Phage Conversion of Shigella flexneri Group Antigens

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A temperate phage, designated Sf6, has been isolated from Shigella flexneri 3a. Characterization of Sf6 revealed that it possesses the capacity for converting the S. flexneri 3,4 group antigen complex to group factor 6. Serological studies and chemical analysis of lipopolysaccharide from converted strains suggest that group factor 6 is a reflection of an acetylation of the preexisting 3,4 antigen complex. Evidence is provided that the 3,4 group antigen complex functions, at least in part, as a cell surface receptor site for Sf6 adsorption.

Bacteriophages possessing the property of somatic antigen conversion have been recovered from certain serotypes of Shigella flexneri. Matsui (19) has converted S. flexneri type-specific antigens 1 and 2 to type-specific antigen 4 with a temperate phage isolated from S. flexneri 4c. This early observation of antigenic conversion is supported by findings of Iseki and Hamano (12) on the conversion of serotypes 1a, 1b, 2a, 3b, and Y to serotype 4 and by the report of Okado et al. (20), showing phage conversion of serotypes 2a, 2b, 4a, X, and Y to type factor 1. More recently, Keyti et al. (13) have described a phage that also functions in factor 4 conversion. Two distinct phages, fII and f7,8, have been recovered from S. flexneri 2b by Giammanco (9) and shown to cause expression of type antigen 2 and group determinant 7,8, respectively. S. flexneri X strains, after lysogenization with fII, behaved serologically like S. flexneri 2b, whereas lysogenization of S. flexneri Y strains resulted in antigenic conversion to the S. flexneri 2a serotype. When Y strains were lvsogenized with f7,8, Giammanco detected a conversion to the S. flexneri X serotype. Additional studies by Giammanco (10) have established that conversion to a type antigen 5 serotype can also be achieved through lysogenization with an appropriate phage.

Our continuing investigations on the genetic control of S. flexneri somatic antigens (4, 7) have recently concerned the genetic determinant of group antigen 6. Our findings have revealed that group factor 6 can be under the genetic control of a temperate phage. This report summarizes some of the properties of this newly isolated phage, its role in converting group factor 3,4 to group factor 6, and the chemical alterations in S. flexneri lipopolysaccharide (LPS) resulting from such antigenic conversion.

MATERIALS AND METHODS

Bacterial strains. The characteristics of S. flexneri and Escherichia coli strains pertinent to this study are described in Table 1. In addition, certain E. coli hybrids were included; their method of preparation has been described previously (4).

Media. Brain heart infusion broth, Penassay broth, and Trypticase soy agar were used for routine cultivation of organisms. The composition of minimal medium, used in conjugation experiments, has been described previously (4); amino acids and other

<table>
<thead>
<tr>
<th>Strains</th>
<th>Species</th>
<th>Persistent characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH10 and Y3</td>
<td>S. flexneri Y</td>
<td>A natural Y variant, lacking a type-specific antigen; produces group factor 3,4</td>
</tr>
<tr>
<td>AB1133-H96</td>
<td>E. coli K-12 hybrid</td>
<td>E. coli K-12 his* hybrid recovered from a mating with S. flexneri 2a Hfr 256 (4); produces group factor 3,4</td>
</tr>
<tr>
<td>W1895</td>
<td>E. coli K-12</td>
<td>Hfr donor strain; transfers chromosome with polarity O—lac—pro—ara—... pur E (5)</td>
</tr>
<tr>
<td>M42-43</td>
<td>S. flexneri 2a</td>
<td>Wild-type strain; produces type-specific antigen 2 and group complex 3,4 (5)</td>
</tr>
</tbody>
</table>
growth factors were added to minimal medium at a final concentration of 25 μg/ml. When required, fermentation characteristics were scored on MacConkey agar base (Difco) supplemented with an appropriate carbohydrate (1%).

**Phage methods.** Procedures for the routine preparation and titration of phages have been described previously (1). Tryptone broth (1%) containing NaCl (5 g/liter) and 0.7% agar was used as a soft-agar overlayer. Just prior to use, molten soft agar was seeded with host cells (statically grown overnight, 37°C) to yield a concentration of about 10⁹ cells per ml. Spot tests to determine phage sensitivity of bacterial strains were performed according to established methods (8). The procedure for kinetic analyses of phage adsorption has been described (6).

**Preparation of antiserum.** Antisera, used in absorption-agglutination experiments, were prepared by immunizing individual rabbits with heat-inactivated (1 h at 100°C) suspensions of appropriate strains. The details of the immunization procedure and the preparation of sera have been described previously (4). Monospecific group factor 6 and group factor 3,4 antiserum were prepared by the absorption protocols of Edwards and Ewing (3).

**Antigen analysis.** The presence of *S. flexneri* somatic antigens was scored by slide and tube agglutination tests. Complete details of these methods have been already described (3, 4).

**Extraction of LPS.** Bacterial cells, grown in brain heart infusion broth under aeration for 36 h at 37°C, were harvested by centrifugation. After a wash, the brain heart infusion cell paste (100 g) was extracted with 500 ml of 45% aqueous phenol at 70°C for 15 min (24). After cooling to 0°C, followed by centrifuging at 30,000 × g for 15 min, the water phase was removed. The remaining interface layer, phenol phase, and cell debris were re-extracted at 70°C with 250 ml of water, cooled, and centrifuged as before. The combined aqueous phases were next dialyzed against running, deionized water for 6 h and concentrated in vacuo at 40°C to a syrup. Crude LPS was precipitated from the concentrate with four volumes of acetone, washed twice with absolute ethanol and ether, and air dried.

**Chemical characterization of LPS.** Total carbohydrate determinations were made on unhydrolyzed samples of LPS, using the phenolsulfuric acid method of Dubois et al. (2). Glucosamine, rhamnose, heptose, and total hexose (i.e., glucose plus galactose) were quantitated by the radiochromatographic method of Koeltzow et al. (14, 15), using 2% LPS solutions that had been hydrolyzed for 6 h at 100°C in 1 N H₂SO₄. Prior to radiochromatography, all H₂SO₄ hydrolysates were treated with HONO, by the method of Shively and Conrad (22), to hydrolyze resistant glycosidic linkages involving glucosamine. The hydroxylamine method of Hestrin (11) was used to quantitate the level of O-acetyl groups present in unhydrolyzed solutions of 2% LPS with ethyl acetate as a standard.

**RESULTS**

**Isolation and host range of phage Sf6.** In the course of characterizing a number of *S. flexneri* 3 strains, it was observed that *S. flexneri* 3a strain 3-19 spontaneously released a temperate phage. This phage, designated Sf6, was recovered by plating culture filtrates of strain 3-19 on *S. flexneri* Y hosts and was found to control antigenic conversion to group factor 6. After being cloned on a *S. flexneri* Y strain, a high-titer phage lysate of Sf6 was prepared by the confluent lysis method (1) for screening studies to determine the host range of Sf6. Overnight broth cultures of the known serotypes of *S. flexneri*, various rough mutants of *S. flexneri*, and a limited number of *S. sonnei*, *S. dysenteriae*, E. coli, and *Salmonella* strains were spread on Trypticase soy agar plates and spotted with drops of a Sf6 lysate (~10¹⁰ plaque-forming units per ml). After overnight incubation at 37°C and an examination for phage sensitivity, it was evident that the host range of Sf6 is limited. Only the *S. flexneri* Y strains were lysed by Sf6. When *S. flexneri* Y strains are employed as hosts in soft-agar overlays, Sf6 produces distinct, turbid plaques (1.5 to 2.0 mm in diameter) with haloes typical of many temperate phages.

*S. flexneri* group antigen 3,4 as an essential part of the surface receptor for phage Sf6. The results of the host range study suggest that *S. flexneri* group antigen 3,4 functions in the adsorption of phage Sf6. *S. flexneri* Y strains, the only serotype lysed by Sf6, represent a variant class of *S. flexneri* that produces only group 3,4 somatic antigen. *S. flexneri* Y strains are defective in their capacity to synthesize an additional type-specific determinant (3, 23).

To establish unequivocally that the group-antigen 3,4 complex functioned, at least in part, as the receptor site for Sf6 adsorption, we studied the behavior of Sf6 on *E. coli* K-12 hybrids which express the *S. flexneri* group 3,4 antigen complex. Previous investigations have demonstrated that the group 3,4 antigen gene(s) is closely linked to the histidine operon of the *S. flexneri* chromosome and that it is possible to transfer this locus to *E. coli* recipients by conjugation (5). Thus, *E. coli* hybrid strain AB1133-H96 was prepared by mating *S. flexneri* 2a Hfr 256 with *E. coli* K-12 AB1133 and selecting for the inheritance of the his-group 3,4 antigen chromosomal segment (14). Unlike the original parent, *E. coli* AB1133, hybrid derivative AB1133-H96 produces the 3,4 group antigen complex of *S. flexneri*. On the basis of agglutinin-absorption tests and immunodiffusion analyses, this hybrid behaves serologically as a *S. flexneri* Y strain (5). This strain was found to be sensitive to lysis by Sf6, plating with an
efficiency approximately that of the natural host, _S. flexneri_ Y (efficiency of plating 0.5 to 1.0). The _E. coli_ parent AB1133 was resistant to Sf6. The results of phage adsorption tests performed with _E. coli_ hybrid AB1133-H96 and parent AB1133 are presented in Fig. 1. Unlike the parent _E. coli_ AB1133, hybrid strain AB1133-H96 efficiently adsorbs phage Sf6. The sensitivity of this hybrid to Sf6 is thus considered to be a direct consequence of its inheritance and expression of the 3,4 group antigen complex.

**Effect of type-specific antigen on Sf6 adsorption.** Included among the serotypes that appear insensitive to lysis by Sf6 were _S. flexneri_ 2a and 4a. Both serotypes express group factor 3,4 but, unlike the Y serotype, also produce an additional type-specific determinant. Since type-specific antigens 2 and 4 result from a glycosylation of the group 3,4 antigen _O_-repeat unit (18, 23), it was considered that such a modification may affect adsorption of Sf6 and hence the lytic infection of these hosts. This interpretation is supported by results from comparative adsorption and sensitivity tests on _S. flexneri_ 2a M42-43 and its Y derivatives.

The type-specific 2 antigen gene(s), which maps in the lac-pro segment of the _S. flexneri_ chromosome, can be removed by replacing this region with the _E. coli_ lac-pro segment (5, 7). Most hybrids of this type produce only the 3,4 group antigen and behave serologically as typical Y derivatives. Thus, _E. coli_ K-12 Hfr W1895 was mated with _S. flexneri_ 2a M42-43, selection being made for recombinants that inherited the donor lac+ chromosomal segment. Such hybrids were then tested for sensitivity to Sf6 by the spot test method. All those that lost the type 2 antigen were fully lysed by Sf6, whereas those that conserved their type-specific antigen did not lyse. The results of adsorption tests performed with _S. flexneri_ 2a M42-43 and one of the Y hybrids, G127-1-2, are presented in Fig. 2. It is apparent that the _S. flexneri_ hybrid lacking type-specific antigen 2, G127-1-2, adsorbs phage Sf6 more efficiently than the native _S. flexneri_ 2a, M42-43. The presence of type-specific 2 antigen thus appears to inter-

![Fig. 1. Adsorption of Sf6 to _E. coli_ K-12 hybrid AB1133-H96 and its parent AB1133. Approximately 2.5 x 10^9 plaque-forming units were added to 2.5 x 10^9 bacterial cells suspended in Penassay broth at 37 C. Periodically samples were diluted into chilled Penassay broth containing CHCl_3, thoroughly mixed, and subsequently assayed in soft agar for unadsorbed phage, with _S. flexneri_ Y strain FH10 as an indicator host.](image1)

![Fig. 2. Adsorption of Sf6 to _S. flexneri_ Y hybrid G127-1-2 and its parent _S. flexneri_ 2a M42-43. The procedure employed is similar to that described in the legend to Fig. 1.](image2)
fere with, but not prevent, this step of Sf6 infection.

**Group antigen conversion by phage Sf6.** The results of serological studies revealed that phage Sf6 functions in *S. flexneri* group 6 antigen conversion. *S. flexneri* Y strains FH10 and F3 and *E. coli* hybrid AB 1133-H96, which agglutinate in group 3,4 antiserum but not in group 6 antiserum, were lysogenized with Sf6 and tested for their agglutination properties. Lysogenization was achieved by depositing drops (0.01 ml) of a Sf6 lysate (10^8 plaque-forming units/ml) on lawns of host cells prepared on Trypticase soy agar and incubating overnight at 37°C. Isolates, prepared from the secondary growth in the area of lysis, were cloned twice and then scored for their lysogenic properties. Clones that expressed immunity to lysys by Sf6 and released Sf6 plaque-forming units were considered to be Sf6 lysogens. Unlike the parental strains, such lysogens were found to agglutinate strongly in group factor 6 serum in addition to retaining a weak reactivity in the group 3,4 antiserum. Slide agglutination tests on over 200 independent Sf6 lysogens established an absolute correlation between Sf6 lysogeny and agglutination in group factor 6 serum. The group 6-converting property of Sf6 was confirmed by agglutinin absorption tests (Table 2). Comparative tube agglutination tests with various antisera were performed with *S. flexneri* Y strain FH10, *E. coli* hybrid AB1133-H96, and Sf6 lysogens of them. Unlike the parental strains, their Sf6 lysogens agglutinated at significant levels in group 6-specific serum. In addition, agglutination in group 3,4 antiserum was observed with these lysogens, indicating the presence of group antigens 3,4. The expression of both group complex 3,4 and group factor 6 by Sf6 lysogens was confirmed by preparing antisera against *E. coli* hybrid AB1133-H96 (Sf6). This antiserum contained agglutinins against group antigens 3,4 (Table 2). Furthermore, after absorption with the nonlysogenic parent AB1133-H96 to remove the 3,4 agglutinins, the antisera behaved as a typical group 6-specific serum. A wild-type *S. flexneri* 1b strain was also included in these tests. This strain, 2381-0, which produces group factor 6 and group antigen 4, agglutinated as expected in all antisera.

**Analyses of LPS.** LPSs were isolated from *S. flexneri* Y strain F3 and a representative lysogenic phage-converted derivative, F3 (Sf6), and analyzed to determine the chemical basis of antigenic conversion to group factor 6. The results of LPS analysis of strains F3 and F3 (Sf6) are summarized in Table 3. It can be seen that both strains yield approximately the same amount of LPS per 100 g of cells (wet weight). In addition, monosaccharide analyses support the conclusion that both strains produce a complete LPS core. The heptose/hexose (glucose plus galactose) ratios for strains F3 and F3 (Sf6) were found to be 1.0:2.2 and 1.0:2.4, respectively. These values are in close agreement with the expected ratio of 1:0.2:0, based on the complete core structure of *S. flexneri* LPS as described by Simmons (23).

Comparison of rhamnose/O-acetyl ratios, on the other hand, yielded a significant difference between the LPS of strain F3 and its phage-converted derivative, F3 (Sf6). The rhamnose/O-acetyl ratios of 1:0:0.1 for F3 and 1:0:0.7 for F3 (Sf6) indicate elevated levels of O-acetyl groups in the phage-converted strain. In addition, the average length of O side chains of the phage-converted strain F3 (Sf6) appears to be shorter than that of F3 (Table 3).

**Table 2. Agglutination reactions of strains converted to group antigen 6 by phage Sf6**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group-specific 3,4</th>
<th>Group-specific 6</th>
<th>Anti-AB1133-H96</th>
<th>Anti-AB1133-H96 (Sf6) absorbed with AB1133-H96</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. flexneri</em> Y FH10</td>
<td>+ (640)</td>
<td>- (&lt;20)</td>
<td>+ (2,560)</td>
<td>+ (640)</td>
</tr>
<tr>
<td><em>S. flexneri</em> Y lysogen FH10</td>
<td>+ (640)</td>
<td>+ (2,560)</td>
<td>+ (640)</td>
<td>+ (2,560)</td>
</tr>
<tr>
<td><em>E. coli</em> hybrid AB1133-H96</td>
<td>+ (640)</td>
<td>- (&lt;20)</td>
<td>+ (5,120)</td>
<td>+ (1,280)</td>
</tr>
<tr>
<td><em>E. coli</em> hybrid lysogen AB1133-H96 (Sf6)</td>
<td>+ (640)</td>
<td>+ (2,560)</td>
<td>+ (2,560)</td>
<td>+ (2,560)</td>
</tr>
<tr>
<td><em>S. flexneri</em> 1b 2381-0</td>
<td>+ (320)</td>
<td>+ (640)</td>
<td>+ (320)</td>
<td>+ (640)</td>
</tr>
</tbody>
</table>

*Prepared according to absorption protocols of Edwards and Ewing (3).
 Tube agglutination titer.
DISCUSSION

*S. flexneri*, an antigenically heterogeneous group of organisms, have been differentiated into various serotypes, with each serotype expressing one of six distinct type-specific antigens (3). The significant serological cross-reactivity among these organisms has been shown to be a reflection of common group antigens shared by this species (3). The three recognized group antigen complexes of *S. flexneri* (group 3,4, group 6, and group 7,8) are expressed either singly or in combination within the six serotypes (3). In addition, two naturally occurring serological variants that lack a type-specific determinant have been described. The *S. flexneri* Y serotype expresses only group complex 3,4, whereas the X serotype produces group complex 7,8 (3). As is the case with other enterics, the O-antigen specificity of *S. flexneri* is determined by the chemical and structural properties of the polysaccharide portion of the LPS cell wall component. Studies of the immunochemistry of *S. flexneri* antigens indicate that the 3,4 group complex serves as the basic underlying O-specific antigen of *S. flexneri* and that subsequent chemical and structural modifications of this O-repeat unit result in changes in antigenic characteristics (18, 23). By correlating antigenic factors with molecular studies, it was shown that the *S. flexneri* 3,4 group complex consists of primary sidechains comprised of a rhamnose/rhamnose/rhamnose/N-acetyl-glucosamine/O-repeat unit (17, 18). Additional structural studies of various *S. flexneri* serotypes indicated that type-antigen specificity resides in α-glucosyl secondary side chains attached to this primary structure and that distinct modifications of such glucose side chains accounted for the type specificities 1, 2, 3, 4, and 5 (18, 23). Because such changes could be accomplished by single enzymes, it has been inferred that the various *Shigella* type antigen-converting phages function, most likely, by coding for specific glucose transferases essential for the incorporation or modification of secondary glucose chains in the primary side chains (18, 23).

As our previous genetic studies have shown, the group 3,4 complex is controlled by chromosomal genes closely linked to the histidine operon of *S. flexneri* (4). There was no apparent involvement of a phage in the genetic determination of group factor 3,4. Such a possibility appears unlikely when one considers the chemical complexity of the 3,4 O-repeat unit and the multienzyme systems needed, presumably, for its synthesis. In contrast, it is evident from our present findings that group antigen 6 expression can be under the genetic control of a temperate phage. Our results support the conclusion that lysogenization with phage Sf6 results in appearance of group antigen 6. Immunological tests indicate that the group 6 factor controlled by Sf6 is typical of that produced by a native *S. flexneri* 1b. Furthermore, it is apparent that lysogenization of hosts with Sf6 does not result in a complete conversion to group antigen 6. Such strains, in addition to expressing factor 6, retained reactivity in group 3,4 antisera.

Our comparative analyses of LPS from a *S. flexneri* Y strain and its Sf6 lysogenic derivative revealed an elevated level of O-acetyl residues in the phage-converted strain. This finding is in agreement with other workers who have involved O-acetyl groups as the chemical determinant of group 6 specificity. Romanowska et al. (21) have shown that removal of O-acetyl groups by mild alkaline hydrolyses of polysaccharides prepared from group 6 LPS results in a full loss of group 6 specificity. In addition, Lindberg et al. (16), on the basis of studying *S. flexneri* 3c and 4b LPS, concluded that acetylation of rhamnose residues of the *S. flexneri* O-repeat unit LPS side chain is responsible for group factor 6 specificity.

<table>
<thead>
<tr>
<th>Organism</th>
<th>LPS (g)/100 g cells</th>
<th>Total carbohydrate</th>
<th>Glucosamine</th>
<th>Hepose</th>
<th>Hexose</th>
<th>Rhamnose</th>
<th>O-acetyl</th>
<th>N^e</th>
</tr>
</thead>
<tbody>
<tr>
<td>F3</td>
<td>1.33</td>
<td>32.6</td>
<td>89.0</td>
<td>21.9</td>
<td>48.9</td>
<td>99.3</td>
<td>10.9</td>
<td>3.2</td>
</tr>
<tr>
<td>F3 (Sf6)</td>
<td>1.21</td>
<td>15.1</td>
<td>54.2</td>
<td>20.6</td>
<td>49.4</td>
<td>25.2</td>
<td>18.3</td>
<td>0.8</td>
</tr>
</tbody>
</table>

*Values are reported in micromoles/100 mg of LPS.

*Expressed in micromoles of glucose equivalents.

*Total hexose = glucose and galactose.

*Assayed by the hydroxylamine method of Hestrin (11).

*^e N is the average number of O-repeat units per side chain. It can be calculated by multiplying the rhamnose/heptose ratio by 2/3 according to the revised *Shigella* O-repeat unit structure published by Lindberg et al. (17).*
The significance of the difference in the length of O side chains of LPS that we observed between strains F3 and F3 (Sf6) remains to be investigated further. It is conceivable that the presence of O-acetyl groups could affect the polymerization of O-repeat units, thus causing a higher percentage of short primary side chains in the LPS of the converted strain.

On the basis of these observations it appears that phage Sf6 functions by converting pre-existing group 3,4 O-repeat units to a group 6 specificity. The significant increase in the level of O-acetyl groups in the LPS of the group 6-converted strain, F3 (Sf6), suggests that this phage determines a specific acetylase which results in the acetylation of rhamnose residues in the 3,4 O-repeat unit and the subsequent expression of the group 6 antigen. Our interpretation of the behavior of phage Sf6 is consistent with that proposed by Giammanco (6) for phage f7,8, which functions in conversion to group antigen complex 7,8. Immunochemical studies comparing Y strains (group 3,4) and X strains (group 7,8) indicated that phage f7,8 functioned by altering the preexistent group determinant (6). It thus appears that modifications to the 3,4 group complex, achieved by enzymes under the control of appropriate phages such as Sf6 and f7,8, are reflected as a conversion from group antigen 3,4 to group factors 6 and 7,8, respectively.

As evidenced by our adsorption studies (Fig. 1), group factor 3,4 functions in the adsorption step of Sf6 infection. Alterations to the group 3,4 factor appear to affect the efficiency with which Sf6 adsorbs to host. S. flexneri 2a, which expresses type-specific antigen 2 as a consequence of the secondary glucosyl side chains attached to rhamnose of the 3,4 O-repeat unit primary side chain, adsorbs Sf6 poorly as compared to a Y derivative of it (Fig. 2). Similar results were recently obtained when we compared E. coli hybrid AB1133-H96 (Y-like) to a group 6-converted derivative of it. The mechanism by which type factor 2 and group factor 6 affect Sf6 adsorption is unknown. Most likely, the active component of the 3,4 group complex which functions in phage adsorption (presumably rhamnose) is modified by glucosylation and acetylation.

At the present time, the precise position of the Sf6 prophage on the chromosome of S. flexneri lysogenic for Sf6 is being determined. In addition to the intrinsic value of Sf6 for achieving antigen conversion, this phage, on the basis of preliminary studies, may prove useful as a genetic tool for achieving both intergeneric transduction between E. coli and S. flexneri and interspecific transductions within the S. flexneri group.

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LITERATURE CITED


