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Insertional Inactivation of Streptolysin S Expression in *Streptococcus pyogenes*

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The inactivation of a genetic determinant critical for streptolysin S production was accomplished by transfer and insertion of the transposon Tn*916* into the DNA of a group A streptococcal strain. The group D strain CG110 was able to efficiently transfer Tn*916* into the group A strain CS91 when donor and recipient cells were concentrated and incubated together on membrane filters. Among tetracycline-resistant transconjugants, nonhemolytic mutants that no longer produced streptolysin S and retained the capacity to produce streptolysin O were discovered. Hemolytic revertants from these mutants regained tetracycline sensitivity; other revertants still retained a tetracycline resistance phenotype. Hybridization studies employing Tn*916* DNA located Tn*916* sequences in *Eco*RI and *Hin*dIII fragments of DNA from mutants devoid of streptolysin S; one carried a single copy of Tn*916*, and the other two carried multiple copies of the transposon.

The 10 megadalton tetracycline resistance (Tet^r) transposon Tn*916* is chromosomally located in *Streptococcus faecalis* DS16 (7, 8). This group D streptococcal transposon is self-mobilizing and can insert into several chromosomal and plasmid locations in group D streptococcal strains; insertions into certain sites of plasmid pAD1 affect the expression of the bacteriocin-hemolysin gene of that plasmid (7). The variant group D strain CG110 conjugatively transfers Tn*916* to other group D recipients with a 10- to 100-fold increased frequency (8). The availability of selectable markers in group A streptococci, which has been limited primarily to plasmid- and chromosome-determined antibiotic resistance, has placed severe constraints on genetic studies in this organism. Transpositional mutagenesis has proven to be invaluable in other bacterial species with rudimentary genetic systems. We report that Tn*916* can be transferred by membrane filter matings from *S. faecalis* CG110 to the group A streptococcal strain CS91. This transposon is able to insert into several sites on the chromosome of this organism, whereupon it can inactivate genes essential for the expression of streptolysin S (SLS). Upon excision of the transposon, SLS is once again expressed, confirming the earlier report of Gawron-Burke and Clewell that excision of Tn*916* can be precise (8).

MATERIALS AND METHODS

Bacterial strains and media. The group A strain used as the transposon recipient, CS91 (M type 12, T type 12), carries an erythromycin and lincomycin resistance plasmid of unknown origin and produces both streptolysin O (SLO) and SLS. The donor *S. faecalis* CG110 is able to transfer Tn*916* at a high frequency in membrane filter mating experiments (8). A second group D streptococcal strain, OG1-RF1(pAM211) was used as the source of a Tn*916*-bearing plasmid; this plasmid pAM211 is a derivative of pAD1 with Tn*916* in the *Eco*RI F fragment (7, 8). This plasmid was used as the ^{32}P-labeled probe for Tn*916* sequences in hybridization experiments. Strains OG1-RF1(pAM211) and CG110 were kindly provided by D. Clewell, University of Michigan, Ann Arbor. Three standard strains known to lack SLS or both hemolysins were used as controls in streptolysin assays. These strains, obtained from L. Wannamaker, University of Minnesota, Minneapolis, have the following streptolysin phenotypes: GT80-220 and GT80-119 produce only SLO, and GT80-232 is unable to synthesize either hemolysin (13, 16).

Nonhemolytic mutants (Hem^-) of strain CS91 and hemolytic revertants (Hem^+) were T typed by the agglutination method of Griffith (9) and M typed by the capillary precipitin method (9, 20) to assure their identity to the group A parental strain CS91.

Streptococcal strains were grown in no. 1 broth (19) for membrane filter mating experiments. The mating mixtures deposited on membrane filters were placed on blood agar plates prepared from a base medium (THBA) composed of 3% Todd-Hewitt broth, 1% yeast extract, 1% neopeptone, and 1% agar (all products from Difco Laboratories) with 5% sheep erythrocytes and supplemented with hyaluronidase (Sigma Chemical Co.) at 6.8 μg/ml final concentration. The selective and differential medium used for the isolation of transconjugants and to screen for nonhemolytic transconjugants was prepared from the base medium as described above but without neopeptone. In addition, this medium contained 2.5% sheep erythrocytes and antibiotics, tetracycline, and erythromycin, each at a final concentration of 5 μg/ml. After 18 h of

incubation at 37°C, surface growth washed from membrane filters with 1 ml of no. 1 broth was added to 40 ml of selective medium and poured into petri plates (150-mm diameter).

Membrane filter mating. The conjugation experiments were performed as described elsewhere (7) with the following changes. The inocula applied to filters were from 6-h broth cultures; the media described above were used, and the plates of selective medium were incubated anaerobically (in GasPak jars) for 20 to 48 h at 37°C. Control filters, each containing the donor or the recipient strain only, were processed identically to filters inoculated with mating mixtures. Transfer frequency was determined by dividing the number of transconjugants by the viable count or recipient cells in the mating mixtures at the end of mating (7).

Detection of SLS and SLO. The hemolytic reactions of all group A streptococcal strains were periodically evaluated on tryptose blood agar base (Difco) containing 4% sheep erythrocytes. Strains were tested for extracellular SLS after overnight growth in brain heart infusion broth (Difco) supplemented with horse serum (SBHI). The culture supernatants were tested for hemolytic activity as described by others (2, 16). Cell-bound SLS activity was detected in a similar manner, except bacteria were grown in Todd-Hewitt broth supplemented with 1% neopeptone, 1% yeast extract, and 1 μg of thiamine per ml. Cells were washed twice with saline and tumbled in horse serum with glass beads at 25°C to extract bound hemolysin (14).

Assays for SLO required that cells be grown overnight in SBHI and suspended in Todd-Hewitt broth supplemented with 1% yeast extract, neopeptone, and glucose and with 1 μg of thiamine per ml, 0.02 M Na_2HPO_4, and 0.02 M KH_2PO_4. After 9 h of incubation at 37°C, these culture supernatants were assayed for SLO as described by Alouf and Raynaud (1) and modified by Skjold et al. (16).

Assessment of reversion frequency. To determine the frequency of beta-hemolytic revertants among Hem^- cells, cultures were first streaked for isolation on THBA containing erythromycin and tetracycline, each at 5 μg/ml. After overnight incubation at 37°C, a single, isolated Hem^- colony was transferred to Todd-Hewitt broth containing 2% yeast extract and tetracycline (5 μg/ml). After growth, dilutions of this culture were plated on THBA plates. After 24 h of incubation at 37°C, the total number of Hem^- colonies and Hem^+ revertants were counted, and the reversion frequency was calculated.

Preparation of DNA and DNA-DNA hybridization. Total group A streptococcal DNA was isolated from cells after a combination of treatments which sensitized them to lysozyme. L-Cysteine (V. Burdett, personal communication) and DL-threonine (3) were added to early logarithm phase cultures to a final concentration of 6 and 20 mM, respectively. After incubation for 1 h at 37°C, glycine was added to a concentration of 5% (15); after 45 min of incubation, these cells were washed three times with 0.2 M sodium acetate (pH 6) (V. Burdett, personal communication). DNA was released from sensitized cells after the incubation with lysozyme (2 mg/ml) for 1 h at 37°C and the subsequent addition of Sarkosyl to a concentration of 1%. The lysate was digested with RNase and pronase in 30 and 60 min, respectively, before concentration by ethanol precipitation at −20°C. Precipitated DNA was further purified by phenol extraction, ether extraction, and ethanol precipitation (4). Plasmid pAM211 was isolated from strain OG1-RF1(pAM211) and purified by ethidium bromide-cesium chloride buoyant density centrifugation (6). Samples (1 μg) of high-molecular-weight chromosomal DNA isolated from CS91, Hem^- mutants, and Hem^+ revertants were digested with restriction endonucleases *Eco*RI and *Hin*dIII by the conditions described by the manufacturer. Samples (10 ng) of plasmid pAM211 were similarly digested. Digests were electrophoresed for 15 h at 50 V in Tris-acetate buffer (0.04 M Tris-acetate, 0.02 M EDTA) on a 0.7% agarose horizontal submerged gel. DNA was transferred from the agarose to nitrocellulose sheets by the method of Southern (17). A ^{32}P-labeled probe was prepared by nick translation of pAM211 DNA, and hybridization was carried out at 68°C for 20 to 24 h; autoradiography followed (4).

RESULTS

Reports by Franke and Clewell (7) of the self-transmissible nature of Tn*916* instigated our efforts to introduce this transposon into genes crucial for the virulence of group A streptococci. First, various strains were tested as recipients, and strain CS91 proved to be superior. Employing strain CS91 with selection for erythromycin and tetracycline resistance (Ery^r, Tet^r), the conjugation frequency ranged from 10^{-7} to 10^{-6}. No spontaneous antibiotic-resistant mutants arose from either the recipient or the *S. faecalis* donor. When conjugation mixtures were plated on THBA media, the frequency of Hem^- transconjugants among the Ery^r Tet^r colonies varied from 10^{-4} to 10^{-3}.

Nonhemolytic colonies were purified by serial passage of isolated colonies on THBA plates. The Hem^- phenotype was confirmed by streak and stab inoculation of standard sheep blood agar, and the antibiotic resistance phenotype was also verified. Three independent Hem^- transconjugants from different mating mixtures were further characterized. These three strains, CS91-23, CS91-71, and CS91-81, were confirmed to have the M type 12 and T type 12 antigens of the original parental strain CS91.

Reversion of Hem^- mutants. Reversion experiments were performed on all three Hem^- mutants in duplicate on four occasions. The frequency of beta-hemolytic colonies in cultures of Hem^- mutants was quite variable, ranging from 2.7×10^{-3} to 8×10^{-7} (Table 1). Beta-hemolytic revertants from experiment 4 were tested for tetracycline sensitivity. Both Tet^r and Tet^s hemolytic colonies were discovered; ca. 50% were Tet^s. To eliminate the possibility that reversion to Tet^s occurred independently of reversion to the hemolytic state, 350 randomly selected Hem^- colonies were screened for tetracycline sensitivity; all were found to be resistant. Two Hem^+ revertants from each Hem^-

TABLE 1. Reversion of nonhemolytic transconjugant strains

Nonhemolytic Tet^r strains	Frequency of hemolytic colonies per CFU (expt)[a]			
	1	2	3	4
CS91-23	5.7×10^{-5}	1.7×10^{-4}	8.0×10^{-7}	1.1×10^{-5}
CS91-71	2.1×10^{-7}	9.6×10^{-4}	2.1×10^{-4}	3.1×10^{-5}
CS91-81	2.7×10^{-3}	4.6×10^{-6}	1.5×10^{-4}	6.4×10^{-6}

[a] Each experiment represents two complete sets of experimental data for each strain: two overnight broth cultures each serially diluted and plated on THBA. Frequency equals the number of hemolytic colonies observed divided by the number of total CFU analyzed.

mutant, one Tet^r and one Tet^s, were purified and confirmed to retain the M type 12 and T type 12 phenotype.

Characterization of the nonhemolytic phenotype. All three mutant strains were nonhemolytic when grown on standard sheep blood agar, whether plates were incubated anaerobically or aerobically. Because group A streptococci produce two hemolysins, SLS and SLO, it was important to identify which had been inactivated by the transposition. Supernatants of stationary phase cultures of strain CS91 and its Hem$^-$ derivatives were assayed for SLO with and without known inhibitors of both lysins. Undiluted and 1:2 dilutions of supernatants reduced with β-mercaptoethanol hemolyzed sheep erythrocytes in the presence of trypan blue (Allied Chemicals), a known inhibitor of SLS (2, 16) (Table 2). The sensitivity of this activity to cholesterol indicates that the lysis measured in these assays was, indeed, due to SLO not SLS or an undefined, nonspecific agent. From these experiments, it was concluded that the mutations had not altered the production of SLO.

To detect SLS, cultures were grown in media containing horse serum (2, 14). None of the nonhemolytic mutant strains CS91-23, CS91-71, and CS91-81 or control strains, known to be SLS defective (13, 16), produced either extracellular or cell-bound SLS. In contrast, strain CS91 and six hemolytic revertants produced both extracellular and bound SLS. This activity was, as expected, inhibited by trypan blue. The number of CFU per milliliter was equivalent for all cultures used in these assays.

The production of DNase by the parent and mutant strains was evaluated on methyl green DNA plates (12) to ensure that the synthesis of other extracellular products was unaltered. This was to rule out the possibility that mutations in some other gene, unrelated specifically to SLS, resulted in the Hem$^-$ phenotype. No difference could be detected in the extracellular DNase produced by these strains (unpublished data). Thus, these insertional mutations had not altered these cells in a more general manner.

Location of Tn*916* sequences in DNA from SLS$^-$ strains. Reversion analysis suggested that Tn*916* can insert into DNA sequences required for the synthesis of SLS. To confirm the nature of these insertions and to develop a rudimentary physical map of genes encoding SLS, hybridization studies were performed to localize Tn*916* in DNA extracted from independent Hem$^-$ mutants and respective Tet^s and Tet^r Hem$^+$ revertants. The *S. faecalis* plasmid pAM211 (7) labeled with ^{32}P by nick translation was employed as a probe for hybridization to *Eco*RI- and *Hin*dIII-digested DNA. Tn*916* lacks an *Eco*RI site and has only one *Hin*dIII site (7, 8); therefore, one *Eco*RI and two *Hin*dIII Tn*916*-chromosomal junction fragments should be detected for each copy of the transposon. Its insertion between *Eco*RI sites increases any fragment by 15 kilobases; therefore, DNA fragments containing Tn*916* were large and poorly discriminated by these electrophoresis conditions. However, SLS$^-$ Tet^r mutant CS91-23 can be shown to harbor one copy of Tn*916* with this enzyme.

TABLE 2. SLO activity of wild-type and mutant streptococci

Assay mixture[a]	Reciprocal of highest dilution exhibiting lytic activity in bacterial strains:			
	CS91	CS91-81	CS91-23	CS91-71
Supernatant only	4	2	2	4
Supernatant and 5.5 μg of trypan blue	2	2	2	4
Supernatant and 25 μg of cholesterol	<1[b]	<1	<1	<1
Supernatant with trypan blue and cholesterol	<1	<1	<1	<1

[a] Assay mixtures contained 0.5 ml of culture supernatant or their dilutions in phosphate buffer with β-mercaptoethanol in a total volume of 1.5 ml of phosphate buffer with or without cholesterol or trypan blue or both. The reaction was started by the addition of 0.5 ml of 5% washed sheep erythrocytes in phosphate buffer. The presence of hemolysis was determined after 1 h of incubation at 37°C and sedimentation of unlysed erythrocytes. The absence of a cell pellet was taken as an indication of complete hemolysis (1, 2).

[b] No hemolysis was detected in undiluted culture supernatants.

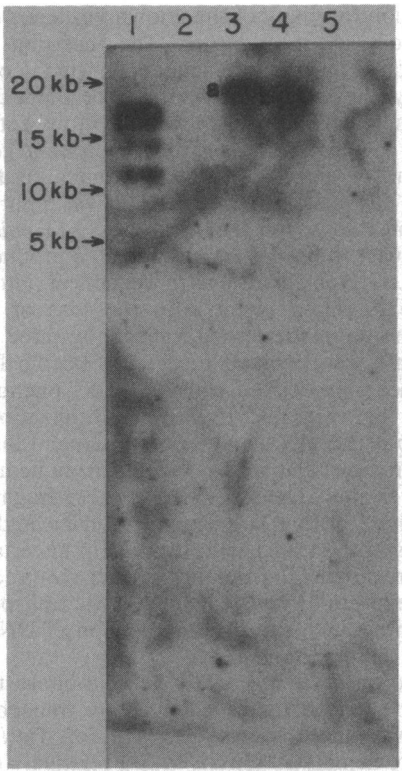

FIG. 1. Autoradiogram of *Eco*RI-digested DNA hybridized to ^{32}P-labeled pAM211. Lanes 1, pAM211; 2, CS91; 3, CS91-23; and 4 and 5, hemolytic revertants of CS91-23 which are Tet^r and Tet^s, respectively.

*Eco*RI digests (Fig. 1, lane 3) demonstrated a large fragment a. A hemolytic revertant of this strain retained Tn*916* but it had shifted to an *Eco*RI fragment of somewhat lower molecular weight, fragment b (Fig. 1, lane 4). Both fragments were greater than 15 kilobases in size. A Tet^s SLS$^+$ revertant had lost all copies of the transposon (Fig. 1, lane 5).

Tn*916* DNA digested with *Hin*dIII more clearly defines the location of Tn*916* because this enzyme cuts the transposon in one site, yielding two junction fragments for each copy (8). DNAs from CS91-23 and two other nonhemolytic mutants and their respective hemolytic revertants were also digested with *Hin*dIII and hybridized to the Tn*916* probe. As expected, strain CS91-23 carried one copy of Tn*916* yielding two junction fragments, a and b; their sum was greater than 15 kilobases (Fig. 2, lane 3). The Tet^r SLS$^+$ revertant of this strain produced junction fragments c and d with altered molecular weights, confirming that reversion has involved movement of the transposon to a new locus.

Hybridization of the probe to *Hin*dIII digests of mutant CS91-71 DNA revealed seven labeled fragments, b and d partially overlap (Fig. 2, lane 6). An expected eighth fragment was not observed; it was either masked by another fragment or simply did not exist. At least two of the three fragments, a, b, and c, identify Tn*916* sequences inserted within hemolytic determinants, since all three are lacking in DNA isolated from a Tet^r hemolytic revertant (Fig. 2, lane 7). The d fragment (lane 7) is apparent in both mutant and revertant strains. Multiple fragments were reproducibly detected in both *Hin*dIII and *Eco*RI digests and do not represent incomplete digestion products. Failure to detect eight fragments and the loss of three fragments rather than two by this revertant could be explained if two copies of the transposon were integrated into hemolytic sequences in tandem, the result of a duplication event, or one copy integrated within the other by a transposition event. Alternatively, two copies of Tn*916* could have been independently segregated and lost by this revertant. The remaining four fragments in the revertant DNA are junction fragments of Tn*916* copies located at two other chromosomal sites. Again, an SLS$^+$ Tet^s revertant of CS91-71 lacked all copies of the transposon (Fig. 2, lane 8).

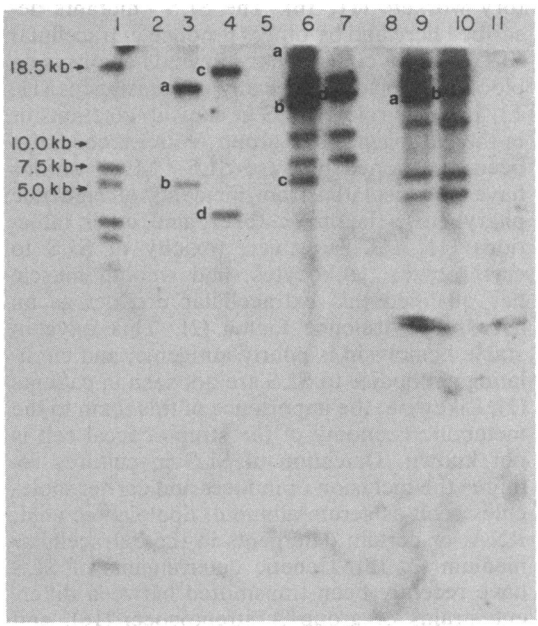

FIG. 2. Autoradiogram of *Hin*dIII-digested DNA from the parental strain CS91, three SLS$^-$ strains, and six SLS$^+$ revertant strains. Lanes 1, pAM211; 2, CS91; 3, 6, and 9, nonhemolytic mutants CS91-23, CS91-71, and CS91-81, respectively; 4 and 5, hemolytic revertants of CS91-23 which are Tet^r and Tet^s, respectively; 7 and 8, hemolytic revertants of CS91-71 which are Tet^r and Tet^s, respectively; 10 and 11, hemolytic revertants of strain CS91-81 which are Tet^r and Tet^s, respectively.

DNA from a third mutant, CS91-81, appears to contain three copies of Tn*916*, although only one junction fragment, a, affecting hemolysis could be identified with certainty (Fig. 2, lane 9). The undetected junction fragment could be masked by another fragment. The Tet^s revertant has lost all copies of the transposon (Fig. 2, lane 11).

Conjugal transfer of Tn*916* into *Streptococcus pyogenes* can result in its insertion into a gene or genes required for SLS synthesis. The three independent nonhemolytic mutants analyzed here readily revert to the hemolytic state when the crucial copy of the transposon is either transposed to a new locus or is excised and lost from the cell. As expected, Tn*916* has the potential to precisely excise with a return of the defective gene to the original state (8).

DISCUSSION

Naturally occurring SLS$^-$ strains of *S. pyogenes* are seldom seen, therefore, SLS is generally considered a conserved trait. The incidence of SLS$^-$ strains in clinical specimens is unknown because SLS is primarily responsible for beta-hemolysis on blood agar plates, and nonhemolytic colonies are ignored by clinical laboratory workers (11, 16). The SLS$^-$ mutants described here and by others produce extracellular SLO in broth cultures but show no hemolysis on blood agar plates under any circumstances (11, 13, 16). The role of SLS in clinical infections or in the pathogenesis of group A streptococci has been questioned because SLS$^-$ SLO$^+$ strains have been isolated from incidents of epidemic pharyngitis, rheumatic fever, and other infections (11, 16). However, toxicity of SLS to erythrocytes, leukocytes, and smooth muscle has qualified this extracellular product as an accessory virulence factor (2). This oxygen-stable hemolysin is poorly antigenic, and circulating antibodies to SLS are not seen in patients (2). Likewise, the importance of this toxin to the metabolic economy of the streptococcal cell is not known. Detection of SLS in cultures requires the inclusion of inducer and carrier molecules such as serum albumin, lipoteichoic acid, RNA, or certain detergents in the extracellular medium (2, 18). Genetic determinants of SLS have recently been transmitted between different strains of group A streptococci (16), and SLS$^-$ mutants have been produced by nitrosoguanidine mutagenesis (13), but the number of genetic loci and their location relative to one another or to other markers is unknown.

Transposon mutagenesis has proven to be a powerful tool for the investigation of genetic systems in which the genes in question are not selectable or when an identifiable phenotype on an agar medium is unavailable. Such is the situation for many of the known virulence traits of group A streptococci. The insertion of a selectable marker into sequences which control the synthesis of the M antigen or the various toxins of this organism would allow detailed study of their importance to virulence and genetic control and facilitate the cloning of these genes for in vitro studies. Here, we report the use of transposon Tn*916* from *S. faecalis* to inactivate the genes required for the production of SLS. Transfer of Tn*916* sequences into the group A strain resulted in the loss of SLS expression in some transconjugants; three such variants were studied in detail. Specific DNA fragments associated with the SLS$^-$ phenotype could be identified by their hybridization to Tn*916* probe DNA and their subsequent loss or shift in molecular weight in DNA from hemolytic revertants. The large size of these fragments and the fact that two mutants carried multiple copies of the transposon resulted in uncertainty with regard to the number of loci required for SLS expression or even whether the transposon had integrated into the same region of DNA in these three mutants.

Two observations are of general interest and may be unique to this conjugative transposon. The seemingly precise excision of Tn*916* to restore hemolytic activity was a relatively common event. Moreover, reversion produced cells either completely devoid of Tn*916* sequences or cells in which the transposon had moved to a new chromosomal location with complete loss of the original copy. This suggests that transposition may involve excision, circularization, and reinsertion of Tn*916* in a manner analogous to the integration of phage λ.

The ^{32}P-labeled probe pAM211 used in these studies encodes a group D streptococcal hemolysin-bacteriocin as well as the Tn*916* sequences (5, 8). It is interesting to note that this DNA failed to hybridize to strain CS91 DNA, indicating that the inactivated hemolysin DNA sequences of this plasmid have limited homology with those encoding the group A streptococcal hemolysins.

Employing various endonucleases, it should be possible to clone hemolysin sequences or any gene adjacent to Tn*916* sequences into *E. coli* by selecting tetracycline resistance clones. Those clones which retain the vector but have lost the transposon will become Tet^s and concurrently return the gene in question to the wild-type state, if required sequences are contained in the cloned fragment. The large size of Tn*916* probably limits this approach to the use of λ or cosmid vectors. A more suitable transposon may be Tn*917*, also described by Clewell and collaborators (5, 6), since it is much smaller in size and can also be introduced into various species of

streptococci. The above approach is feasible only if antibiotic resistance and transposon excision proves to function in *E. coli*.

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