13) (Fig. 2). As will be discussed, these data suggest that while the entire regulatory region demonstrates transcriptional activity, activation of an individual promoter may require sequence elements that are widely separated across the entire region.

pVIT201 integrates into Tn*916* via a single homologous recombination event. Since at this time it is possible to transform only a limited number of streptococcal strains with linear DNA, an additional pVIT vector that can integrate into Tn*916* via a single homologous recombination event and thus allow transformation of circular DNA was developed. Designated pVIT201 (Fig. 3A), this vector shares many of the same features of the vectors described above, including *cat*86C2-L22 and ΩKm-2, the latter of which provides a kanamycin resistance determinant and a strong transcriptional terminator upstream of the promoter cloning sites. A novel feature is the inclusion of a 1.3-kb fragment (*tet*M*) that lies internal to the coding region of the *tet*M gene of Tn*916* (details of the construction are provided in the legend to Fig. 3). Because the ColE1 replicon of pVIT201 is not capable of autonomous replication in streptococci, transformation of circular DNA results in kanamycin-resistant transformants that arise from homologous recombination between *tet*M* present on pVIT201 and *tet*M of a resident chromosomal copy of Tn*916* (Fig. 3B). Since *tet*M* lies internal to the *tet*M coding sequence, homologous recombination results in the integration of the entire plasmid, including the promoter-*cat*86 fusion and ΩKm-2, as well as insertional inactivation of *tet*M (Fig. 3B). Thus, recombination into the correct locus of Tn*916* can be

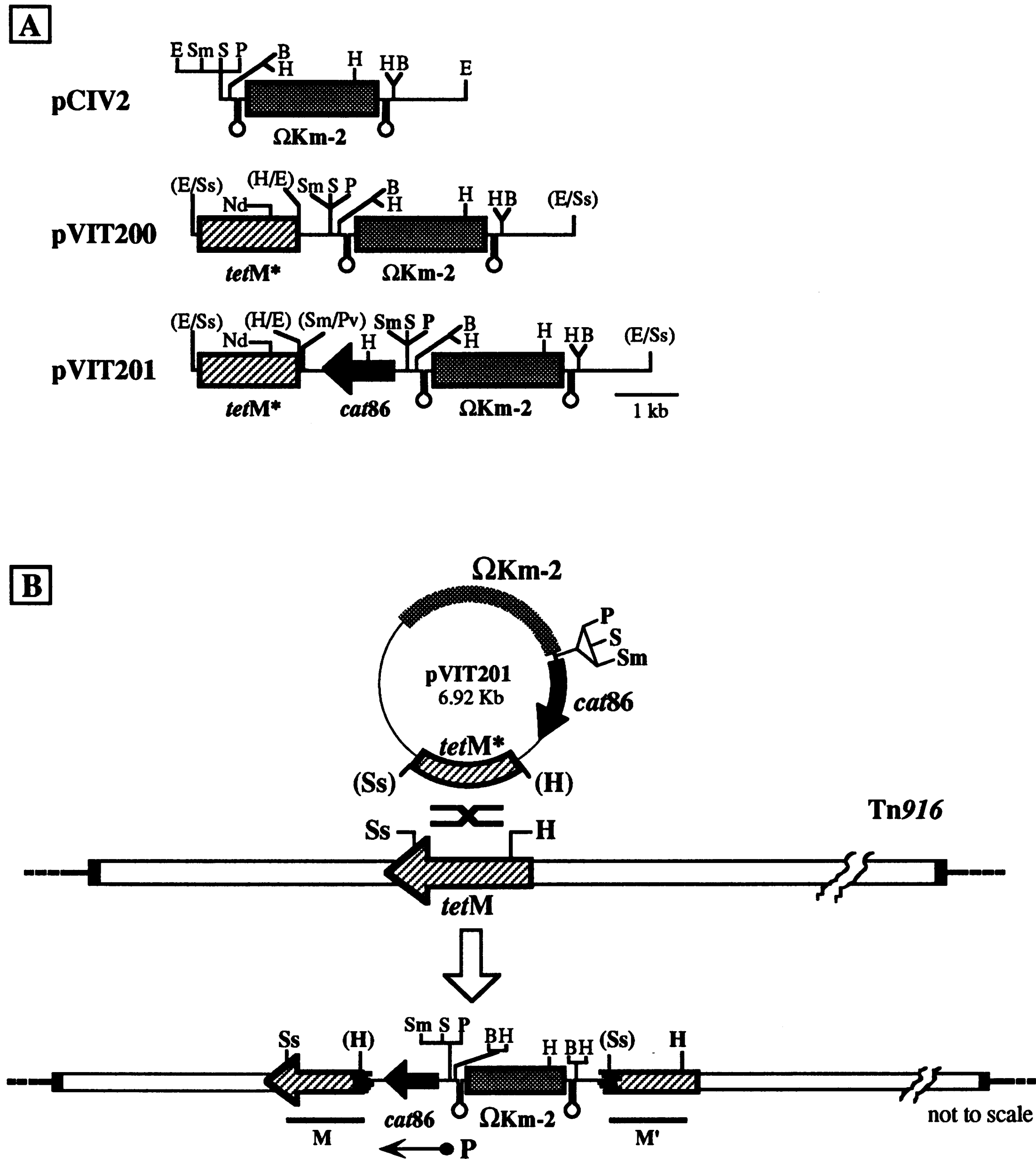


FIG. 3. Construction and strategy for use of pVIT201. (A) A pVIT vector was constructed that can integrate into Tn*916* via a single homologous recombination event. The chimeric plasmid pCIV2 (shown linearized at an *Eco*RI site) is derived by deletion of the β-lactamase gene and the insertion of ΩKm-2 into the *Bam*HI site of pUC18 (23). Insertion of a 1.3-kb *Hin*dIII-*Sst*I fragment from pAM120 (9), which contains sequences internal to the *tet*M gene (indicated as *tet*M*), into the *Eco*RI site of pCIV2 produced pVIT200. A 1.4-kb *Sma*I-*Pvu*II fragment of pBR322-L22, which contains *cat*86C2-L22, was inserted into the *Sma*I site of pVIT200 to generate pVIT201. The location and direction of transcription of *cat*86 are indicated by the closed bar and arrow. The unique restriction sites available for cloning promoters are indicated in bold type. (B) The strategy for using pVIT201 for promoter analysis involves first cloning the promoter into the vector in *E. coli*. The chimeric plasmid is purified and used to transform a Tn*916*-containing host to kanamycin resistance. Because the ColE1 replicon of pVIT201 will not replicate in gram-positive hosts, homologous recombination between *tet*M* and *tet*M (as shown by the X between pVIT201 and Tn*916*) results in the integration of the entire plasmid into Tn*916*. Since *tet*M* lies internal to the *tet*M coding region (the location of *tet*M and direction of transcription are indicated by the striped bar and arrow on the figure, respectively) integration results in tetracycline-sensitive, kanamycin-resistant transformants. If an intermediate host is transformed, the chimeric transposon can be transferred to *S. pyogenes* by conjugation for analysis of the cloned promoter (indicated by the line and arrow labeled P). The region that becomes duplicated upon integration of pVIT201 is shown by the thin solid bars below the diagram labeled M and M', respectively. Restriction site: Nd, *Nde*I. Other restriction sites and indications are as described in the legends to Fig. 1 and 2.

TABLE 2. Activity of *emm*6.1 promoter from *S. pyogenes*

Plasmid DNA	Transformation of *B. subtilis* (CFU)[a]		Transfer to *S. pyogenes*[b]		Isolate	CAT sp act[c]
	Total	Em[s] (%)	Donor	Frequency		
pMGC21[d]	349	349 (100)	RTG104			
			A	3.3×10^{-8}	RTG239	1,710 ± 215
			B	1.9×10^{-8}	RTG421	5,210 ± 130
			C	1.6×10^{-8}	RTG422	4,700 ± 610
pVIT162.1	143	143 (100)	RTG103	1.1×10^{-8}	RTG238	<1.0
None			JJA100	4.6×10^{-8}		

[a] Linearized plasmid DNA was used to transform JJA100 to kanamycin resistance. Individual colonies were then replica plated onto medium which contained erythromycin.
[b] One erythromycin-sensitive (Em[s]) derivative of JJA100 transformed with the indicated plasmid DNA was mated with JRS4 with selection for tetracycline and kanamycin (or tetracycline and erythromycin for Tn*916*-J4). The letter indicates an independent mating conducted with the same *B. subtilis* donor.
[c] One transconjugant from each of the indicated matings was analyzed. CAT-specific activity is reported as micromoles of chloramphenicol acylated min^{-1} mg of $protein^{-1}$ (mean ± standard errors of the means).
[d] Contains the *emm*6.1 promoter cloned into pVIT162.1.

distinguished from other recombination events by the former's tetracycline-sensitive phenotype.

To test the utility of pVIT201, a fragment containing the promoter for protein F, a fibronectin-binding surface protein of *S. pyogenes* (12), was inserted into the vector in the appropriate orientation to transcribe *cat*86 (see Materials and Methods). The resulting plasmid (pCIV6) and vector pVIT201 transformed *S. pyogenes* JRS15, a derivative of JRS4 that contains a single Tn*916* insertion, to resistance to kanamycin at a frequency of approximately 10/μg DNA. Of seven transformants analyzed, six (86%) were sensitive to tetracycline. Since integration generates a partial duplication of the *tet*M sequences (Fig. 3B), we anticipated that the tetracycline-sensitive phenotype would be unstable. Therefore, all transformants were further analyzed under constant kanamycin selection. The chimeric transposons were proficient for conjugal transfer as demonstrated by their ability to transfer resistance to kanamycin to *E. faecalis* OG1RF at frequencies of 4.5×10^{-8} (pVIT201 derived) and 1.0×10^{-8} (pCIV6 derived). The protein F promoter was active in the *S. pyogenes* transformants (405 ± 25 μmol of chloramphenicol acylated min^{-1} mg of $protein^{-1}$), thus demonstrating that pVIT201 can be used for promoter analysis.

To further evaluate pVIT201, segments of the *mry* regulatory region were cloned into the vector (see Materials and Methods) and used to transform JRS15 for determination of CAT-specific activities (Table 3). No activity at levels any greater than that observed for the pVIT201 vector alone was obtained from a construct which contained only P_2 (pCIV8) (Table 3). However, a fragment which contained both P_1 and P_2 demonstrated considerable activity (pCIV9) (Table 3). These data are consistent with those obtained by using the other pVIT vectors.

TABLE 3. Analysis of *mry* promoters with pVIT201

Plasmid DNA	Relevant *mry* region[a]	Promoter(s)	Isolate[b]	CAT sp act[c]
pVIT201	None	None	NO100	<1.0
pCIV8	*Dra*I-*Dra*I	P_2	NO105	<1.0
pCIV9	*Ssp*I-*Hpa*I	P_1, P_2	NO113	164 ± 63

[a] Relevant segment of the *mry* regulatory region cloned into pVIT201 (Fig. 2).
[b] Circular DNA was used to transform JRS15 to kanamycin resistance. Individual colonies were then replica plated onto medium containing tetracycline.
[c] One tetracycline-sensitive (Tc[s]) isolate from each transformation was analyzed. CAT-specific activities are reported as micromoles of chloramphenicol acylated min^{-1} mg of $protein^{-1}$ (mean ± standard error of the mean).

DISCUSSION

Previous work showing that foreign DNA can be inserted into Tn*916* without affecting the element's ability to undergo conjugative transposition (21, 32) has suggested that this element can be developed as an *E. coli*-streptococcal shuttle vector (shuttle transposon) for analyses of gene regulation. However, several factors have made this approach impractical: (i) direct cloning of a DNA fragment into Tn*916* in *E. coli* is made difficult by the large size of Tn*916* (16.4 kb) and any plasmid that contains Tn*916*, (ii) analysis of constructs is complicated by the fact that Tn*916* is unstable and excises at a high frequency from plasmids in *E. coli*, and (iii) it is often necessary to screen several potential donors when an intermediate host is utilized because the ability of any host to donate Tn*916* can vary over a broad range (10^5-fold), which may be influenced by the chromosomal location of the element (6).

To make the use of Tn*916*-based shuttle transposons practical, we have utilized a strategy that involves introducing a short segment(s) of Tn*916* into an *E. coli* plasmid that cannot replicate in a gram-positive host. The resulting vectors are small, stable plasmids in *E. coli*, a feature which facilitates molecular cloning and mutagenesis procedures. When used to transform a gram-positive host that contains a copy of Tn*916*, homologous recombination between the sequences shared by transposon and vector results in the integration of vector DNA into Tn*916*. Thus, these vectors have been designated the pVIT vectors. Because the regions of Tn*916* chosen to promote integration lie in nonessential loci of the element, the chimeric transposons remain fully competent for conjugative transposition. This latter feature, in conjunction with the ability of Tn*916* to function in almost all gram-positive organisms, suggests that the pVIT vectors will be useful for gene analysis in many additional bacterial species, including those that do not have well-developed genetic systems.

In this report, we have developed specialized pVIT vectors for the high-resolution analysis of *mry* and other streptococcal promoters. A DNA region which contains the transcriptional control elements of a streptococcal gene is inserted into a pVIT vector to direct transcription of a promoterless CAT gene. The construct is purified from *E. coli* and then introduced directly into *S. pyogenes* or into a transformable intermediate host for subsequent conjugal transfer to *S. pyogenes*.

Promoter activity can then be quantitated by determination of CAT-specific activities. This method offers numerous important advantages which complement those of existing vectors that are available for analyses of gene regulation in streptococci. Among these are the fact that the promoter under analysis is assayed at low copy number. Current vectors for the construction and analysis of transcriptional fusions in streptococci utilize high-copy-number plasmids (14, 27). However, the normal regulation of many genes is disrupted by the presence of multiple copies of the promoter and other regulatory elements of those genes. Undesirable high expression, gene dosage effects, and unpredictable variation in copy number are other potential artifacts that can be associated with plasmid vectors. With the pVIT vectors, the promoter is analyzed in a chromosomal environment. Currently utilized plasmid vectors employ members of the promiscuous replicon family which replicate via a rolling circle mechanism (for a review, see reference 35). This mechanism differs from that of the chromosome and may have unpredictable effects on gene expression.

The fact that Tn*916* is fully proficient for conjugative transposition following integration of a pVIT vector extends the repertory of gene analysis techniques available to strains of *S. pyogenes* that are difficult or impossible to transform. In this case, the chimeric transposon can first be used to transform a convenient intermediate host (e.g., *B. subtilis*), and then the promiscuous nature of conjugative transposition (donor and recipient do not have to be of the same strain, species, or genus) can be used to mobilize the construct into *S. pyogenes*. Activity of the promoter under analysis can then be determined as long as care is taken to control for the number of copies of the chimeric transposon that the recipient has acquired. However, it is also possible that the activity of some promoters will be influenced by local variations in topology, such as variations in supercoiling between specific chromosomal loci. This may result in different levels of promoter activity depending on where in the chromosome the chimeric transposon is located. In this case, comparisons of different promoter constructs should be conducted when they are present at the identical locus. Since it has been shown that the presence of Tn*916* in a recipient host does not impede the transfer of a second copy of the element (22), the chimeric transposons can be introduced into an *S. pyogenes* host that contains a copy of Tn*916*-J4. While transposition of the incoming chimeric element to a new locus is the most frequent event, a significant number of transconjugants (up to 13%) will have arisen from homologous recombination between the entering and resident transposons (22). Monitoring kanamycin-resistant transconjugants for sensitivity to erythromycin will identify strains in which the incoming copy of the promoter-CAT fusion-bearing Tn*916* derivative has replaced the resident copy of the *erm*-containing Tn*916*-J4 by homologous recombination and thus will allow the comparison of all promoter constructs at the same locus.

A more direct method to control for both transposon copy number and location is to avoid an intermediate host and to directly transform a Tn*916*-J4-bearing *S. pyogenes* host with linearized DNA. While this process occurs at low frequency and is currently available for only a limited number of *S. pyogenes* strains (10, 34), it does offer the additional advantage of simplicity. Transformation of linear DNA would be the method of choice for using the pVIT vectors in other, more readily transformable streptococcal or gram-positive species. A pVIT vector (pVIT201) that can integrate via a single homologous recombination event and thus allow *S. pyogenes* to be transformed by circular DNA was also developed. Since circular DNA presents no free ends to cellular exonucleases, it often transforms bacteria at higher frequency. While we did not observe integration of pVIT201-based vectors at any higher frequency than that obtained with linear DNA in the *S. pyogenes* strains used in this study, we have successfully transformed several strains with circular DNA that were not possible to transform with linear DNA (data not shown). Thus, this method should make the pVIT approach applicable to a greater range of strains. In addition, we are currently developing a pVIT vector that will utilize a recently described temperature-sensitive streptococcal replicon (26), which will extend the pVIT approach to all strains of *S. pyogenes* that can be transformed by plasmid DNA.

We developed the pVIT vectors primarily for analysis of the regulation of *mry*. We utilized the ability of the pVIT vectors to construct partial diploid strains in order to analyze each *mry* promoter independently. Fragments derived from the entire regulatory region or those which just encompassed P_1 or P_2 were introduced via pVIT vectors into the chromosome at a locus unlinked to the native *mry* locus. Thus, the native locus was unaffected and was available to provide a source of Mry for activation of the cloned promoters. Parallel studies were conducted with both the pVIT vectors which integrate DNA into the *Bst*XI site and the vector that is introduced into the *tet*M gene of Tn*916*. Consistent with all previous data, cloned regions which contained both P_1 and P_2 demonstrated promoter activity. However, no fragment analyzed that contained only P_1 or P_2 generated appreciable activity in either type of pVIT vector. These results are also consistent with a previous study which utilized a plasmid-based reporter-fusion vector to report that only very low promoter activity was associated with a region immediately upstream of *mry* (27). Comparison of the region analyzed in that study with the present study indicates that the region analyzed previously contained only P_2.

While these data do not invalidate the model proposed for *mry* regulation, they do suggest that it must incorporate additional levels of complexity. For example, these data may suggest that activation requires cooperativity between the promoters. In addition, we have observed considerable altered mobility of DNA fragments representing the *mry* regulatory region in polyacrylamide gel electrophoresis that are suggestive of intrinsic DNA curvature (10). The region of greatest curvature is located in the extremely A+T-rich (77% A and T residues) sequences located between P_1 and P_2 (see reference 23 for the presentation of the sequence). These observations may suggest that important control elements for activation of the promoters, which could include binding sites for Mry or other regulatory proteins, are widely separated on the linear sequence and are brought into conjunction by a mechanism that involves DNA looping. Numerous other well-characterized promoters contain widely separated regulatory elements that function through mechanisms involving DNA looping (for a review, see reference 20). The curved region itself is not sufficient to activate either promoter individually, as fragments that contain all or most of this region and only one of the two promoters are not active. Alternative models involving processing of a single primary transcript which is initiated from P_1 may also be unlikely, since these models would predict that P_1 can function independently. Thus, additional experimentation will be required to generate a clear understanding of the mechanism of *mry* regulation in *S. pyogenes*. The availability of the pVIT vectors should prove invaluable for further studies designed to test the role of DNA looping and other controlling elements in these regulatory phenomenon.

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