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Autonomous Expression of the *slo* Gene of the Bicistronic *nga-slo* Operon of *Streptococcus pyogenes*

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A recent model for cytolysin-mediated translocation in *Streptococcus pyogenes* proposes that NAD-glycohydrolase is translocated through streptolysin O-generated pores into a host cell (J. Madden, N. Ruiz, and M. Caparon, Cell 104:143–152, 2001). This model also assumes that the NAD-glycohydrolase (*nga*) and streptolysin O (*slo*) genes that code for these products are organized in an operon-like structure expressed from a single promoter only (*nga*). We expand this model by showing that *slo* possesses its own autonomous promoter, which is located 155 bp upstream of the *slo* gene. Under experimental conditions in which *S. pyogenes* is grown in THY medium, the strength of the *slo* promoter, as measured by the activity of a *lacZ* reporter gene, resulted in low but highly reproducible values. Finally, we demonstrated that *sloR*, a *S. pyogenes* gene that closely resembles the *Clostridium perfringens pfoR* gene, exerts a negative effect on the expression of the *slo* gene.

Streptolysin O (SLO) is an extracellular protein produced by most strains of *Streptococcus pyogenes*. It belongs to a large group of highly conserved cholesterol-dependent cytolysins found in species of four genera, including *Streptococcus*, *Clostridium*, *Listeria*, and *Bacillus* (2). SLO exerts its cytolytic function by forming large homopolymeric pores in the membranes of the targeted cells. The pores are formed by binding of SLO monomers to cholesterol receptors in the cell membrane followed by their aggregation into supramolecular complexes containing up to 50 monomers (11, 13).

In contrast to rather extensive research on the SLO toxin and its mode of action, little information is available on the regulation of the *slo* gene. The original studies of Kehoe, Timmis, and colleagues were centered mainly on the cloning, sequencing, and analysis of the *slo* gene in comparison with other cytolysin-coding genes. However, these studies also provided some information on the regulatory role of a certain DNA segment in the upstream region of the *slo* gene (6, 7). Their deletion and transposon mutagenesis analysis of the *slo* region produced three distinct phenotypes: Slo^+, Slo^-, and $Slo^\pm$, the last of which was obtained by transposon insertion into the chromosome region later shown to be occupied by the *nga* gene and deletions which cover the *nga* promoter but not the *slo* promoter (Fig. 1). These results suggested the existence of a weak *slo* promoter (6) located, as has been predicted, 155 bp upstream of the structural gene (7). The most recent report by Caparon and colleagues describes for the first time a cytolysin-mediated translocation system in gram-positive bacteria which is a functional equivalent to the type III secretion system found in gram-negative bacteria (8). Their data support a model in which the effector NAD-glycohydrolase, encoded by *nga* (*spn*), the gene immediately upstream of the *slo* gene (4) (Fig. 1), is transported through the SLO pore into the host cell (8). Results of that study also suggest that the *slo* gene is a part of a bicistronic *nga-slo* operon transcribed from a promoter immediately upstream of the *nga* gene, implying that they are regulated in common at the transcriptional level (8) (Fig. 1). Here we show that the regulation of these two products is possibly more complicated and that the *slo* gene, in accordance with the reports cited in references 6 and 7, may also possess its own independently regulated promoter.

To test the authenticity of the previously predicted *slo* promoter (7), a set of strains carrying fragments of different lengths of the *slo* upstream region fused to the *lacZ* reporter gene of vector pCAMP17 was constructed. The pCAMP17 vector contains the attachment site for and the integrase gene of *S. pyogenes* phage T12, which enable it to integrate irreversibly into a specific site in the *S. pyogenes* chromosome (5). The fragments were obtained by PCR amplification of chromosomal DNA from strain NZ131 (M49) with mutagenic oligonucleotides containing *Pst*I and *Bam*HI restriction sites. Upon treatment with *Pst*I and *Bam*HI enzymes, the fragments were cloned into the *Pst*I- and *Bam*HI-treated vector pCAMP17. The *Bam*HI restriction site is found at locations inside both the *lacZ* and *slo* genes, and the obtained products encompass the ribosome binding site, the ATG initiation codon, and the first four codons of the *slo* gene (Fig. 2). All constructs (data not presented) were confirmed by DNA sequence analysis before they were integrated into the chromosome of strain NZ131 (the resulting strains: OK88, OK89, OK103, OK129, OK130, and OK153) (Fig. 2). The DNA recombinant techniques and measurement of β-galactosidase (β-Gal) activity were performed as described previously (10). The advantage of this system is that the *slo* upstream DNA in all constructs ends far below the hypothetical Spy166 gene promoter (4) (Fig. 1). Also, the *int* gene on pCAMP17 is positioned in an opposite orientation to that of the *lacZ* reporter gene (Fig. 2). Consequently, all detected activity of the reporter gene should be attributed to the putative *slo* promoter. The results demonstrate low but reproducible expression of the *lacZ* gene in all deletion mutants, with the exception of control strain NZ131, with no plasmid (data not presented), and strain OK103, in which the −35 sequence of the putative *slo* promoter is deleted

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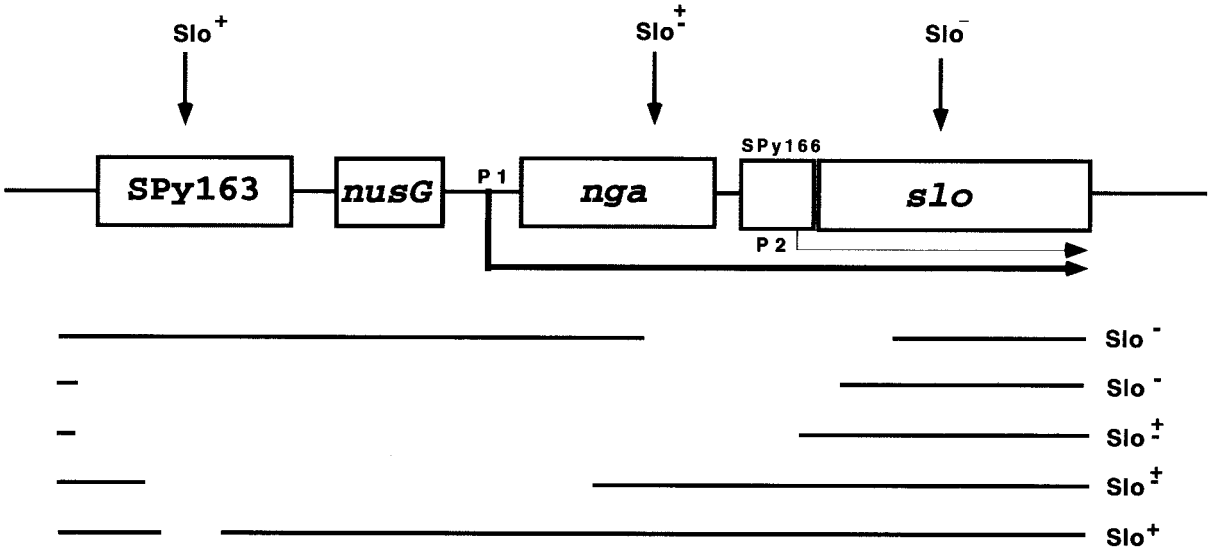

FIG. 1. Schematic representation of the *nga-slo* region of the *S. pyogenes* chromosome (4). Arrowed horizontal lines represent transcripts emerging from the *nga* (P1, thick line) and *slo* (P2, thin line) promoters. Transposon inserts (vertical arrows) and horizontal interrupted lines (with gaps representing deleted material), both with specific phenotypes related to SLO synthesis, were adapted from Kehoe and Timmis (6) and superimposed on the current *nga-slo* region map (4).

(zero β-Gal activity) (Fig. 2). The low activity values obtained may be the result of sensitivity of the *slo* promoter to local DNA conformations (pCAMP17 inserts into the chromosome at the opposite site of the chromosome relative to the native site of the *slo* gene, more specifically between the open reading frames Spy1289 and Spy1290 (4), lack of a specific environmental signal, sharing of a hypothetical positive regulator with the native *slo* regulatory region, weakness of the *slo* promoter, or the combined effects of several factors. We conclude that expression of the reporter gene in strains with β-Gal activity is not the result of some spurious transcript coming from vector DNA and that the *slo* gene possesses its own genuine promoter as predicted before (7).

A similar experiment to that carried out with plasmid constructs as described above was performed with a set of mutants in which the reporter *lacZ* gene was inserted into the chromosome at its natural site. For that purpose, a mutant of pCAMP17 (pCAMP17-1) which had lost the ability to inte-

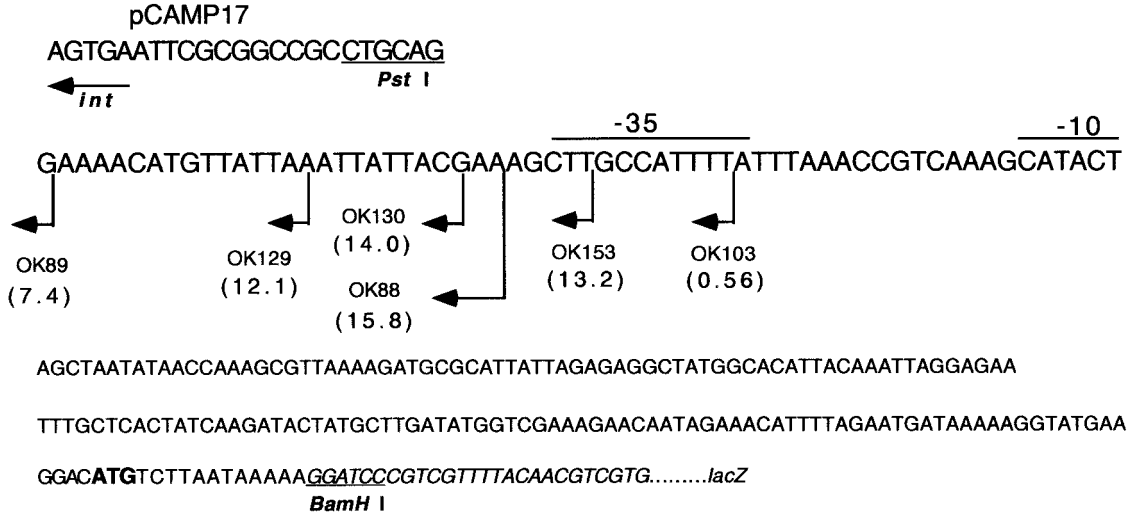

FIG. 2. Schematic representation of the *slo* gene upstream region with constructed deletion ends indicated (vertical lines with arrows oriented to the left). The material to the left of deletion ends is spliced to the *Pst*I site of the pCAMP17 DNA (upper row), and the bottom three nucleotide sequences are contiguous to the −10 box. Numbers in parentheses denote average values in Miller units obtained in three independent measurements. Lines above the main nucleotide sequence represent the predicted −10 and −35 promoter sequences of the *slo* gene (7). The initiation codon of the *slo* gene is given in bold letters, and the *lacZ* DNA sequence is given in italics. The *Pst*I site in the pCAMP17 vector and *Bam*HI site at the junction of the *slo* and *lacZ* DNA sequences are underlined. For a more detailed description of pCAMP17-based constructs and their mode of integration into the chromosome, see the text.

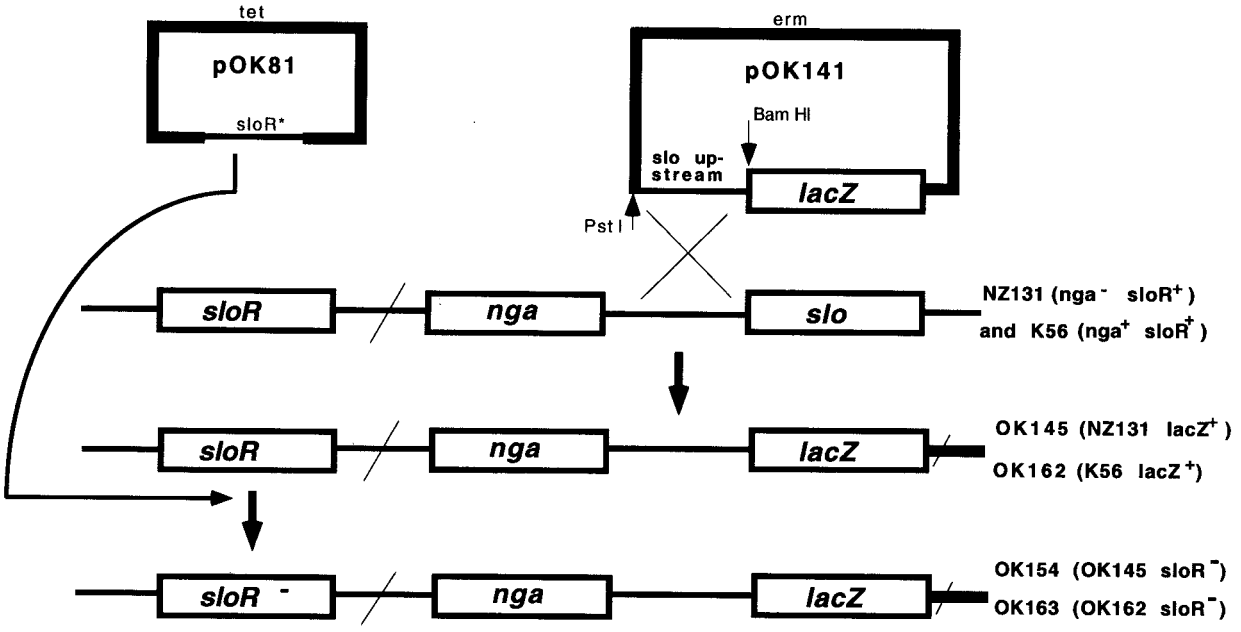

FIG. 3. Construction of strains OK145 and OK162 by integration of the recombinant plasmid pOK141 carrying the *slo* upstream region into the *slo* regions of the recipients NZ131 and K56, respectively. In the second step, the *sloR* derivatives of strain OK145 and strain OK162 (strains OK154 and OK163, respectively) were created by insertional inactivation by using the recombinant plasmid pOK81. The designation *sloR** indicates that pOK81 carries an incomplete copy of the *sloR* gene (see the text). As indicated for the NZ131 genotype, strain NZ131 and its derivatives (OK145 and OK154) harbor the *nga* mutant allele. The scheme of strains OK154 and OK163, which are related to the *sloR* allele, is presented in simplified form, without inserted plasmid material and with two incomplete copies of the *sloR* gene. For details, see the text. The drawing is not to scale.

grate at its specific chromosomal site (5) was used. As in the previously described construction procedure (Fig. 2), a piece of the *slo* upstream region (approximately 0.6 kb) was amplified by PCR, treated with *Pst*I and *Bam*HI enzymes, and ligated into the pCAMP17-1 mutant vector. The construct obtained (pOK141) (Fig. 3) was inserted into strain NZ131 by transformation. Confirmation that insertion in the chosen Erm^r transformant occurred at the *slo* locus was accomplished by PCR with appropriate primers. In this way, the *lacZ* reporter and *slo* upstream region in the new strain OK145 were brought into continuity with the immediate upstream genes and the rest of the chromosome (Fig. 3). Previous results indicate that the *nga* gene is inactive in strain NZ131 (our unpublished results and reference 1). The nature of the *nga* mutation in strain NZ131 is unknown. However, similar absolute values of β-Gal activity obtained for deletion strains devoid of any incoming transcripts from the upstream DNA (Fig. 2) and strain OK145 (Fig. 4), as opposed to the β-Gal activity values obtained for strain OK162 with a wild-type allele of the *nga* gene, point to the polar nature of the *nga* mutation in strain NZ131 and its derivative OK145. These data suggest that all, or almost all, transcripts coming into the *lacZ* gene originate from the *slo* promoter.

It has been shown that the *phoR* gene of *Clostridium perfringens* may regulate expression of the *phoA* gene as an inducer (12), although the hypothesis regarding the mechanism of action of the PhoR protein in regulation of the *phoA* gene was later questioned (3). The *phoA* gene encodes perfringolysin O, which together with SLO belongs to a single family of CDC pore-forming cytolysins (13). Our analysis of the *S. pyogenes* genome demonstrated a high degree of DNA sequence homology between the *sloR* gene (Spy146) of *S. pyogenes*, which maps to a location approximately 19 kb from the *slo* gene (4), and the *phoR* gene from *C. perfringens.* To test whether the *sloR* gene influences expression of the *slo* gene, the activities of β-Gal were compared for strains OK145 (*nga sloR*$^+$) and OK154 (*nga*

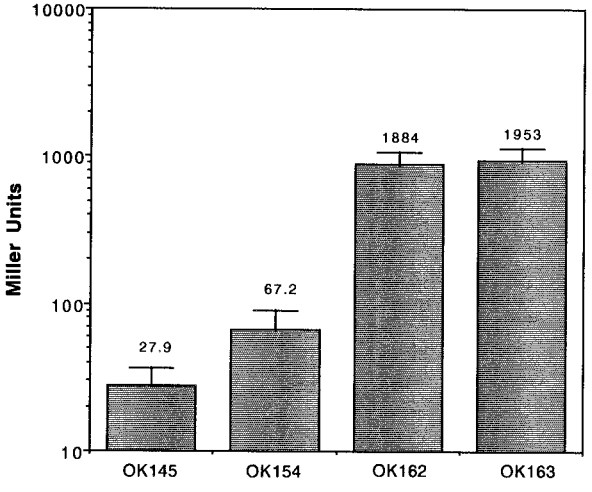

FIG. 4. Comparison of *lacZ* reporter gene activities among strains with the wild-type or mutated alleles of the *sloR* gene. For a description of strain genotypes, see Fig. 3. The numbers above the columns denote average values in Miller units obtained in 10 (strains OK145 and OK154) or 5 (strains OK162 and OK163) independent experiments.

sloR). Strain OK154 was made by insertional inactivation as follows. The central part (around 800 bp) of the approximately 1-kb-long *sloR* gene was amplified with appropriate primers by using DNA from strain NZ131 as a substrate, cloned into pGEM-T Easy vector (Promega Corp.), and recloned into the insertional vector p7tet (9). The resulting construct (pOK81) (Fig. 3) was inserted by transformation into the OK145 recipient, and its insertion into the *sloR* gene was verified by PCR with appropriate primers (data not presented; Fig. 3). The resulting data demonstrate a 2.4-fold-higher level of activity of the reporter gene in strain OK154 (*sloR*) compared to that of the OK145 strain with the wild-type allele of the *sloR* gene (Fig. 4), indicating the repressor role of the SloR protein in regulation of the *slo* gene. This result is in contrast to the inducer role of *pfoR* gene in *C. perfringens* (12). As mentioned, a more detailed study of the role of the *pfoR* gene in the regulation of expression of the *phoA* gene (3) and the solution of the problem posed thereby await the isolation and analysis of a *pfoR* mutant in *C. perfringens.*

A pair of mutants with the same characteristics as those described above was made by using strain K56, which, in contrast to NZ131, possesses the wild-type allele of the *nga* gene (OK162 [nga^+ $sloR^+$] and OK163 [nga^+ *sloR*]) (Fig. 3). As shown in Fig. 4, the β-Gal activities detected for these strains attained much higher values than those for strains OK145 and OK154. Also, no substantial difference was observed between strains carrying $sloR^+$ or *sloR* alleles (Fig. 4). The levels of expression for the strains in the NZ131 background (OK145 versus OK154) differed significantly ($P < 0.001$), while those for the strains in the K56 background (OK162 versus OK163) did not ($P > 1$). These data confirm that the *nga* and *slo* genes are organized in an operon structure expressed from the strong common promoter upstream from the *nga* gene (8). They also indicate that the product of the *sloR* gene, which has been shown to be a DNA binding protein (12), does not affect transcription from the *nga* promoter but does affect expression of the *slo* promoter, which confirms ipso facto the previous results (Fig. 2) and points to the existence of an autonomous *slo* promoter. We explain the lack of significant differences in β-Gal activity between strains OK162 and OK163 and strains OK145 and OK154 by the relative strengths of the *nga* and *slo* promoters; that is, the overwhelming majority of mRNA coming into the *lacZ* gene in these strains possibly originates from the *nga* promoter, obscuring the effect of the *sloR* gene product at the weak *slo* promoter. That the *sloR* gene does not affect transcription from the *nga* promoter was confirmed in an independent experiment, in which no difference was found in NADase activity between strains K56 and OK156 (K56 *sloR*) (results not presented). The results presented here are in agreement with earlier findings (6, 7) and in contrast with the report of Caparon and colleagues, whose data indicated that a polar mutation in the *nga* gene reduces expression of the *slo* gene to undetectable levels (8). A detailed analysis of RNA transcripts in this region, including determination of the status of the hypothetical gene Spy166 (4), should provide an explanation for these disparate views.

The genetic data we present support the hypothesis of the existence of an internal *slo* promoter within the operon-like organized *nga* and *slo* genes which is regulated in a negative manner by the product of the *sloR* gene. The weak activity of this promoter does not necessarily reflect its potential under different physiological circumstances; it only reflects its activity during growth in Todd Hewitt broth with 0.2% yeast extract (THY) medium. The biological implication of the existence of an autonomous *slo* promoter is that it would increase genetic flexibility by providing the cell with a potential to divorce the SLO and NGA synthesis processes when necessary. That is, it is not difficult to imagine the SLO toxin as having another function in the infection process and pathogenesis besides its role in the NGA translocation system. Further studies of the regulatory mechanisms are needed to elucidate the functional relationship between these two genes.

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