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Phage Influence on the Synthesis of Extracellular Toxins in Group A Streptococci

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Phage conversion of group A streptococci to produce streptococcal exotoxins was shown to occur more widely than has been previously reported. Toxigenic conversion was found in 19 newly constructed lysogenic and pseudolysogenic strains resulting in synthesis of exotoxin types A and B. Conversion was accomplished by a positive conversion effector, which was a phage characteristic expressed by the prophage and vegetatively reproducing phage. Exotoxin production was determined by the rabbit skin test and by countercurrent immunoelectrophoresis with type-specific antisera. New lysogens and pseudolysogens were constructed with strains which failed to produce at least one exotoxin type. Phages were obtained from toxigenic strains isolated from cases of scarlet fever. Conversions were consistent and repeatable; loss of the recently introduced phage was accompanied by loss of the newly acquired toxin productivity. Conversion resulted in production of additional exotoxin type or types and never affected existing toxin synthesis. Converting phages were characterized by electron microscopy of negatively stained preparations and were all found to be of morphological class B1. All phage nucleic acid was double-stranded DNA. Though similar in structure, each converting phage had a different host range, and the nine new converting phages identified here did not react with antiserum prepared against the originally reported converting phage.

Although the mechanism of phage-directed alteration of bacterial phenotypes is well understood at the molecular level in many models, little is known about the phage-directed toxigenic conversion of *Streptococcus pyogenes*. The "scarlet fever toxin" produced by many strains of group A streptococci and discovered by Dick and Dick in 1924 (11) has been variously termed streptococcal pyrogenic exotoxin (29), erythrogenic toxin (26), scarlatinal toxin, and Dick toxin (2). Presently, three types of this toxin are recognized, termed exotoxin types A, B, and C (29). They elicit the same group of responses when tested in experimental animals although they are biochemically and serologically distinct. These toxins are pyrogenic, T-cell mitogenic, and rash evoking. Each exotoxin type enhances susceptibility to endotoxin shock and causes a breach of the blood-brain barrier permitting entry of toxic products and bacteria (23).

Conversion of nontoxigenic streptococcal strains to toxin production by a filterable factor from cultures of toxigenic strains was first reported by Cantacuzene and Boncieu (9) in 1926 and Frobisher and Brown (13) in 1927 and was confirmed in 1949 by Bingel (6). These early reports apparently identify the phage-host relationship as pseudolysogeny by present definitions, because toxin production ceased after growth in antiserum (5, 12). Lysogeny, characterized by the presence of a prophage in every member of the culture, contrasts with pseudolysogeny, or the carrier state, in which there is a persistent viral infection of some members only of the culture and in which the prophage is not formed. This carrier state is cured by growth of the culture in antiserum.

In 1964, Zabriskie reported the conversion of one host, $T25_3$, to type A exotoxin production by lysogeny with either of two serologically related phages, T12gl and 3GL16 (30). These observations have been confirmed in a preliminary report of the present communication (21), by Johnson et al. (15), and by McKane and Ferretti (20). Colon-Whitt et al. (10) and Johnson et al. (15) have also reported the conversion of group A strains to type C exotoxin production. Alouf (1) has recently reviewed the status of work with this toxin and other streptococcal toxins.

In the present communication we report on the toxigenic conversion of a number of different bacterial strains with phages isolated from strep-

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tococcal strains associated with clinical cases of scarlet fever. Additionally, new information is presented concerning the carrier state (pseudolysogeny) and type A exotoxin production, as well as characterization of some of the properties of phages involved in toxigenic conversion.

MATERIALS AND METHODS

Bacteria. Thirty-one strains of *S. pyogenes* were used in this research. The primary laboratory strains included strains $T25_3$ and $T25_3$(T12) (27), K56 (17), SM60 (19), T18P and T19 (23), and NY5 (25). Twenty-two strains (Table 1) were clinical isolates from unrelated cases of scarlet fever (generously provided by Lewis W. Wannamaker). All of the strains were tested for exotoxin production, and each strain was tested for phage, with each of the other 30 strains being used in turn as phage indicator strains. The strains isolated from cases of scarlet fever, designated here as AS 104 to AS 125, were primarily used as a source of phage, in addition to their use in phage detection and host range studies. The standard laboratory strains were used as sources of exotoxins and phages and as indicators and phage recipients in the construction of new lysogens (28). Stock cultures of streptococcal strains were maintained lyophilized or held at −70°C after growth in Todd-Hewitt broth (Difco Laboratories) and suspension in 2% skimmed milk (Difco).

Media. Serum Todd-Hewitt (STH) broth and agar media, used for the growth of phage indicator cultures, were prepared in the following manner. A solution of 3% (wt/vol) Todd-Hewitt broth (Difco) and 2.2 mM K_2HPO_4, including 1.5% (wt/vol) agar when required, was autoclaved and cooled to 50°C; this was completed with sterile supplements of 5% (vol/vol) horse serum (GIBCO Diagnostics) and 1.8 mM $CaCl_2$. STH soft agar used in overlays was prepared similarly, but with a reduction of agar concentration to 0.7% (wt/vol) and with omission of $CaCl_2$.

Peptone broth (P broth), selected for best promoting phage production and recovery, consisted of 6% (wt/vol) proteose peptone no. 3 (Difco), 0.05 M NaCl, and 2.5 mM Na_2HPO_4, which was aseptically supplemented, after autoclaving, with 0.05% (wt/vol) glucose, 5% (vol/vol) horse serum, and 1.8 mM $CaCl_2$ (27). The final pH was 7.1.

Tests for the production of streptococcal exotoxin were performed on culture filtrates of strains grown in a supplemented diffusate of Todd-Hewitt broth. The diffusate was prepared by placing a 30% (wt/vol) aqueous solution of Todd-Hewitt broth in Spectrapore no. 1 dialysis tubing (8,000-molecular-weight cutoff) and dialyzing it against a fivefold-greater volume of distilled water for 18 h at 4°C. The external diffusate was supplemented with 2.2 mM K_2HPO_4 and autoclaved before completion with the addition of sterile glucose to a final concentration of 0.05% (wt/vol).

Preparation of exotoxin samples. A 9% inoculum (vol/vol) of a logarithmically growing test culture in Todd-Hewitt broth diffusate was placed into prewarmed (37°C) Todd-Hewitt broth diffusate medium. The cultures were incubated for 7 h and then centrifuged at 20,000 × *g* for 15 min at 4°C in a Sorvall RC2-B high-speed centrifuge. The supernatants, kept constantly cool, were sterilized by filtration through membrane filters (type MF-HA; Millipore Corp.) having a mean pore size of 0.45 μm. The filtrates were subjected to ethanol fractionation to purify any exotoxins present by the method of Kim and Watson (16).

TABLE 1. Group A streptococcal strains isolated from cases of scarlet fever

Strain	Other designation	M type
AS 104	GT 73·010	NT[a]
AS 105	GT 76·019	3
AS 106	GT 8453	4
AS 107	GT 8627	2/22
AS 108	GT 8841	4
AS 109	GT 8996	4
AS 110	GT 9316	4
AS 111	GT 70·1500	NT
AS 112	GT 73·366	NT
AS 113	GT 8316	22
AS 114	GT 8588	NT
AS 115	GT 8774	4
AS 116	GT 8928	22
AS 117	GT 9093	NT
AS 118	GT 72·118	4
AS 119	GT 74·677	49
AS 120	GT 8406	49
AS 121	GT 8626	2/22
AS 122	GT 8840	4
AS 123	GT 8961	NT
AS 124	GT 9094	NT
AS 125	GT 72·121	22

[a] NT, Not typed.

Exotoxin assay methods. To provide continuity with earlier publications (30), the rabbit skin test was used to assay for type A streptococcal exotoxin. Culture filtrate preparations were injected intradermally into the shaved back of a New Zealand white rabbit. The test sites were observed at 24 and 48 h. Positive tests were indicated by an erythematous reaction site extending outside the 1-cm bleb produced upon filtrate injection.

Countercurrent immunoelectrophoresis (CIE) was used to identify exotoxins as type A, B, or C, with specific rabbit antisera kindly contributed by Dennis Watson (University of Minnesota Medical School) and Clifford Houston (14). CIE was performed according to standard requirements (3). CIE gels were inspected for immunoprecipitin lines immediately and after 24 and 48 h of incubation at 4°C. The gel slabs were washed, pressed, and dried by the method described by Axelsen et al. (4). Dried gels were stained with 0.5% amido black, 43% ethanol, and 10% glacial acetic acid (18). Stained films were destained in an aqueous acid-alcohol solution of the same composition as that in the stain solution. The gels were dried in an oven at 65°C and then examined and filed.

Bacteriophage assays. Strains to be tested for phage were grown in P broth at 30°C overnight. To obtain the phage lysate of spontaneous induction, the culture was centrifuged at 9,750 × *g* for 15 min at 4°C, and the supernatant was filtered through membrane filters (type MF-HA; Millipore Corp.) having a mean pore size of 0.45 μm. The 31 strains were each induced with mitomycin C also by the following method. A 0.1-ml

amount of an overnight P broth culture of the test strain at 30°C was placed into a Klett tube containing 10 ml of prewarmed P broth. The culture was incubated at 37°C for 1.5 h, then treated with 0.2 μg of freshly prepared sterile mitomycin C solution per ml, and incubated for an additional 3 h. Klett values were determined at 30-min intervals throughout the procedure to document the response of the culture to mitomycin C. The cultures were centrifuged and filtered as described previously. Phage lysates were immediately refrigerated at 4°C or stored in 1-ml volumes at −70°C in a Revco freezer. Refrigerated preparations were discarded after 24 h. Frozen preparations, once thawed for use, were held at 4°C and discarded after 24 h.

Indicator lawns were prepared by inoculating 2.5 ml of STH soft agar at 45°C with 0.1 ml of an overnight STH broth culture at 30°C. The overlays were mixed, poured smoothly over an STH agar plate, and permitted to solidify. They were used within 10 to 20 min after becoming firm.

Culture supernatants were tested for the presence of phage by the application of a 0.03-ml drop of supernatant onto an inoculated soft-agar overlay. After the drops had dried, the plates were inverted and incubated overnight at 30°C and inspected the next day for plaques indicating the presence of phage.

Construction of lysogens. Streptococcal strains found not to produce one or more of the exotoxin types were used as phage recipients and, as such, were used to inoculate a soft-agar lawn. Phages isolated from strains producing the required toxin type were spotted onto the soft-agar lawn. After plaque formation during overnight incubation at 30°C, the plaque contents were picked and transferred to a fresh STH agar plate. This inoculum was streaked out for isolation of colonies. The clones which arose were tested for lysogeny.

Identification of lysogens. The clones were inoculated in a grid pattern onto each of two STH agar plates, the second of which contained a soft-agar overlay of the isogenic parental strain. After overnight incubation at 30°C, the plates with soft-agar lawns were inspected by indirect light. A clear halo surrounding a superimposed colony marked the phage-producing clones. The identical clone was then selected from the designated area on the first of the duplicate plates and streaked for isolation on a fresh STH agar plate. A minimum of three such transfers followed by identification of phage-carrying clones was made for each newly constructed lysogen.

Preparation of rabbit antiphage serum. Differential centrifugation of a sterile, filtered phage lysate was performed for two cycles with a Sorvall RC2-B high-speed centrifuge; centrifugation at 30,000 × *g* for 2 h followed by resuspension of the pellet in streptococcal phage buffer and centrifugation at 5,000 × *g* for 30 min constituted each cycle. Streptococcal phage buffer contained 0.15 M NaCl, 10 mM Tris, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1 mM spermine, adjusted with 1 M HCl to pH 7.5.

A preparation of phage T12gl (which was one of the original converting phages cited by Zabriskie [30]) was used to immunize a New Zealand white rabbit. The first immunization consisted of a total volume of 0.6 ml, of which 0.1 ml was intravenously injected and 0.5 ml was intracutaneously injected, 0.1 ml into each of five sites. One week later, 0.7 ml was injected intracutaneously in 0.1-ml amounts into each of seven sites. The animal was bled 3 weeks after the last immunization. The serum was separated from the clot and stored in small volumes at −70°C. Purified and concentrated samples of 10 phages, including phage T12gl, were tested for reactivity with this antiserum by CIE.

Characterization of phage nucleic acid. The Bradley nucleic acid stain procedure was used to identify the nucleic acids of the phages (7).

RESULTS

Phage association of primary strains and constructed strains. The 31 primary strains listed in Table 1 and above were all shown to be lysogenic as evidenced by either spontaneous or mitomycin C induction of phage. Each phage strain obtained after induction had homologous immunity with the producing strain, and each phage strain had a unique host range within the 30 indicator strains tested. There was no correlation between phage host ranges and streptococcal M type or T type.

Phages obtained from bacterial strains known to produce type A exotoxin were infected into bacterial strains that did not produce type A exotoxin in order to construct new phage-host combinations; 18 lysogens and 1 pseudolysogen were obtained and are listed in Table 2. Lysogen designation reflected the composite origin of the new strain; the bacterial recipient strain is listed first, followed by the phage donor designation in parentheses (5). Pseudolysogens were indicated by separation of the recipient designation and the phage designation by a midline point instead of parentheses. Phages were identified by the designation of the native host strain.

Characterization of each phage-host combination revealed that the constructed lysogens released the newly acquired phage by spontaneous induction and responded to mitomycin C with phage induction, producing greater quantities of phage. The newly acquired phage was able to lyse the isogenic parental strain, but the new lysogens had homologous immunity to the phage produced. The pseudolysogenic clones had the following characteristics of pseudolysogens: colonies were cratered and spotted with pocks at the colony margin and scattered through the colony, cultures failed to respond to inducing amounts of mitomycin C with induction but responded with slowed growth and release of fewer phages (1.7×10^4 phages were produced, compared with the usual induction titer of 10^7 or 10^8), and homologous immunity to the phage being produced was not present. Seventeen constructed lysogens were maintained on STH agar slants and tested monthly for the presence of phage. There was a continued presence of phage in the strains for 3 months; at the end of the 4th month, 75% of the clones could no longer be

TABLE 2. Summary of toxin production by constructed lysogens

Strain[a]	Exotoxin type[b]		
	A[c]	B	C
$T25_3$	−	+	+
$T25_3(110)_1$	+	+	+
$T25_3(119)_1$	+	+	+
$T25_3(119)_2$	+	+	+
$T25_3(119)_4$	+	+	+
$T25_3(119)_5$	+	+	+
$T25_3(120)_1$	+	+	+
$T25_3(120)_3$	+	+	+
$T25_3(120)_1'$	+	+	+
$T25_3(120)_2'$	+	+	+
$T25_3(120)_3'$	+	+	+
$T25_3(124)_1$	+	+	+
K56	−	+	+
$K56(T12)_1$	+	+	+
$K56(107)_1$	+	+	+
$K56(107)_2$	+		
$K56(107)_3$	+	+	+
$K56(110)_1$	+	+	+
$K56(111)_1$	+	+	+
$K56(111)_2$	+	+	+
$K56(119)_1$	+	+	+
$K56(120)_1$	+	+	+
9440	−	+	+
$9440(113)_1$	+	+	+
$9440(120)_1$	+	+	+
$9440(120)_1$[d]	−	+	+
$9440(124)_1$	+	+	+
SM60	−	+	+
$SM60(T19)_1$	+	+	+
$SM60(T19)_2$	+	+	+
$SM60(NY5)_1$	+	+	+
$SM60(NY5)_2$	+	+	+
$SM60(NY5)_3$	+	+	+
$SM60(107)_1$	+	+	+
T18P	−	−	+
$T18P\text{-}T19_1$	+	+	+
$T18P(T19)_2$	+	+	+
$T18P\text{-}T19_3$	+	+	+
$T18P\text{-}T19_4$	+	+	+
$T18P(T19)_5$	+	+	+
$T18P\text{-}T19_6$	+	+	+
$T18P(120)_1$	+	+	+
$T18P(120)_2$	+	+	+
$T18P(120)_3$	+	+	+

[a] Subscript number identifies the specific clone tested. Prime subscript numbers denote lysogens constructed several months after construction of the first group.

[b] Identified with type-specific antisera. +, Positive reaction; −, negative reaction; blank indicates that the test was not performed.

[c] Reaction confirmed by the erythematous skin test in rabbits.

[d] Clone spontaneously cured of phage.

demonstrated to harbor the phage. This indicated that the artificially constructed lysogens were most often unstable.

Exotoxin synthesis by primary strains and constructed lysogens. All strains were assayed for the ability to produce streptococcal exotoxins by CIE with specific antisera and partially fractionated exotoxin preparations as described above. Type A exotoxin assays were always also performed with the erythematous rabbit skin test. Partial fractionation of exotoxin samples served as a concentration step and to remove or reduce protease activity. These procedures gave consistent and repeatable results. The results of these assays are presented in Table 2. Two to five clones of each constructed lysogen and pseudolysogen were tested and found to have acquired toxin synthetic capacity which was not present in the isogenic parental strain. In every primary strain and constructed strain, toxin synthesis was never found in the absence of phage. Conversion was consistently and repeatedly associated with lysogeny in several independently arising lysogens or pseudolysogens of each phage-host combination. Loss of the recently introduced phage was always accompanied by loss of the newly acquired toxin synthesis capacity. In no instance did establishment of lysogeny result in cessation of the original exotoxin production.

Bacterial strains $T25_3$, K56, SM60, and 9440 were all converted to type A exotoxin production by a number of different phages. Strain T18P, which produces no detectable type A or type B exotoxin, was also converted to produce both exotoxins by two different phages. This is the first report of phage conversion of a group A streptococcus to type B exotoxin production. The positive results we obtained with strains K56 and $T25_3$ for the production of type B and C exotoxins do not agree with those reported by Johnson et al. (15). This discrepancy may be attributable to differences in the exotoxin preparation procedure or assay method.

Characteristics of converting phages. The streptococcal phages were found to be delicate and easily disrupted by physical and chemical procedures tolerated by other phages. They required storage and handling in 10 mM Tris, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.1 mM spermine, pH 7.5. Other buffers caused a 50% diminution in phage numbers every 4 h at 4°C. High-speed centrifugation, CsCl density gradient ultracentrifugation, and membrane dialysis concentration resulted in unacceptable loss of particle viability and thus proved to be inefficient methods for the harvesting of large quantities of phage. Purified samples of several converting phages (NY5, T12, AS 120, AS 111) were obtained by centrifugation, and staining by the Bradley method (7)

revealed that all contained duplex DNA strands.

Antiserum prepared against phage T12 reacted with phage T12 in CIE experiments but did not react with any of the other nine phages identified here as converting phages. Thus, none of the other converting phages were antigenically related to phage T12.

Inspection of group A streptococcal phages by electron microscopy revealed a morphology similar to the Bradley class B1 (8). The polyhedral heads appeared isometric. The tails were long, unsheathed, and noncontractile. There was no collar, nor were other structures visible. Uranyl acetate stained the tail, producing a striated appearance that indicated a helical structure.

DISCUSSION

The results obtained in this study expand considerably the number and scope of strains involved in the toxigenic phage conversion of group A streptococci. The determination of the generality of toxigenic phage conversion is important in revealing whether this is solely a laboratory phenomenon or whether it could have significance in natural infections. Since lysogeny is almost universal among group A streptococci (10), the process of accurately identifying phages, the degree of relatedness among phages, and the exotoxin types is essential if phage conversion is considered to be important in natural infections.

We have constructed a group of 18 lysogens and 1 pseudolysogen from five bacterial recipient strains and 10 phages. Four of the recipients had not been reported to be converted before this study (21), and nine of the phages had not been reported to be converting phages. Three of the five bacterial recipient strains had not been subjected to any mutagenizing treatment, thus removing the possibility that a phage-associated change in phenotype might be attributed to a mutation in the host.

All of the phages were able to convert the bacterial recipient strains to produce type A exotoxin once the lysogen was formed. In the conversion of strain T18P (exotoxin type $A^-B^-C^+$) to exotoxin production by phage AS 120 from a strain of exotoxin type $A^+B^+C^+$, lysogeny resulted in the synthesis of two new exotoxin types. Conversion by phage T19, which originated from a host of exotoxin type $A^+B^+C^-$, resulted in lysogens and a pseudolysogen which produced exotoxins A, B, and C. In this instance, the phage T19 brought both of the exotoxin production characteristics of its native host to the new host, as did phage AS 120. It is significant to note that conversion by phage T19 did not result in the loss of type C exotoxin synthesis even though the phage donor strain failed to produce type C exotoxin.

Although every phage used in this research was able to convert the new host to type A exotoxin production, the research of McKane and Ferretti (20) showed that the establishment of a phage association alone was not sufficient to result in toxigenic conversion. McKane constructed two K56 lysogens with phages isolated from streptococcal strains causing glomerulonephritis and found that they were not converted to type A exotoxin production. In contrast, with phages isolated from toxigenic strains, most of which were isolated from cases of scarlet fever, all six K56 lysogens produced in the present research were shown to be converted to type A exotoxin production.

The present work resolves the apparent discrepancy between the early reports of pseudolysogenic conversion (5, 12) and later reports of lysogenic conversion by showing that both lysogenic and pseudolysogenic conversion occur in toxigenic conversion of *S. pyogenes*. In phage-directed conversion in group A streptococci, we conclude that a positive conversion effector is expressed by the prophage, the vegetative phage, and as McKane has shown, by the virulent mutant phage, resulting in type A and B exotoxin production phenotypes. The nature and mechanism of this positive conversion effector remain to be determined.

The present research indicated that the phage conversion of *S. pyogenes* followed the model of *Corynebacterium diphtheriae* (24), inasmuch as the conversion effector is expressed by the prophage, the vegetative phage, and the virulent mutant phage. Although the location of the structural gene (or genes) for streptococcal exotoxins cannot be determined yet, as it has been in *C. diphtheriae*, similarities of toxigenic conversion extend further. Conversion of *C. diphtheriae* can be accomplished by many serologically unrelated phages, as well as related phages, but cannot be accomplished by every phage able to establish lysogeny.

Attempts to characterize the converting phages indicated similarities in some properties but differences in others. For example, although the host range of each phage was unique and probably indicated that each phage was different, these studies were not fully determinative of such a final conclusion since there were many host factors that could result in the host range results. All of the phages were found to be in the Bradley morphological B1 classification, an observation consistent with the report by Nugent and Cole (22) that all streptococcal phages were in the same group B1 classification. However, the nine newly identified converting phages were serologically distinct from phage T12 since

antiserum specific for T12 failed to react with the other nine phages. Thus, the phages are related morphologically and by nucleic acid characteristics but are unique and of at least two serological types.

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