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Cloning and Expression in *Escherichia coli* of the Streptolysin O Determinant from *Streptococcus pyogenes*: Characterization of the Cloned Streptolysin O Determinant and Demonstration of the Absence of Substantial Homology with Determinants of Other Thiol-Activated Toxins

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A gene bank of *Streptococcus pyogenes* Richards was constructed in *Escherichia coli* by using the bacteriophage replacement vector λL47.1, and hybrid phage expressing streptolysin O (SLO) were identified among the recombinants. DNA sequences encoding SLO were subcloned from an *slo*$^+$ hybrid phage into a low-copy-number vector plasmid to yield an *slo*$^+$ hybrid plasmid, pMK157. This plasmid contains 5.6 kilobase pairs of cloned streptococcal DNA sequences, is stable, and expresses SLO at easily detectable levels in *E. coli*. Transposon γδ insertion mutants and in vitro-generated deletion mutants of pMK157 were isolated and analyzed. This analysis showed that a single gene is sufficient for production of SLO in *E. coli* and allowed this *slo* gene to be mapped to within ±100 base pairs. Two forms of the *slo* gene product, with molecular weights of 68,000 and 61,000, were detected in *E. coli* minicells harboring *slo*$^+$ plasmids and by immunoblotting of *E. coli* whole cells harboring *slo*$^+$ plasmids. Southern blotting hybridization experiments with the cloned SLO DNA sequences as probes failed to demonstrate homology between the cloned SLO determinant and DNA isolated from bacteria expressing thiol-activated cytolysins related to SLO.

Streptolysin O (SLO) is a cytolytic, extracellular protein produced by most strains of *Streptococcus pyogenes* and many strains of groups C and G streptococci (for reviews, see references 1, 13, 29, and 34). It is one of 15 biologically and immunologically related toxins, known as the thiol-activated (oxygen-sensitive) cytolytic toxins, produced by gram-positive bacterial species belonging to four different genera, namely, *Streptococcus*, *Bacillus*, *Clostridium*, and *Listeria*. SLO is the best characterized of the thiol-activated cytolysins with respect to its physical and chemical properties and mode of action. There is good evidence that SLO binds to cholesterol in the target cell membrane (1, 10), and, at least at high concentrations of toxin, this leads to the formation of C-, S-, and closed-ring-shaped structures visible by electron microscopy (11, 29). These interactions result in the formation of large functional pores, allowing the release of high-molecular-weight cytoplasmic molecules (9, 29). Despite extensive studies on the interaction of SLO with a variety of natural and artificial membranes, the precise nature of SLO-induced membrane lesions is not understood.

SLO is produced by group A streptococci both in vitro and in vivo, but the factors which determine the synthesis and release of SLO are not understood (1). The tissue-damaging and cytolytic properties of SLO (1) and the ability of sublytic concentrations of SLO to inhibit chemotaxis of human neutrophilic leukocytes (2, 33) suggest that SLO may contribute to the pathogenesis of group A streptococcal infections in humans. High titers of anti-SLO antibodies which are indicative of a recent group A streptococcal infection of the pharynx and a good response to streptococcal antigens are a characteristic feature of rheumatic heart disease, an often fatal nonsuppurative sequel to streptococcal pharyngitis. Together with the marked cardiotoxicity of SLO (1), this has led some workers to speculate that SLO might contribute to the onset of rheumatic heart disease (13, 34). However, there is no direct evidence concerning the role of SLO in group A streptococcal infections or their nonsuppurative sequels.

Studies on the structure, mode of action, synthesis, release, and contribution to pathogenicity of SLO would be greatly facilitated by the ability to isolate and characterize genetically well-defined mutations within the gene that encodes this toxin. As is the case with most gram-positive bacteria, genetic studies of group A streptococci are hampered by a lack of methodology and would be simplified if the determinants of interest could be isolated in a genetically well-defined background. Although plasmid cloning vectors have recently been developed for use in *Streptococcus sanguis* (5, 23), it would be difficult to use this system to clone determinants for which there is no direct selection or which are not known to be located on easily manipulated plasmid or phage replicons. For these reasons we chose to clone the SLO determinant in *Escherichia coli*, where well-developed mutagenesis and expression systems could be used to characterize the cloned DNA sequences. In this paper, we report the cloning, expression, and characterization of the SLO determinant from *S. pyogenes* Richards in *E. coli* and examine the relationship between the SLO determinant and the determinants of other thiol-activated cytolysins.

MATERIALS AND METHODS

Bacterial strains, growth conditions, plasmids, and bacteriophages. The bacterial strains used are described in Table 1. Nutrient agar consisted of antibiotic medium 3 (Difco Laboratories, Detroit, Mich.) containing 1.0% (wt/vol) Bacto-Agar (Difco). Blood agar plates were made by the

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TABLE 1. Bacterial strains

Strain	Relevant characteristics	Source or reference
E. coli K-12		
LE392	F⁻ *hsdR514 supE44 supF58 lacY1* or Δ (*lacI24*) *6 galK2 galT22 metB1 trpR55* λ⁻	24
WL95	*lac thr leu thi tonA hsdR hsdM*; P_2 lysogen	21
LC707	F⁺ *asnA31 asnB32*	G. Churchward
ED2196	*his trp nal*	35
BHB2688	λ *imm434* c*I*1857 b_2 *red3 E*am4 *S*am7	15
BHB2690	λ *imm434* c*I*1857 b_2 *red3 D*am15 *S*am7	15
DS410	*minA minB*	19
S. pyogenes Richards	*slo*⁺, M-type 3 (original)	J. Duncan
S. pneumonia La2372	Produces SLO-related hemolysin	Hôpital Cantonal, Lausanne, Switzerland
B. cereus La1138	Produces SLO-related hemolysin	Hôpital Cantonal
L. monocytogenes	Produces SLO-related hemolysin	S. Kaufmann
C. welchii	Produces SLO-related hemolysin	D. Fernie
C. histolyticum	Produces SLO-related hemolysin	D. Fernie
C. novyii	Produces SLO-related hemolysin	D. Fernie

addition of 2.5% (vol/vol) washed, packed erythrocytes to nutrient agar, cooled to below 50°C, before the plates were poured. Todd-Hewitt broth (Difco) was used for *Streptococcus* cultures; *Clostridium* strains were grown in thioglycolate medium (Oxoid Ltd., Basingstoke, United Kingdom), *Listeria monocytogenes* and *E. coli* DS410 were grown in brain heart infusion broth (Difco), and other strains were grown in Luria broth (25). *Bacillus cereus* cultures were incubated at 30°C, and all other cultures were incubated at 37°C. For anaerobic cultures an anaerobe jar containing an Oxoid Gas Generating Kit was used. Where appropriate, antibiotics were added to media at the following concentrations: kanamycin, 50 μg/ml; tetracycline, 20 μg/ml; and nalidixic acid, 40 μg/ml. The media and growth conditions for λ bacteriophages have been described by Miller (25).

The low-copy-number vector plasmid pLG339 has been described by Stoker et al. (31). The bacteriophage λ replacement vector λL47.1 has been described by Loenen and Brammar (21). Other plasmids and phage are described in the text.

General procedures. Whole-cell DNA was purified as described by Saito and Miura (27) from cells lysed as described by Clewell and Frank (6) in the case of *Streptococcus* strains or as described by Hansen and Olsen (14) in the case of other strains. The procedures used to purify large quantities of plasmid DNA and bacteriophage λ DNA and for agarose and polyacrylamide gel electrophoresis have been described previously (18, 19). For the rapid screening of recombinant plasmids, DNA was isolated as described by Holmes and Quigley (16). Transformation of *E. coli* strains with DNA was performed by the procedure of Cohen et al. (7). Restriction endonucleases and DNA T4 ligase were purchased from Genofit S.A. (Geneva, Switzerland) or New England Biolabs (Schwalbach, Germany) and used as described by Maniatis et al. (24). Restriction endonuclease-generated DNA fragments were purified from agarose gels by electrophoresis onto and elution from DE81 filters (Whatman) as described by Dretzen et al. (8). For the construction of gene banks, λ packaging mixes were prepared from *E. coli* BHB2688 and BHB2690 and ligated DNA molecules were packaged in vitro as described by Hohn (15). The procedures used for the analysis of plasmid-encoded polypeptides in *E. coli* minicells have been described previously (19).

Southern blotting and DNA hybridization. Restriction endonuclease-cleaved DNA was transferred onto nitrocellulose filters as described by Southern (30). Labeling of DNA with [^{32}P]dCTP (New England Nuclear Corp., Germany) by nick translation and DNA hybridization procedures has been described by Jeffreys and Flavell (17).

Immunoblotting. For immunoblotting, polypeptides were transferred by electrophoresis from sodium dodecyl sulfate-polyacrylamide gels to nitrocellulose filters as described by Towbin et al. (32) and subsequently treated by a modification of the procedures of Towbin et al. (32) and Renart et al. (26) (J. Kistler, personal communication). The filters were shaken gently for 1 to 2 h at room temperature in a solution containing 10 mM Tris-hydrochloride (pH 7.5), 2 mM EDTA, 150 mM NaCl, 0.05% (vol/vol) Triton X-100, and 0.5% (wt/vol) bovine serum albumin to saturate protein-free sites on the nitrocellulose filters. Anti-SLO serum, generously provided by J. Alouf (Institute Pasteur, Paris, France), was then added, and shaking at room temperature was continued for a further 2 h. The filters were then washed twice for 15 min each time with a buffer containing 10 mM Tris-hydrochloride (pH 7.5), 2 mM EDTA, 150 mM NaCl, 0.1% (vol/vol) Triton X-100, and 0.1% (wt/vol) bovine serum albumin before the addition of 1 μCi of ^{125}I-protein A (New England Nuclear Corp.) per 25 ml. Shaking was continued for a further 1 to 2 h before the filters were washed twice for 20 min each time with a high salt buffer (10 mM Tris-hydrochloride [pH 7.5], 2 mM EDTA, 1 M NaCl, 0.1% [vol/vol] Triton X-100, and 0.1% [wt/vol] bovine serum albumin), rinsed with 10 mM Tris-hydrochloride (pH 7.5), and dried at 37°C. The filters were then autoradiographed using Kodak X-Omat (SO-282) film.

Titration of hemolytic activity. Titrations were carried out in microtiter wells containing a total reaction volumn of 100 μl. One hemolytic unit was defined as the smallest amount (highest dilution) of toxin in phosphate-buffered saline (PBS) which produced visibly detectable lysis of 50 μl of a 1.7% (vol/vol) erythrocyte suspension in PBS in 30 min at 37°C. Where appropriate, inhibitors were added to the toxin before the addition of erythrocytes. Cholesterol was prepared as a 100 mg/ml suspension and trypan blue as a 5 mg/ml stock solution; these were diluted 1/20 or 1/100 into toxin. Protein determinations were carried out as described by Lowry et al. (22).

RESULTS

Cloning of the *S. pyogenes* SLO determinant in *E. coli*. A gene bank of *S. pyogenes* Richards was constructed in *E.*

coli by using the bacteriophage λ replacement vector λL47.1. Restriction endonuclease *Sau*3A-generated partial digest products of total-cell DNA isolated from strain Richards were ligated with λL47.1 DNA which had been digested to completion with the restriction endonuclease *Bam*HI, and the ligation products were packaged in vitro to generate intact phage particles. Streptococcal DNA inserts of sizes between 4.7 and 19.6 kilobase pairs (kb) can be inserted into the vector DNA to generate packagable hybrid λ DNA molecules (21). The cloning strategy involves replacement of the central 6.5-kb *Bam*HI fragment of the vector which encodes the λ *gam* gene with foreign DNA and thus generates gam^- hybrid λ phage. Since gam^+ λ phage cannot grow on *E. coli* strains lysogenized by the bacteriophage P2 (21), recombinant phage among the packaged ligation products were selected directly by plating on *E. coli* WL95 (Table 1). In control experiments neither wild-type λ phage nor the vector phage λL47.1 formed plaques when plated at high multiplicities of infection on *E. coli* WL95. About 9,000 independent recombinant phage plaques selected on *E. coli* WL95 were pooled and stored without further amplification.

Recombinant phage expressing *slo* were detected in the gene bank by plating the phage on *E. coli* LE392 at a density of ca. 300 plaques per plate and overlaying the plaques with 1% (wt/vol) agarose in PBS, containing 1 mM dithiothreitol and 2.5% (vol/vol) packed washed rabbit erythrocytes. After incubating the plates at 37°C for 4 to 6 h, small zones of hemolysis were detected above ca. 1 of 3,000 plaques. These hemolytic (Hly^+) plaques were purified and shown to produce hemolysis when rabbit erythrocytes were replaced with either sheep or human erythrocytes in the agarose overlay. This hemolysis was completely inhibited when cholesterol or anti-SLO serum was included in the soft phage agar upon plating of the phage but was unaffected by trypan blue (an SLS inhibitor). This inhibition pattern was subsequently confirmed in quantitative microtitration assays when the Hly determinant was subcloned from one of the Hly^+ recombinant phage into *E. coli* plasmid vectors (see below). These results indicate that the hemolytic activity associated with the Hly^+ phage is due to the expression of *slo*.

Subcloning the SLO determinant into plasmid vectors. The DNA of one of the Hly^+ recombinant phage, termed λR3, was isolated, and the cleavage sites in λR3 for a number of restriction endonucleases were mapped. Phage λR3 contains 6.4 kb of cloned streptococcal DNA sequences. Attempts to subclone these sequences into the *Sph*1 site of the high-copy-number vector plasmid pBR322 (3) yielded highly unstable Hly^+ pBR322 hybrid plasmids of the desired construction which caused *E. coli* LE392 host cells to lyse readily, resulting in the selection of spontaneous Hly^- deletion derivatives (data not shown). This instability problem was overcome by using a low-copy-number plasmid, the pSC101-based replicon pLG339 (31), as the vector.

Cleavage of λR3 DNA with the restriction endonucleases *Bgl*II and *Sph*I generates a 6.2-kb DNA fragment which contains most of the cloned streptococcal DNA sequences of λR3, in addition to 600 base pairs (bp) of vector DNA sequences (Fig. 1). Phage λR3 DNA, digested to completion with *Bgl*II and *Sph*I, was ligated with pLG339 DNA which had been cleaved with the restriction endonucleases *Bam*HI and *Sph*I, and the ligation products were transformed into *E. coli* LE392, selecting for the Kam^r determinant of pLG339. A hybrid plasmid, named pMK157, which contains the 6.2-kb *Bgl*II-*Sph*I fragment of λR3 inserted between the *Bam*HI and *Sph*I sites of pLG339, was identified by screening the plasmids in the resulting Km^r Tet^s transformants. This plasmid is stably maintained in *E. coli* hosts, although cells harboring pMK157 grow slowly, forming small colonies on blood agar plates after 18 h of incubation at 37°C. These colonies are surrounded by small (ca. 1 mm) zones of

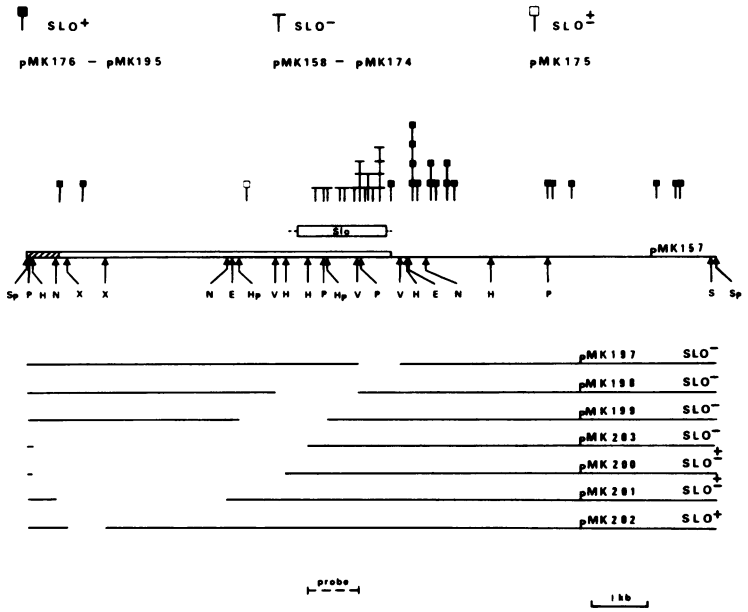

FIG. 1. Structure of pMK157 and its derivatives. The central box represents the *Sph*I-*Bgl*II DNA fragment cloned from the slo^+ hybrid phage λR3 into the vector pLG339, which is represented by the line. This cloning fused the *Bgl*II site of the insert DNA with the *Bam*HI site of the vector, resulting in the loss of both sites in the product, pMK157. The lower arrows indicate the cleavage sites in pMK157 for the following restriction endonucleases: E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; N, *Nco*I; P, *Pvu*II; S, *Sal*I; Sp, *Sph*I; V, *Eco*RV; and X, *Xba*I. The lower horizontal lines represent the pMK157 deletion derivatives, and the pMK157::γδ insertion mutants are represented by the upper vertical lines. The lower broken line corresponds to the 860-bp *Hin*dIII-*Eco*RV fragment used as a probe in hybridization experiments.

hemolysis, which are absent when the blood agar plates are impregnated with anti-SLO serum before inoculation, suggesting that pMK157 expresses *slo*. Cells pelleted from late-exponential-phase broth cultures of *E. coli* LE392(pMK157) were resuspended in PBS and lysed by sonication, and the levels of hemolytic activity in the lysates were assayed in quantitative microtitration assays. The sonic lysate contained ca. 16 U of hemolytic activity per mg of protein. This activity was completely inhibited by cholesterol or anti-SLO serum but was unaffected by trypan blue. These results clearly indicate that pMK157 expresses *slo* in *E. coli*. The SLO activity expressed by *E. coli* LE392(pMK157) remains predominantly cell associated. The levels of SLO observed leaking from colonies on blood agar plates are apparently too low to be detectable in cell-free supernatants of liquid cultures.

Mapping the SLO determinant of plasmid pMK157. The cleavage sites in pMK157 DNA for a number of restriction endonuclease are depicted in Fig. 1. To identify and map the cistrons involved in *slo* expression, transposon γδ insertion mutants and deletion mutants of pMK157 were isolated as follows. Mobilization of the pLG339-based hybrid plasmid pMK157 by the *E. coli* F-factor occurs by γδ-mediated cointegration of the two plasmids. Resolution of the cointegrate in the recipient cell results in the formation of pMK157::γδ insertion derivatives (12, 31). Plasmid pMK157 was transformed into F^+ *E. coli* LC707, and equal volumes of exponential-phase cultures of *E. coli* LC707 (F, pMK157) and *E. coli* ED2196 (Nal^r) were mated at 37°C for 45 min, after which Km^r Nal^r transconjugants were selected. Partially purified plasmid DNA from individual transconjugants was transformed into *E. coli* LE392, selecting for the pMK157 Km^r determinant. The insertion sites of the transposon γδ in plasmids isolated from these transformants were mapped by using restriction endonucleases *Eco*RI, *Sal*I, and *Pvu*II (Fig. 1).

Surprisingly, only one γδ insertion mutation (pMK175) located within the central 3.5 kb of cloned streptococcal DNA sequences of pMK157 was obtained. The reason for this is not understood, although it may reflect a degree of regional specificity for transposon γδ insertion, which is known to be the case for a number of other transposons (20).

Specific deletion mutants within the cloned streptococcal DNA sequences of pMK157 were constructed in vitro by deleting particular restriction endonuclease fragments. Plasmid pMK157 DNA was partially digested with the restriction endonucleases *Eco*RV (to construct pMK197 and pMK198), *Hin*dIII (to construct pMK200 and pMK203), or *Nco*I (to construct pMK201). Partial digest products were separated by agarose gel electrophoresis, and DNA fragments of appropriate sizes were recovered from the agarose gels, ligated, and transformed into *E. coli* LE392, selecting for the vector Km^r determinant. Plasmids with the desired deletions were identified by screening the plasmids in the resulting Km^r transformants. Similarly, PMK199 and PMK202 were isolated after complete digestion of pMK157 DNA with the restriction endonucleases *Hpa*I and *Xba*I, respectively, ligation, and transformation of *E. coli* LE392.

The pMK157 mutants were tested for their ability to produce SLO and the polypeptides expressed by pMK157, and selected mutants were analyzed by using the *E. coli* minicell system (Fig. 1 and 2). Mutants designated slo^+ in Fig. 1 express levels of cholesterol or anti-SLO-inhibitable hemolytic activity similar to pMK157 in the *E. coli* host LE392. Only 3 of 20 of the slo^+ pMK157::γδ insertion mutations were found to be located within the cloned streptococcal DNA sequences. Two of these map within a 600-bp region at one end of these sequences, and the third is located at the opposite end, close to the streptococcal DNA-vector DNA junction. The properties of these mutants suggest that the complete *slo* structural gene lies within the cloned streptococcal DNA sequences of pMK157 and does not extend beyond the ends of these sequences to produce a fusion polypeptide with hemolytic activity. *E. coli* LE392 harboring the slo^+ deletion mutant pMK202 produced normal levels of SLO, as assayed in sonicated cell lysates but, unlike other SLO^+ mutants, produced reduced hemolysis on blood agar plates. In addition, *E. coli* LE392 harboring pMK202 did not exhibit the slow growth rate characteristic of cells harboring pMK157. The deletion suffered by pMK202 has probably abolished or decreased expression of a product which affects the *E. coli* cell envelope, causing slower growth and increased leakage of SLO.

All (17 of 17) of the slo^- pMK157::γδ insertions map within a 1.4-kb region, close to one end of the cloned streptococcal DNA sequences of pMK157. DNA sequences either within or immediately adjacent to this region are deleted in each of the four slo^- deletion derivatives of pMK157 (Fig. 1). Thus, at least 1.4 kb of DNA sequences is required for expression of SLO by pMK157. Two of the pMK157 deletion derivatives (pMK200 and pMK201) and one pMK157::γδ insertion mutant (pMK175) expressed SLO at levels which were too low to be quantified in 30-min microtitration assays. However, unlike slo^- mutants, hemolysis could be clearly detected under colonies of *E. coli*

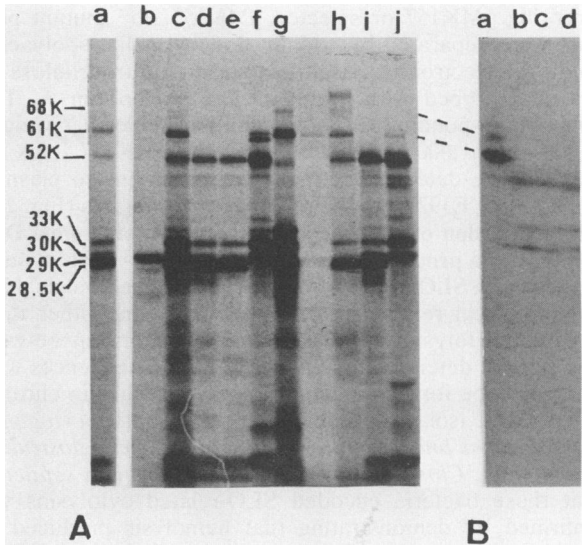

FIG. 2. (A) Autoradiographs of ^{35}S-labeled polypeptides expressed in *E. coli* minicells by pMK157 and its derivatives. Tracks b to j were deliberately overexposed to facilitate visualization of the faint 68,000-dalton SLO polypeptide band. A normal exposure of the pMK157 track is shown on the left. Tracks: a, pMK157, normal exposure; b, pLG339, vector; c, pMK157; d, pMK197; e, pMK198; f, pMK199; g, pMK200; h, pMK202; i, pMK158; j, pMK175. The two new polypeptides of ca. 62,000 and 60,000 daltons observed in track f are probably the product of fused cistrons and a degradation derivative of this product. (B) Immunoblot of LE392 cells harboring pMK157 and derivative plasmids. Tracks: a, pMK157; b, pMK197; c, pMK198; d, plasmid-free LE392. Other SLO^+ and $SLO^\pm$ mutants tested (pMK175 and pMK200) yield the same result as pMK157 (track a). The faint lower bands, visible in all tracks including the plasmid-free host, may reflect nonspecific binding of ^{125}I-protein A to *E. coli* polypeptides.

DS410 harboring pMK200, pMK201, or pMK175 after 2 days on blood agar plates, and this hemolysis was inhibited in areas of the blood agar which had been impregnated with anti-SLO serum before inoculation. In addition, prolonged incubation (>3 h) of erythrocytes with sonic lysates from *E. coli* LE392 cells harboring these mutant plasmids resulted in hemolysis which was inhibited by anti-SLO serum. These mutants are designated *slo*$^{\pm}$. Deletion mutant pMK200 retains only 1.9 kb of cloned streptococcal DNA sequences, which is consistent with the location of the *slo* gene indicated by insertion and deletion mutants of pMK157 and allows the *slo* gene to be localized as designated in Fig. 1.

The polypeptides expressed in *E. coli* minicells by pMK157 and selected derivatives are shown in Fig. 2. Minicells containing pMK157 express at least six polypeptides in addition to a vector-encoded polypeptide of 29,000 daltons. Two of these polypeptides, with molecular weights of 68,000 and 61,000, could not be detected in *E. coli* minicells harboring any of the *slo*$^{-}$ pMK157 mutants tested but were expressed in *E. coli* minicells harboring the *slo*$^{\pm}$ deletion mutant pMK200. Approximately 1.7 and 1.5 kb of DNA sequences are required to encode polypeptides of molecular sizes 68,000 and 61,000, respectively. Since pMK200 retains only 1.9 kb of cloned streptococcal DNA sequences and expresses both of these polypeptides, it is clear that they must be encoded by the same DNA sequences and are likely to represent two forms of the *slo* gene product. Further evidence which indicates that 68,000- and 61,000-dalton forms of SLO are produced in *E. coli* harboring the cloned *slo* determinant was provided by immunoblotting experiments. Total cell polypeptides of *E. coli* LE392 harboring pMK157 or selected pMK157 *slo*$^{-}$ mutant plasmids were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and probed with anti-SLO and ^{125}I-protein A. Two bands corresponding to polypeptides of molecular weights 68,000 and 61,000 were detected in the pMK157 track but could not be detected in tracks corresponding to plasmid-free *E. coli* LE392 or to the pMK157 *slo*$^{-}$ mutants (Fig. 2B).

Hybridization of *slo* gene sequences to chromosomal DNA from bacteria producing hemolysins that cross-react immunologically with SLO. To determine whether the biological and immunological relationship between SLO and other thiol-activated cytolysins reflects a close relationship between their genetic determinants, cloned *slo* DNA sequences were used to probe for homologous DNA sequences in chromosomal DNA isolated from *B. cereus*, *L. monocytogenes*, *Streptococcus pneumonia*, *Clostridium welchii*, *Clostridium histolyticum*, *Clostridium novyi*, and *Clostridium septicum*. That these bacteria encoded SLO-related cytolysins was confirmed by demonstrating that hemolysis produced by these bacteria grown under anaerobic conditions was inhibited by anti-SLO serum. Plasmid pMK157 DNA was digested with the restriction endonucleases *Hin*dIII and *Eco*RV, and the digest products were separated by agarose gel electrophoresis. A 860-bp *Hin*dIII-*Eco*RV fragment consisting of DNA sequences located entirely within the *slo* gene (Fig. 1) was purified from the gel. This fragment was labeled with [^{32}P]dCTP by nick translation and used as the probe in hybridization with Southern blots of *Eco*RI-digested chromosomal DNA isolated from the strains listed above. Under hybridization conditions which allow up to 20% bp mismatch between probe and target DNA sequences, no homology could be detected between the *slo* DNA sequences and chromosomal DNA from bacteria producing SLO-related hemolysins, whereas in the control tracks, homology between the probe and *S. pyogenes* Richards chromosomal DNA was readily detectable.

DISCUSSION

The *E. coli* bacteriophage λ vector system used to clone *slo* has recently been used to clone the *Staphylococcus aureus* α-toxin (18) and the M-proteins from a number of *S. pyogenes* strains (M. Kehoe, T. Poirier, E. H. Beachey, and K. N. Timmis, unpublished data). In each case, the cloned determinants were expressed at easily detectable levels in the gram-negative host, and the detection of their products was simplified by their extracellular location in phage plaques. Thus, this system should prove of general use for the genetic characterization of virulence determinants of gram-positive bacteria.

Recombinant λ phage expressing *slo* were detected at a frequency of ca. 1 in 3,000 in the *S. pyogenes* gene bank, which is lower than the frequency expected (1 in 900) from statistical considerations. It was noted that the *slo*$^{+}$ hybrid phage λR3 grows slowly, producing low phage titers in plate lysates. Pooling of the recombinant phage plaques selected on *E. coli* WL95 would reduce the ratio of such slow-growing phage with respect to other phage in the pooled gene bank, and this may account for the reduced frequency at which *slo*$^{+}$ hybrid phage were detected. The slow growth rate of λR3 may be due to the expression of a product which is antagonistic to *E. coli* cells. This would explain the instability of *E. coli* cells harboring high-copy-number hybrid plasmids containing the cloned streptococcal DNA sequences of λR3, whereas the low-copy-number *slo*$^{+}$ hybrid plasmid pMK157 was stably maintained in *E. coli*, although cells harboring this plasmid do grow slowly.

To characterize the SLO determinant cloned in the hybrid plasmid pMK157, transposon γδ insertion mutants and deletion mutants of pMK157 were isolated and analyzed. This analysis allowed the SLO determinant to be mapped to within a 1.8 kb region of the cloned streptococcal DNA sequences. These sequences encode a 68,000-dalton polypeptide which accounts for ca. 1.7 kb of their coding capacity. Thus, it is likely that a single cistron is sufficient to express functionally active SLO in *E. coli*. Mutations in DNA sequences adjacent to this cistron reduced but did not abolish SLO activity. The simplest explanation for these mutants is that deletions pMK200 and pMK201 have removed a promoter involved in expression of the *slo* cistron of pMK157 and that the γδ insertion mutant pMK175 exhibits a polar effect on expression from this promoter. However, no consistent differences were noted on autoradiographs between the intensities of *slo* polypeptides labeled in minicells harboring *slo*$^{+}$ plasmids and in minicells harboring *slo*$^{\pm}$ plasmids. The difference in the level of *slo* activity expressed by *slo*$^{+}$ and *slo*$^{\pm}$ plasmids may be less than 10-fold, and care must be taken in interpreting levels of expression from the intensities of polypeptide bands which have been labeled in minicells. A second possibility that the mutations suffered by the *slo*$^{\pm}$ plasmids may affect pMK157-encoded cistrons involved in activating the *slo* gene product cannot at present be ruled out.

Two forms of the *slo* gene product, with molecular sizes of 68,000 and 61,000, were detected both in *E. coli* minicells and by immunoblotting of *E. coli* whole cells harboring *slo*$^{+}$ or *slo*$^{\pm}$ hybrid plasmids. The 61,000-dalton polypeptide band was consistently more intense than the 68,000-dalton polypeptide band, which often required prolonged exposure of the gels to be detected. This suggests that the 68,000-dalton polypeptide is rapidly processed or degraded to the 61,000-

dalton form in *E. coli*. It is not presently known whether both forms of the *slo* gene product detected possess cytolytic activity. Interestingly, recent studies on the polypeptides associated with hemolytic activity recovered from nondenaturing polyacrylamide gels of *slo* partially purified from *S. pyogenes* culture supernatants identified polypeptides of 69,000, 60,000, and 47,000 daltons as being associated with the hemolytic fractions (M. Lutz, H. Hasser, and F. Fehrenbach, personal communication). The 69,000- and 60,000-dalton polypeptides identified by these workers appear to be remarkably similar in size to the 68,000- and 61,000-dalton SLO polypeptides detected in *E. coli*. Thus, SLO may undergo a similar proteolytic cleavage in *E. coli* and *S. pyogenes*. A second explanation would be the existence of alternative translational initiation or termination sites within the *slo* gene, resulting in the expression of different-sized SLO antigens. There is evidence for the existence of alternative translational initiation sites in the case of a number of other bacterial genes (4, 28).

The genetic determinants of the biologically and immunologically related thiol-activated cytotoxins might be expected to share a considerable degree of DNA sequence homology. No homology was detected between the cloned *slo* DNA sequences and DNA isolated from bacteria expressing cerolysin, pneumolysin, listeriolysin, perfringolysin, histolyticolysin, or edematolysin. These hybridization experiments were performed at a stringency of ca. 80%. Similar experiments using a cloned cerolysin determinant as the probe failed to detect DNA sequence homology between the cerolysin determinant and the determinants of SLO or listeriolysin (W. Goebel, personal communication). Thus, if homology exists between the DNA sequences encoding SLO (or cerolysin) and the determinants of other thiol-activated cytolysins, it is less than that which can be easily detected by Southern blot hybridization experiments. A comparison of the nucleotide sequences of a number of thiol-activated cytotoxin determinants will be required to define relationships between these toxins more precisely.

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