Transduction of the Genetic Determinant for Streptolysin S in Group A Streptococci

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The genetic determinant for streptolysin S production (SLS+) was successfully transduced to two naturally occurring nonhemolytic strains of group A streptococci (Streptococcus pyogenes), an M-type 18 strain associated with an outbreak of rheumatic fever and an M-negative variant of a type 49 strain isolated from a skin lesion. Attempts to transduce this determinant to a nonhemolytic M-type 68 strain and a nonhemolytic T-type 12 strain were not successful. Transduction was accomplished with a double temperature-sensitive mutant bacteriophage. Cellular antigen characters and the phage sensitivity of the transductants remained unaltered. The donor strain also transduced streptomycin resistance well when the nonhemolytic type 49 strain was used as a recipient. There was no evidence of cotransduction of the determinants for streptolysin S and streptomycin resistance.

Beta-hemolysis has long been a major marker in the initial recognition of pathogenic streptococci in mixed bacterial cultures. The occurrence of nonhemolytic variants of group A streptococci (Streptococcus pyogenes) has been reported (see review by Chapman [2]) and, though they are apparently rare, may add considerably to the difficulties of identification and isolation of group A streptococci from clinical material.

Production of streptolysin S is primarily responsible for the zone of beta-hemolysis surrounding colonies of group A streptococci grown on blood agar plates (9, 15). Knowledge concerning the transfer or loss of the genetic determinant for streptolysin S in vitro might ultimately contribute to our understanding of the genetic control in nature of this streptococcal product which plays such a key role in diagnostic bacteriology.

Little information is available about the genetic control of hemolysin production in streptococci. Nonhemolytic mutants of group A streptococci have been produced by exposure to penicillin and to nitrosoguanidine (15). The locations of the genetic determinants for the hemolysins of group A streptococci have not been mapped. In group D streptococci, the genetic information for hemolysis synthesis has been found to reside on a plasmid and has been transferred between group D strains by conjugation (14, 23). Since transduction of several different characters between strains of group A streptococci has been successful (24), we have attempted to transfer the genetic determinant for streptolysin S production (SLS+) by this mechanism from a hemolytic (SLS+) donor to four nonhemolytic (SLS−) group A strains in our collection.

MATERIALS AND METHODS

Hemolytic (SLS+) donor strain. Strain GT-9278 (Str+, SLS+), a strain which exhibited typical beta-hemolysis when grown aerobically or anaerobically on sheep blood agar and produced streptolysin S in serum broth medium, was used as the donor strain in these transduction experiments. It had previously been selected as a streptomycin-resistant (Str+) mutant of an M-negative variant of an M-type 49, phage subtype III strain (19). This strain was SLS+ and produced streptolysin O (was SLO+).

Nonhemolytic (SLS−) recipient strains. All four recipient strains (80-119, 80-220, 80-232, and 81-035) investigated as possible recipient strains were nonhemolytic when grown on sheep blood agar, either aerobically or anaerobically, and failed to produce streptolysin S when grown in serum broth medium. Three strains (80-119, 80-220, and 81-035) produced streptolysin O in broth culture, but this was not discernible on anaerobic blood agar cultures; this observation is unexplained but is in keeping with reports of earlier workers (9, 15).

Strain 80-119 (Colindale 69/3903), M-type 18, was strongly mucoid on blood agar. It was isolated from a throat culture during an outbreak of pharyngitis on a United States Air Force base (10). This outbreak was associated with a number of cases of rheumatic fever. This strain was SLS− and SLO−.

Strain 80-220 (Colindale 79/5107), M-type 49, was

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isolated in the United Kingdom from a skin sore. A glossy, M-negative variant was used in the experiments reported here because it had proved superior to its M-positive parent as a recipient for transduction of Str^r. It was SLS^- and SLO^-.

Strain 80-232 (Colindale 79/1022), M-type 68, another very mucoid strain, was isolated in the United Kingdom from a severe infection of the eye. It was both SLS^- and SLO^-.

Strain 81-035 (Colindale 869), T-type 12, M-nontypable, type 66 by serum opacity reaction (SOR) typing (26), was isolated in the United Kingdom during World War II. This strain was SLS^- and SLO^+.

Media. Blood agar plates contained 3.3% tryptose blood agar base (Difco) plus 6% sheep erythrocytes. Todd-Hewitt broth was modified by adding 2% neo-peptone (Difco) to a 3% solution of commercial Todd-Hewitt base (Difco) (1).

Bacteriophage. A double temperature-sensitive mutant of T-d, strain, dubbed phage A25 was used in the transduction of Str^r and SLS^-^. This bacteriophage is designated A25tsl-2 (12).

Propagation of bacteriophage A25tsl-2. The transducing phage A25tsl-2 was propagated in no. 1 broth (25) at 29.5°C for 6 h. Phage titers were determined on agar plates prepared from N6 broth medium (21) and incubated at 29.5°C.

Transduction of Str^r. The methods used for transduction experiments of Str^r were similar to those previously described (25), with the exceptions that only 0.1 ml of the recipient culture was used, no UV irradiation was employed, and plates and transduction mixtures were incubated at 37.5°C.

Transduction of streptolysin S production (SLS^+). The recipient strains were incubated for 18 h at 37°C in modified Todd-Hewitt broth for the type 18 and 68 strains and in no. 1 broth for the type 49 and T-type 12 strains. A 0.1-ml sample of each of the recipient culture was mixed with 0.5 ml of the donor phage preparation and incubated for 30 min at 37°C. To test the possible inhibitory effects of streptococcal encapsulation, in some experiments 1 mg of hyaluronidase (Sigma) was added directly to the transduction mixture or was present for 30 min with the sample of the recipient culture for 10 min at 37°C.

For each nonhemolytic recipient strain, a range of samples of the transduction mixture was plated in blood agar to determine the amount that would produce optimal visibility of hemolytic transductant colonies, i.e., the least masking of hemolytic transductant colonies by the background of nonhemolytic (nontransduced) colonies. In general, the best results with the type 49 recipient were obtained by plating 0.06 ml of a 1:10 dilution, and the best results with the type 18 recipient were found by plating 0.03 to 0.15 ml of a 1:10 dilution. These amounts were added to 50 ml of 3.3% tryptose blood agar base (Difco) containing 4% sheep erythrocytes. The solution was mixed well, poured into a petri dish (150 by 15 mm), and incubated at 37°C for 24 h under 20% CO₂ tension. Transductant colonies were identified initially by evidence of clear hemolysis on the transductant plates, and the production of beta-hemolysis was confirmed by picking colonies to pure culture on sheep blood agar plates. The specificity of the transduced hemolytic marker was confirmed by inhibition tests for streptolysin S and for streptolysin O. To determine whether there were any spontaneous variants of the recipient strains, control plates containing equivalent samples of the recipient strain alone (i.e., without transducing phage) were prepared and incubated in the same manner. To determine whether any contaminating donor streptococci were in the transducing phage preparation, control plates containing equal volumes of the transducing phage preparation alone were prepared and incubated in the same manner. To rule out contamination, in some experiments transducing phage preparations were preincubated for 30 min with 100 µg of DNase per ml (final concentration; Sigma).

Confirmation of the nature of hemolysins by specific inhibitors. Production of streptolysin S was verified if the hemolytic activity in a culture supernatant of brain heart infusion containing 5% horse serum was inhibited by trypan blue (Allied) at a 5-µg/ml final concentration (8). The presence of streptolysin O was confirmed when the hemolytic activity was inhibited by the addition of cholesterol (Sigma; 17 µg/ml, final concentration) to a Todd-Hewitt culture supernatant (20).

Detection of SOR. The production of SOR was examined both in culture supernatants and in Lancefield hot-acid extracts (26). It is characteristic of M-negative variants of M-positive, SOR-positive strains (e.g., the type 49 strains used here as donor and recipient strains) not to yield SOR in culture supernatants or Lancefield hot-acid extracts. Although this was not done in this study, some evidence of opacity in M-negative variants may be found when whole cells or wall-membrane fractions are incubated in horse serum (26).

T and M typing. All strains of group A streptococci were classified according to M and T antigens by standard methods (27).

Phage typing. Phage typing of streptococci was performed as reported previously (19) with a few modifications. The modified phage typing plates, designated no. 749, were prepared as follows: an aqueous mixture of 4% proteose peptone no. 2 (Difco), 0.04 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer, and 0.03 M NaCl was adjusted to pH 7.1 with 5 N NaOH, and 1% agar (Difco) was added. After autoclaving, 0.006 M glucose, 0.0036 M CaCl₂, 0.001 M MgSO₄, 5% horse serum, and 68 µg of hyaluronidase per ml (final concentration; Sigma) were added, and the medium was then distributed in plastic petri dishes. Stock phages and cultures were stored at -70°C. Lawn inocula were diluted 1:5. Plates were incubated for 18 to 24 h at 37°C.

RESULTS

Transduction of streptomycin resistance (Str^r). To have a known effective transducing system for these nonhemolytic strains before attempting transduction of SLS^+", it was decided first to establish a transduction system with streptomycin resistance (Str^r) as a marker. Because it had proved so successful in other transduction studies (11, 13, 18, 21), a double temperature-sensitive phage (A25tsl-2) was used as a vector in these transduction experiments. Of the strains screened as possible donors, GT-9278 (Str^r, SLS^-) was the most successful, yielding >6,000 transductants of the Str^r marker at a transduc-
TABLE 1. Transduction of streptolysin S marker from donor strain GT-9278 (Str⁺, SLS⁺) to two of four nonhemolytic (SLS⁻) recipient strains by bacteriophage A25ts1-2

<table>
<thead>
<tr>
<th>Recipient strains (Str⁺, SLS⁻)</th>
<th>Serological classification</th>
<th>Total (avg/plate) no. of colony-forming transductants (SLS⁺)</th>
<th>Transduction frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>T</td>
<td>M</td>
<td>SOR</td>
</tr>
<tr>
<td>80–220(gl)⁺</td>
<td>14</td>
<td>49–b</td>
<td>Negc</td>
</tr>
<tr>
<td>80–119</td>
<td>18</td>
<td>18</td>
<td>Neg</td>
</tr>
<tr>
<td>80–232</td>
<td>1</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>81–035</td>
<td>12</td>
<td>NT⁺</td>
<td>66d</td>
</tr>
<tr>
<td>a-gl, Glossy variant.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b-M-negative, SOR-negative variant of M-type 49 SOR-positive strain.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-See the text.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-By specific inhibition of SOR.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e-NT, Not typable.</td>
<td></td>
<td></td>
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</table>

Transduction frequency of 4 × 10⁻⁶ (ratio of transductants to PFU). This donor strain was used in attempts to transduce the SLS⁺ marker.

**Transduction of streptolysin S production (SLS⁺).** With the Str⁺, SLS⁺ donor strain GT-9278 and the transducing phage A25ts1-2, transduction of SLS⁺ was attempted with nonhemolytic (SLS⁻) recipient strains of four different serotypes (Table 1). Hemolytic transductant colonies were identified in blood agar pour plates without streptomycin. The ability to produce streptolysin S was transduced to both the type 49 and the type 18 recipient strains, the former at a higher frequency. No transductants were obtained in a number of experiments in which the type 68 and T-type 12 strains were used as possible recipients. The exposure of the recipient bacterial culture to hyaluronidase did not affect the rate of SLS⁺ transduction. Nineteen transductants (16 of type 49 and 3 of type 18) were selected for further testing.

When grown under conditions favoring production of streptolysin S (in serum broth), hemolytic activity was produced by the donor strain and by subcultures of all of the 19 transductant colonies tested, but not by the original recipient strains. The resulting hemolytic activity of the donor strain and of the transductant colonies was inhibited by trypan blue (a specific inhibitor of streptolysin S) and not by cholesterol (a specific inhibitor of streptolysin O).

Tests were also made on culture supernatants of the donor and recipient strains and of the transductant colonies grown under conditions favoring streptolysin O production. The donor strain, all of the 19 transduced colonies tested, and 3 of the 4 recipients exhibited hemolytic activity in their supernatants that was inhibited by the addition of cholesterol. Since no transduction was found with the SLO⁻ recipient strain (type 68), it was not possible to determine whether the SLO⁺ determinant was cotransduced with SLS⁺.

**Controls for transduction of the SLS⁺ marker.** Control plates containing equal volumes of the transducing phage preparation alone showed no hemolytic or nonhemolytic streptococci. Except for the T-12 strain, spontaneous hemolytic variants were never seen in phage-free control preparations of the nonhemolytic strains, nor in many platings of these strains done in the laboratory for other purposes. The T-12 strain did show a rare hemolytic variant, but no evidence of transduction was obtained with this strain as a recipient. Preincubation of the transducing phage preparation with DNase did not alter the results obtained.

**Serological cellular markers in the SLS⁺ transductants.** A number of the SLS⁺ transductants were examined for specific antigen markers (T, M, and SOR proteins) and compared with the donor and recipient strains (Table 2). In all instances, the serological markers for cellular antigens of the transductants were the same as the original recipient strains. In the experiments in which the recipient strain had a different T antigen (T-18) from that of the donor strain (T-14), there was no evidence of cotransduction of the determinant for the T antigen.

**Phage typing and streptomycin sensitivity of the SLS⁺ transductants.** Phage typing of the donor and recipient type 49 strains clearly demonstrated that they were different strains (Table 2). Sixteen of the SLS⁺ transductants were phage typed and found to have the same lyric pattern and subtype as the SLS⁻ recipient. As expected, the type 18 recipient and its transductants were not lysed by any of the phages used in the phage typing system developed for type 49 strains.

The strains that were tested for phage type were also tested for sensitivity to streptomycin (Table 2). All of the strains except the GT-9278 (Str⁺) donor were sensitive to streptomycin, which indicates that the Str⁻ and SLS⁺ markers are not linked and adds to the evidence that this
is true transduction and not contamination with the donor strain.

**DISCUSSION**

The kind of hemolysis produced by streptococci is dependent on the culture conditions (5–7, 17), but even under the most favorable conditions non-beta-hemolytic group A streptococci may be missed in cultures from body sites generally yielding mixed flora (e.g., throat and skin lesion cultures).

Strains of group A streptococci producing very poor beta-hemolysis under aerobic conditions and beta-hemolysis under anaerobic conditions are encountered not infrequently. This provides a strong argument for incubating primary culture plates anaerobically (6) or at least for making a stab into the medium to provide semianaerobic conditions.

Pinney et al. (16) have observed that SOR-positive strains are poorly hemolytic when grown aerobically on horse blood agar, whereas SOR-negative strains give good hemolysis. The results of this study suggested that the production of diffusible SOR factor interferes with extraction of streptolysin S by serum and thereby leads to poor hemolysis on blood agar. The addition of RNase-digested RNA to the medium greatly improved the hemolysis produced by these strains.

Group A streptococci that are nonhemolytic under anaerobic as well as aerobic conditions (e.g., those used as recipients in these experiments) may pose special difficulties in identification by the clinical laboratory. These strains also fail to produce soluble hemolysin in serum broth and are therefore streptolysin S negative (SLS−). Although such strains are apparently rare in nature, their true prevalence may be difficult to ascertain, and they may be overlooked until clinical problems suggest a closer scrutiny of primary culture plates.

Several clinically significant episodes involving nonhemolytic group A streptococci have been described in the literature. Coburn and Pauli (3) described an epidemic in children and nurses on a pediatric ward. Both hemolytic and nonhemolytic variants of the same T-type 12 strain appeared to be involved in this epidemic. Subcultures of the nonhemolytic variants were only weakly hemolytic when grown under anaerobic conditions. Colebrook et al. (4) studied nonhemolytic T-type 12 streptococci (now known to be M-type 66, SOR type 66) recovered from septic grafts among patients on a plastic surgery ward. These strains were nonhemolytic, both aerobically and anaerobically, and were apparently serologically the same as hemolytic strains isolated on the same ward during the early months of this epidemic. Cases of acute nephritis associated with nonhemolytic type 12 infections have been identified in Japan (C. H. Rammelkamp, Jr., personal communication), but it is not certain whether these were tested for hemolysis under anaerobic conditions.

Of special interest is the well-documented outbreak of throat infections associated with rheumatic fever due to a highly mucoid, nonhemolytic group A, M-type 18 streptococcus (10). This strain (80-119) is one of the two strains in which transduction attempts were successful in this study. The atypical cultural appearance of this strain probably resulted in some delay in the institution of appropriate therapeutic and prophylactic measures.

Conversion of hemolytic streptococci to "nonhemolytic" variants and vice versa has been reported to occur on passage in experimental animals and on subculture in the laboratory (7, 22). However, the aerobically cultured nonhemolytic variants referred to in these reports grew as hemolytic strains when cultured anaerobically.

Our studies show that, at least experimentally, bacteriophages may play a role in the transfer of genetic information controlling production of streptolysin S. These transduction studies were
more difficult than those done earlier in this laboratory with antibiotic resistance or bacteriocin markers (18, 21, 25) because no selective agent is available to separate SLS + from SLS - streptococci by growth inhibition or bactericidal properties. The success of these experiments was dependent upon the ability to detect hemolytic transductants in the presence of large numbers of nonhemolytic colonies. This required careful adjustment of dilutions of the transductant mixtures in four plates.

In the SLS transduction experiments the transductants tested retained the streptomycin sensitivity marker. Thus, there was no evidence of linkage of the Str d determinant with that for SLS +.

There was also no evidence of cotransduction of the cellular antigen markers or of the marker(s) for phage lytic patterns. In the experiments in which a strain with heterologous M and T antigens (M-18, T-18) was used as a recipient, all of the transductants kept the cellular antigen markers of the recipient strain. Phage typing proved very useful in the transduction experiments in which the cellular antigens were the same for the donor and recipient strain. It clearly differentiated the type 49 SLS + donor strain (phage type III) from the type 49 SLS - transductant (phage type II) and provided additional evidence that the recipient strain (phage type II) and its transductants (phage type II) are related.

The apparent rarity of SLS - strains in nature suggests that the production of streptolysin S is a highly conserved property of group A streptococci. The reason for this is not clear. There is no known survival advantage to the organism conferred by the production of this hemolysin, and the occurrence of an outbreak of pharyngitis and rheumatic fever caused by an SLS - strain (10) suggests that streptolysin S may not be essential for communicability, virulence, or rheumatogenecity.

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LITERATURE CITED


TRANSDUCTION OF STREPTOLYSIN S 187


