# The Gene for Type A Streptococcal Exotoxin (Erythrogenic Toxin) is Located in Bacteriophage T12

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The infection of Streptococcus pyogenes T25₃ with the temperate bacteriophage T12 results in the conversion of the nontoxigenic strain to type A streptococcal exotoxin (erythrogenic toxin) production. Although previous research has established that integration of the bacteriophage genome into the host chromosome is not essential for exotoxin production, the location of the gene on the bacteriophage or bacterial chromosome had not been determined. In the present investigation, recombinant DNA techniques were used to determine whether the gene specifying type A streptococcal exotoxin (speA) production is located on the bacteriophage chromosome. Bacteriophage T12 was obtained from S. pyogenes T25₃(T12) by induction with mitomycin C, and after isolation of bacteriophage DNA by phenol-chloroform extraction, the DNA was digested with restriction enzymes and ligated with Escherichia coli plasmid pHP34 or the Streptococcus-E. coli shuttle vector pSA3. Transformation of E. coli HB101 with the recombinant molecules allowed selection of E. coli clones containing bacteriophage T12 genes. Immunological assays with specific antibody revealed the presence of type A streptococcal exotoxin in sonicates of E. coli transformants. Subcloning experiments localized the speA gene to a 1.7-kilobase segment of the bacteriophage T12 genome flanked by SalI and HindIII sites. Introduction of the pSA3 vector containing the speA gene into Streptococcus sanguis (Challis) resulted in transformants that secreted the type A exotoxin. Immunological analysis showed that the type A streptococcal exotoxin produced by E. coli and S. sanguis transformants was identical to the type A exotoxin produced by S. pyogenes T25₃(T12). Southern blot hybridizations with the cloned fragment confirmed its presence in the bacteriophage T12 genome and its absence in the T25₃ nonlysogen. Therefore, the gene for type A streptococcal exotoxin is located in the bacteriophage genome, and conversion of S. pyogenes T25₃ to toxigenicity occurs in a manner similar to the conversion of Corynebacterium diphtheriae to toxigenicity by bacteriophage beta.

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Type A streptococcal exotoxin is a biologically active product secreted by bacteriophage-infected group A streptococci. This exotoxin, often referred to as Dick toxin (1), streptococcal pyrogenic exotoxin (45), scarlet fever toxin (1), and erythrogenic toxin (44), is one of three immunologically distinct streptococcal exotoxins, referred to as types A, B, and C.

The streptococcal exotoxins are responsible for a wide range of biological effects, including pyrogenicity (7, 27, 45), erythematous skin reactions (10, 11, 19), which may be due to enhancement of delayed hypersensitivity (39), enhanced susceptibility to endotoxin shock (27, 40, 41, 44), alteration of the blood-brain barrier (40), cardiotoxicity (25, 41, 45), T-cell mitogenicity (2, 21, 22, 28, 34, 37), depression of clearance function of the reticuloendothelial system (8, 15), and alteration of antibody response to sheep erythrocytes (9, 14, 16, 17).

The possibility of bacteriophage involvement in type A streptococcal exotoxin production was introduced in 1927 when Frobisher and Brown reported that a filterable agent from streptococci associated with scarlet fever could induce exotoxin production when introduced into streptococci not previously associated with scarlet fever (12). Bingel (6) later confirmed these results, and in 1964, Zabriskie (46) reported that infection of nontoxigenic Streptococcus pyogenes T25₃ with bacteriophage T12 resulted in the formation of a lysogen which secreted type A streptococcal exotoxin (erythrogenic toxin).

The molecular mechanism to account for this phage-mediated toxigenic conversion has not been illustrated over the years, although Nida and Ferretti (35) and Johnson et al. (25) demonstrated that toxigenic conversion was not limited to the host T25₃-phage T12 system and that the phage used for lysogenic conversion determines the type of toxin produced by the lysogen.

Possible interactions between phage T12 and host S. pyogenes T25₃ that lead to type A exotoxin production have been suggested and include: (i) the bacteriophage contains the gene for type A exotoxin and a structural component of the phage such as the protein capsid is the toxin; (ii) the bacteriophage contains the gene, and a nonstructural gene product is actively synthesized during lysogeny; (iii) the bacterial chromosome contains the gene for type A exotoxin, and integration of the phage DNA into the host chromosome splits a gene coding for a nontoxic product, resulting in a toxic molecule which is the type A exotoxin; (iv) the bacterial chromosome-specified type A exotoxin is activated or derepressed by a phage product; and (v) a transposon contains the gene for type A exotoxin.

The first possibility was previously excluded owing to lack of immunological reactivity between the bacteriophage components and antibody to type A exotoxin and also lack of similarity between phage proteins and toxin proteins when compared by polyacrylamide gels (33). Similarly, the third possibility was dismissed when it was determined that integration of the phage genome into the host chromosome was not essential for expression of the toxin gene (33). In this communication, we report the results of studies

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with recombinant DNA techniques which show that the gene for type A streptococcal exotoxin (speA) is located on the genome of bacteriophage T12. A similar conclusion was reached by Johnson and Schlievert in a recent report (24).

## MATERIALS AND METHODS

**Bacteria, bacteriophages, and plasmid vector.** S. pyogenes T25₃(T12) (46) obtained from two separate sources was used as a source of bacteriophage T12. The bacterial lysogen designated T25₃(T12) was obtained from Dennis Watson from the University of Minnesota in 1972 and had been maintained lyophilized in this laboratory since that time. Cured T25₃ was obtained from Patrick Schlievert of the University of Minnesota and was designated T25₃c to indicate that it had been cured by Alba Colon-Whitt as previously reported (23). S. pyogenes NY-5 type 10 (43), which produces type A, B, and C toxins, served as a source of the type A streptococcal exotoxin. The plasmid vector pHP34 (38) was used for cloning the phage T12 DNA, and plasmid pBR322 was used for the subcloning experiments. Escherichia coli HB101 was the bacterial host which was transformed by the pHP34-phage T12 and the pBR322-phage T12 chimeric plasmids. The Streptococcus-E. coli shuttle vector pSA3 was constructed in this laboratory and was kindly provided by My Lien Dao. Streptococcus sanguis Challis 57 (5) served as the recipient for transformation of the pSA3-phage T12 chimeric plasmids.

**Type A streptococcal exotoxin and specific antibody.** Purified type A streptococcal exotoxin produced by S. pyogenes NY-5 type 10 was generously provided by Dieter Gerlach. Specific type A streptococcal exotoxin antibody was provided by Clifford Houston (20) and Dieter Gerlach (13).

**Media.** The medium used for bacteriophage propagation was a supplemented proteose peptone broth, designated P broth (35). Streptococcal strains were grown and maintained on a standard Todd-Hewitt medium (35) supplemented with 5% horse serum and 1.8 mM CaCl₂. E. coli HB101 was grown in LB broth (30). When necessary, ampicillin and tetracycline were added to the LB broth to a final concentration of 50 and 12.5 μg/ml, respectively. E. coli transformants were grown in LB broth containing 50 μg of ampicillin per ml and S. sanguis transformants were grown in BHI (brain heart infusion) broth containing 10 μg of erythromycin per ml when testing for type A streptococcal exotoxin. The medium used for exotoxin production by S. pyogenes NY-5 type 10 was BHI medium supplemented with 0.05% (wt/vol) glucose.

**Enzymes.** Restriction enzymes were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and Boehringer-Mannheim, Federal Republic of Germany. Restriction enzyme digests were carried out according to the manufacturer's specifications. T4 DNA ligase was purchased from Amersham (Takara Shuzo, Japan).

**Gel electrophoresis.** Agarose gel electrophoresis was conducted through 1% agarose (type II, medium EEO) gels prepared in a Tris-acetate buffer composed of 0.04 M Tris-acetate and 0.002 M EDTA and adjusted to pH 8.0 (32). The gels were stained with ethidium bromide (0.5 μg/ml), and the DNA bands were visualized by UV light. Lambda DNA digested with HindIII served as molecular weight standards.

**Preparation of plasmid DNA.** Large scale isolation of plasmid from E. coli was performed according to the cleared lysate method described by Behnke and Ferretti (4), with minor modifications. Briefly, 100 ml of cells were grown overnight, washed, and suspended in 10 ml of a 15% sucrose solution (50 mM EDTA, 50 mM Tris, 15% sucrose [pH 8.5])

and treated with 3 ml of lysozyme at 6 mg/ml in sucrose solution. After incubation at room temperature for 30 min, the cells were lysed with 0.75 ml of 20% sodium dodecyl sulfate, and 3.5 ml of 5 M NaCl was added to precipitate the protein and chromosomal DNA. The plasmid DNA was purified with two cycles of dye-buoyant density gradient centrifugation, dialyzed extensively against 10 mM Tris–1 mM EDTA, and stored at 4°C.

Streptococcal chromosomal DNA was isolated by the method of Behnke and Ferretti (4). Miniplasmid isolations from transformants were performed by the method of Holmes and Quigley (18).

**Preparation of bacteriophage T12 DNA.** S. pyogenes T25₃c(T12) was inoculated into 10 ml of P broth and incubated overnight at 30°C. The culture was diluted 1:100 in 1 liter of prewarmed P broth and incubated for 1.5 h at 37°C. Phage T12 was induced by the addition of mitomycin C to a final concentration of 0.2 μg/ml and incubation at 37°C for 3 h. After the induction period, the remaining bacteria and cellular debris was pelleted by centrifugation, and the supernatant was filtered through a membrane filter (0.45 μm pore size; Millipore Corp., Bedford, Mass.) to ensure removal of contaminating bacteria. Phage T12 was pelleted by ultracentrifugation at 200,000 × g for 1 h. The pellet was suspended in 10 mM Tris–1 mM EDTA (pH 7.5), and the protein coat was removed by three extractions with an equal volume of phenol-chloroform. The DNA solution was washed once with chloroform and precipitated overnight at −20°C with 2 volumes of 95% ethanol. The DNA was pelleted and suspended in 10 mM Tris–1 mM EDTA, pH 7.5.

**Construction of pHP34-phage T12 chimeric plasmids.** Bacteriophage T12 DNA and plasmid pHP34 were digested with EcoRI and SalI, mixed, and ligated overnight with 1 U of T4 DNA ligase at 14°C as described by Behnke and Gilmore (5). For subcloning of the type A exotoxin gene, 200 ng of the chimeric plasmid pA2 containing the 4.75-kilobase (kb) insert was digested with SalI, and HindIII and subsequently ligated to 200 ng of SalI and HindIII-digested pBR322. E. coli HB101 was transformed with the ligated DNA according to the procedure of Mandel and Higa (31).

To introduce the type A exotoxin gene into S. sanguis, the 1.7-kb fragment containing the type A exotoxin gene was isolated by the melted agarose method (26) and ligated to SalI and EcoRI-digested pSA3. Recombinant plasmids were transformed into S. sanguis Challis 57 by the method of LeBlanc and Hassel (29) with the modifications of Behnke (3).

**Sonification of transformants and testing for type A exotoxin production.** E. coli transformants were inoculated into 100 ml of LB broth containing 50 μg of ampicillin per ml and incubated overnight at 37°C. The cells were pelleted by centrifugation, and the supernatant was concentrated by precipitation with 4 volumes of cold 95% ethanol for 48 h at 4°C (25). After precipitation, the ethanol was decanted, and the precipitate was suspended into 0.5 ml of 10 mM Tris (pH 8.0). To test for cytoplasmic or periplasmic toxin, the E. coli cell pellet was suspended in 0.5 ml of 10 mM Tris, pH 8.0, and incubated with 0.25 mg of lysozyme per ml for 30 min at 37°C. Cells were disrupted with three 15-s bursts with a sonic machine (Sonicator model W-275R, Heat Systems-Ultrasonics, Inc.). The resulting cell lysates were centrifuged at 6,000 × g for 15 min at 4°C to pellet the cellular debris, and the supernatant was tested for type A exotoxin. The Ouchterlony double-diffusion method (36) was used for detection of type A streptococcal exotoxin in concentrated culture medium and cell lysates. Specific type A streptococcal exotoxin

antibody was placed in wells opposite wells containing the cell lysates or culture medium. The Ouchterlony plates were incubated for 4 h at 37°C and observed for lines of identity by indirect lighting.

*S. sanguis* transformants were inoculated into 100 ml of BHI broth containing 10 μg of erythromycin per ml and incubated overnight at 37°C. The cell-free culture medium was concentrated by ethanol precipitation, and the resulting precipitate was suspended in 0.5 ml of 10 mM Tris (pH 8.0). The concentrated culture medium was tested for type A streptococcal exotoxin by the Ouchterlony double-diffusion method described above.

**Biological activity of the cloned type A streptococcal exotoxin.** The cloned type A streptococcal exotoxin was tested for biological activity by the erythematous skin test as described by Houston and Ferretti (20). Briefly, two 2.5- to 3-kg New Zealand White rabbits were sensitized by footpad injection with 300 μg of purified type A streptococcal exotoxin suspended in Freund incomplete adjuvant 3 weeks before testing. The backs of skin test rabbits were shaved, and 50 μl of the type A exotoxin material from *S. sanguis* transformants containing the 1.7-kb phage DNA fragment was injected intradermally into the skin with a 1-ml tuberculin syringe. Type A exotoxin (50 μl from *S. pyogenes* NY-5 type 10), toxin from *S. pyogenes* T25₃(T12) (50 μl), and concentrated culture medium from *S. sanguis* containing pSA3 (50 μl) were injected individually and served as positive and
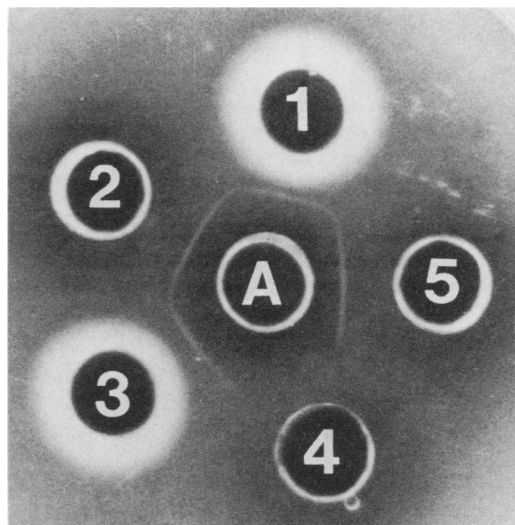


FIG. 2. Ouchterlony double-diffusion assay of sonicates from *E. coli* transformant pA2. Center well contains type A streptococcal exotoxin antibody. Wells: 1, Sonicates from *E. coli* transformant pA2; 2, type A exotoxin from *S. pyogenes* NY5-10; 3, sonicates from *E. coli* transformant p1179; 4, sonicate from nontransformed *E. coli* HB101; 5, type A exotoxin from *S. pyogenes* T25₃(T12).

negative controls, respectively. The rabbit skins were observed at 24 and 48 h for the erythematous skin response.

**Southern hybridization.** Southern transfer, nick translation, and in situ hybridization of plasmid containing the 1.7-kb phage T12 fragment to the nitrocellulose filters was performed by the method of Southern (42) as described by Maniatis et al. (32). Briefly, phage T12 DNA and T25₃c nonlysogen DNA were digested with *Eco*RI and *Sal*I, and the DNA fragments were separated by agarose gel electrophoresis. The separated fragments were transferred to nitrocellulose paper, and the transferred fragments were hybridized with a [³²P]ATP-labeled p1179 probe (containing the 1.7-kb fragment). Autoradiography of the nitrocellulose paper revealed hybridization patterns.

**Construction of lysogens.** The *S. pyogenes* lysogen T25₃c(T12) was constructed by infecting the bacterial strain T25₃c with the bacteriophage T12 [obtained from T25₃(T12)] by the methods of Nida and Ferretti (35).

## RESULTS

**Cloning of the type A streptococcal exotoxin gene from bacteriophage T12 into pHP34.** The lysogenic strain of *S. pyogenes* T25₃(T12) in our collection was the same as that described by Zabriskie (46), and when induced with mitomycin C, it yielded phage DNA that was different in size and restriction enzyme digestion patterns than that reported by Johnson and Schlievert (23). However, construction of the T25₃c(T12) lysogen with phage T12 obtained from the original lysogenic strain gave phage upon induction that appeared to be identical to that described by Johnson and Schlievert (23).

Bacteriophage T12 was isolated from *S. pyogenes* T25₃c(T12) after induction with mitomycin C and the DNA from this phage was obtained by phenol extraction. Bacteriophage T12 DNA was digested with the restriction endonucleases *Sal*I and *Eco*RI and the resulting fragments were ligated to *Sal*I- and *Eco*RI-digested pHP34. After transformation into *E. coli* HB101, the transformants were screened for recombinant plasmids. The HB101 transformants con-
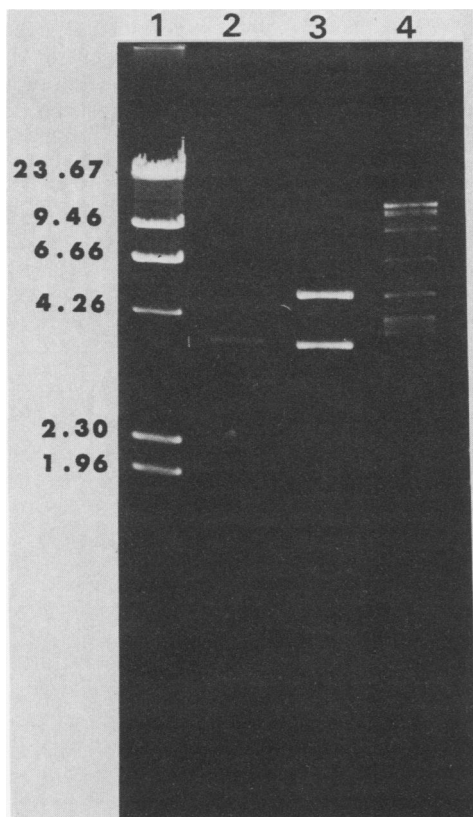


FIG. 1. Agarose gel of plasmid from *E. coli* transformant pA2 which contains the gene for type A streptococcal exotoxin. Lanes: 1, λ DNA digested with *Hind*III; 2, plasmid pHP34 digested with *Sal*I and *Eco*RI; 3, recombinant plasmid pA2 (containing 4.75-kb phage insert) digested with *Sal*I and *Eco*RI; 4, bacteriophage T12 DNA digested with *Sal*I and *Eco*RI.

FIG. 3. Physical map of 4.75-kb phage T12 DNA fragment in *E. coli* transformant pA2. Symbols: ——, Phage T12 DNA fragment; ▬, pHP34 vector DNA. Location of the type A exotoxin gene (*speA*) was determined by subcloning experiments.

taining the chimeric plasmids were analyzed for the presence of secreted or nonsecreted type A streptococcal exotoxin with specific antiserum by the Ouchterlony double-diffusion method. Cell extracts of the transformant clones were obtained by sonication and were applied to wells opposite a well containing specific type A streptococcal exotoxin. One of the transformants (designated pA2) which contained a 4.75-kb (Fig. 1) fragment of the bacteriophage T12, synthesized the type A streptococcal exotoxin as evidenced by a line of identity with the type A exotoxin secreted by *S. pyogenes* NY-5 type 10 (Fig. 2). Culture supernatants obtained from this *E. coli* transformant (designated pA2) were also tested with specific antibody; however, we did not find any evidence of exotoxin presence in the extracellular fluid.

The chimeric plasmid pA2 was digested with various restriction enzymes to construct a physical map of the 4.75-kb phage T12 DNA insert, as shown in Fig. 3.

**Subcloning of the type A streptococcal exotoxin gene.** Digestion of pA2 with *Sal*I and *Hind*III, produced a 1.7-kb fragment that could be potentially subcloned into pBR322. Digestion of pA2 with *Sal*I and *Hind*III and subsequent ligation to *Sal*I- and *Hind*III-digested pBR322 resulted in plasmids that contained the 1.7-kb insert (designated p1179). Cell-free extracts of these transformants were analyzed by the Ouchterlony double-diffusion method with specific type A exotoxin antibody, and it was determined that the 1.7-kb *Hind*III and *Sal*I fragment produced type A exotoxin that gave a line of identity with type A exotoxin produced by the pA2 plasmid (containing the 4.75-kb phage T12 fragment) and that produced extracellularly by *S. pyogenes* NY-5 type 10. The plasmid p1179 was subsequently digested with *Sal*I and *Eco*RI, and the 1.7-kb phage T12 fragment was ligated to the *Streptococcus-E. coli* shuttle vector pSA3 (M. L. Dao and J. J. Ferretti, personal communication) and was transformed into *S. sanguis* Challis 57. This 1.7-kb *Sal*I and *Eco*RI fragment contains 31 base pairs from pBR322, which represents the distance between these two restriction endonuclease sites on plasmid pBR322. Transformants containing the chimeric plasmid designated pSA32 produced extracellular type A exotoxin that was immunologically identical to type A exotoxin produced by the *E. coli* transformants pA2 and p1179 and type A exotoxin secreted by *S. pyogenes* NY-5 type 10.

**Southern hybridizations.** To verify that the cloned gene for type A streptococcal exotoxin, designated *speA*, is indeed located on the phage T12 genome and is absent from the *S. pyogenes* T25₃ nonlysogen, DNA-DNA hybridization experiments were performed by the method of Southern. The 1.7-kb fragment from plasmid p1179 was used as a probe and was hybridized to phage T12 DNA and *S. pyogenes* T25₃ nonlysogen chromosomal DNA that had been double digest-

ed with *Sal*I and *Eco*RI, electrophoretically separated in agarose gels, and transferred to nitrocellulose filters. The autoradiographs from these experiments (Fig. 4) revealed that the 1.7-kb fragment hybridized only to the phage T12 DNA and did not hybridize to the T25₃ nonlysogen chromosomal DNA, or to bacteriophage lambda DNA. These experiments confirmed that the cloned gene for type A streptococcal exotoxin in plasmid pA2 originated from phage T12 and was not present in the nonlysogen.

**Biological activity of the cloned type A exotoxin gene.** The type A streptococcal exotoxin produced by *Streptococcus sanguis* transformants containing a 1.7-kb phage T12 DNA insert had biological activity as measured by the erythematous skin reaction in two presensitized rabbits. Maximum skin reactivity was observed at 48 h postinjection of the test material. Concentrated culture medium of *S. sanguis* Challis transformed with pSA3 (without the 1.7-kb phage T12 insert) did not produce an erythematous skin reaction at 24 or 48 h.

## DISCUSSION

The results of these studies provide insight into the molecular mechanism of bacteriophage T12 conversion of *Streptococcus pyogenes* T25₃ to toxigenicity. By cloning the bacteriophage T12 DNA into the *E. coli* plasmid pHP34 and examining for expression of the bacteriophage T12 genes in *E. coli*, we were able to determine that the gene for type A streptococcal exotoxin is located in the bacteriophage T12 genome.

Southern hybridization of the cloned bacteriophage T12 fragment to the T25₃c nonlysogen genome and the bacteriophage T12 genome confirmed these results and revealed that the gene for type A exotoxin is not present in the T25₃c nonlysogen chromosome. As expected, the autoradiographs also indicated that the bacteriophage induced from the T25₃c(T12) lysogen contained the gene for the type A
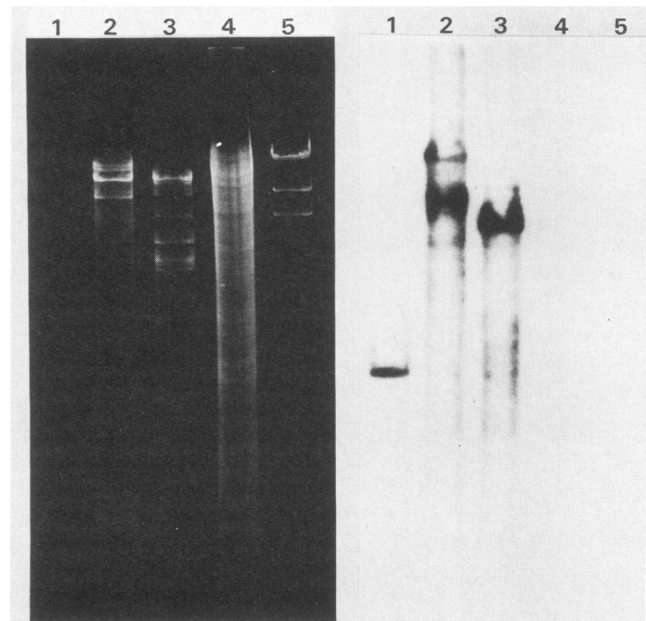


FIG. 4. Southern hybridization of labeled p1179 DNA to an agarose gel containing phage T12 DNAs and T25₃ chromosomal DNA. Lanes, 1, 1.7-kb fragment flanked by *Sal*I and *Hind*III sites; 2, T12 DNA digested with *Sal*I; 3, T12 DNA digested with *Sal*I and *Eco*RI; 4, T25₃c DNA digested with *Sal* and *Eco*RI; 5, λDNA digested with *Hind*III.

exotoxin. The specificity of the hybridization reaction was affirmed since the cloned phage fragment did not hybridize to chromosomal DNA or the phage lambda DNA. Thus, the mechanism of phage conversion to toxigenicity in *S. pyogenes* is similar to that of *C. diphtheriae*, in which the gene for toxin production is located on the β-prophage genome.

Subcloning experiments have localized the type A exotoxin gene to a 1.7-kb segment of the phage T12 genome flanked by *Sal*I and *Hind*III sites. This segment has a coding capacity for a 56,666-dalton protein and therefore would easily encode an 8,500-dalton protein, the estimated minimal size of the type A exotoxin molecule (20). Also, this 1.7-kb fragment most probably supplies the promoter region for the type A exotoxin gene since *Sal*I and *Eco*RI digestion of pHP34 removed the promoter for the tetracycline gene. Expression of the type A exotoxin gene in the absence of the tetracycline promoter suggests that the promoter for the type A exotoxin gene is intact and functioning.

Apparently, the type A streptococcal exotoxin is not secreted from *E. coli* HB101, since the type A exotoxin is present only in sonicates and is absent from concentrated culture medium of transformants. However, introduction of the type A exotoxin gene into *S. sanguis* Challis 57 via the *Streptococcus-E. coli* shuttle vector pSA3 results in transformants that produce extracellular type A exotoxin. Therefore, the cloned *speA* gene contains the sequences necessary for export of the protein out of the *Streptococcus sanguis* cell. Since the exact mechanism of secretion of the type A streptococcal exotoxin is not known, the lack of secretion of the exotoxin from *E. coli* probably reflects the differences in processing or secretion in the gram-negative *E. coli* and gram-positive *S. pyogenes* systems. Furthermore, the cloned type A exotoxin produced by *S. sanguis* transformants is biologically active and induces the erythematous skin response characteristic of type A streptococcal exotoxin in presensitized rabbits.

Although these experiments have demonstrated that the gene for type A streptococcal exotoxin is located on the bacteriophage T12 genome, they do not eliminate the possibility that the type A streptococcal exotoxin gene is located on a transposon, and transposition from the chromosome to the phage genome (or vice versa) occurs. Southern hybridization of the cloned toxin gene to numerous strains of streptococci associated with scarlet fever and phage induced from these clinical strains should allow examination of this possibility. The discovery of streptococcal strains associated with scarlet fever, in which the chromosome contains the *speA* gene and whose temperate phage do not encode the *speA* gene, would support this hypothesis. Nucleotide sequence data of the 1.7-kb fragment may reveal the presence of insertion sequences that may allow transposition. Finally, numerous phage-host systems will need to be examined to determine whether the phage T12-host T25₃ system is ubiquitous among strains of *S. pyogenes* associated with scarlet fever.

In conclusion, information obtained from this study indicates that phage T12 conversion of *S. pyogenes* T25₃ is analogous to the phage conversion system of *C. diphtheriae* by β-prophage. Other phage-host systems will have to be examined to determine whether the same interaction occurs among other clinical group A streptococcal lysogens.

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