Serological Relationships of *Salmonella* A Bacteriophages Isolated from *Salmonellae* in Different Kauffmann-White Groups

LEONARD R. BULLAS AND ROBERT L. NUTTER

Department of Microbiology, Loma Linda University, Loma Linda, California 92354

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Six different *Salmonella* group A phages from salmonellae in Kauffmann-White groups B, C₁, C₂, and D were examined serologically. Those phages which were specific for a particular somatic antigen were found to be serologically very similar. Antiserum against a phage with one specificity was able to neutralize a different phage with the same specificity but unable to neutralize, in the normal way, a phage with a different specificity. Phages mixed with heterologous phage antiserum responded with an "inhibition response" in which there appeared to be a neutralization of the phage infectivity for the first 10 min, followed by a reversal of the neutralization until, by 20 or 25 min, there was no apparent neutralization. This response was interpreted to indicate that the adsorption antigens, probably situated on the tail fibers, were different for phages with different specificities but sufficiently similar so that heterologous antibodies could react with the antigens; but the antigen-antibody complex was quickly disassociated, resulting in a modification of the antibody molecules but no change in the specificity sites of the antigen. A subgrouping of the *Salmonella* A phages based on their antigenic specificity is suggested.

We recently described a number of *Salmonella* bacteriophages which were placed into three groups designated A, B, and C (11). Group A phages isolated from different *Salmonella* serotypes, belonging to several Kauffmann-White (K-W) groups, were found to be morphologically identical, consisting of an hexagonal head about 55 nm in diameter with a short tail about 15 nm in length (3, 11). We were led to the conclusion that such phages were probably widespread among the salmonellae. Group A phages were also found to lyse only smooth strains of *Salmonella* which possessed group-specific antigens. Thus, for example, A phages isolated from *S. typhimurium* lysed only *Salmonella* possessing antigen 12, A phages from *S. potsdam* lysed only *Salmonella* with antigen 7, and A phages from *S. muenchen* lysed only *Salmonella* possessing antigen 8. There were no serological cross-reactions of the A phages with any of the B or C phages. Boyd (4, 5) showed that the A phages isolated from different lysogenic cultures of *S. typhimurium* were serologically very similar but could be subdivided on the basis of minor serological variations. In this paper, we present results of a serological investigation of a selected

series of A phages of *Salmonella* from K-W groups B, C₁, C₂, and D.

MATERIALS AND METHODS

Bacteria and bacteriophages. Six different group A *Salmonella* bacteriophages were used. They were isolated from and propagated on *Salmonella* in the K-W groups B, C₁, C₂, and D. The K-W group B phage was P22 which was kindly supplied by L. W. Cohen, Pomona College, Claremont, Calif., together with its propagating culture of *S. typhimurium* LT2. From K-W group C₁ there were three group A phages: phages P4 (ATCC 25957-B₂) and P9c (ATCC 25957-B₄), the same phages as used previously (11), both isolated from lysogenic cultures of *S. potsdam* and propagated on *S. potsdam* SP2 (ATCC 25957); bacteriophage G-Cl, isolated from the lysogenic culture of *S. gelsenkirchen* stock 534 and propagated on *Salmonella choleraesuis* var. Kunzendorf stock 153, both cultures having been obtained from the Center for Disease Control, Atlanta, Ga. Bacteriophage M3 was isolated from a lysogenic culture of *S. muenchen* of K-W group C₂ and propagated on *S. muenchen* SM24. Bacteriophage B1 was isolated from a lysogenic culture of *S. blegdam* of K-W group D and propagated on *S. blegdam* SB2. *S. muenchen* SM24 and *S. blegdam* SB2 are identical to the cultures used by Atkinson and Bullas (2).

Phage P22 and phage B1 adsorbed specifically to smooth strains of *Salmonella* possessing somatic antigen 12. Phages P4, P9c, and G-C1 adsorbed only to smooth strains of *Salmonella* possessing somatic antigen 7. Phage M3 adsorbed only to smooth *Salmonella* cultures with somatic antigen 8. Each phage showed a high level of thermal resistance, the majority of the particles in an L-broth lysate of each phage surviving 85 C for 30 min.

All phages were resistant to inactivation by chloroform. By electron microscopy, all phages were found to be morphologically similar, resembling P22. In addition, none of the phages showed any serological relatedness to other *Salmonella* bacteriophages which did not have all of the previous properties. Furthermore, all phages except P4 and P9c were vectors of generalized transduction. The six bacteriophages therefore possessed properties which clearly placed them into the A group of *Salmonella* bacteriophages (11). Bacteriophage lysates were prepared by inoculation of exponentially growing cultures of the appropriate propagating strain growing in L broth with phages from a single isolated plaque of the bacteriophage. Lysates were sterilized by the addition of chloroform after centrifugation to remove cell debris. The agar-layer technique described by Adams (1) was used in phage assays.

Media. L broth and L agar were routinely used for growth and plating of bacteria and bacteriophages (9).

Serology. Antisera against phages were prepared with rabbits. Sterilized, high-titer lysates were prepared, and 1 ml of these lysates was administered by ear vein twice a week until a total of at least 10 ml of each lysate had been administered. Animals were bled 3 days after administration of the injection, and the serum was collected. The neutralization test was the same as that described by Adams (1). The sample of antiserum prepared against phage P22 was kindly supplied by L. W. Cohen, Pomona College.

An antiserum which gave a typical neutralization curve with a specific phage was referred to as "homologous"; an antiserum to which a phage did not react with a typical neutralization curve was referred to as "heterologous."

RESULTS

Cross-neutralization tests were done by using antisera against the three phages P22, B1, and P4 with all six phages. In these tests, the numbers of plaque-forming units were determined after a standard period of 15 min of exposure to the antisera at 37 C. When there was neutralization of the phage, the *K* value was determined. Results of these tests, with the various *K* values, are shown in Table 1.

Table 1 indicates that there is a very close serological relationship between *S. typhimurium* phage P22 and the *S. blegdam* phage B1 and a similar relationship among the *S. potsdam* phages P4 and P9c and the phage propagated on *S. choleraesuis*, G-C1. The *S. muenchen* phage M3 was serologically unrelated to any of the other

TABLE 1. Antiserum neutralization tests showing *K* values with antisera against phage P22, P4, and B1 with phages P22, P4, P9c, G-C1, M3, and B1

Phage	Propagating strain of <i>Salmonella</i>	K-W group	Antiserum against phage		
			P22	P4	B1
P22	<i>S. typhimurium</i> LT2	B	188	0	48
P4	<i>S. potsdam</i> SP2	C ₁	0	388	0
P9c	<i>S. potsdam</i> SP2	C ₁	0	253	0
G-C ₁	<i>S. choleraesuis</i> 153	C ₁	0	236	0
M3	<i>S. muenchen</i> SM24	C ₂	0	0	0
B1	<i>S. blegdam</i> SB2	D	124	0	48

phages. It is of significance that the serologically similar phages P22 and B1 were the only phages in this group which were specific for somatic antigen 12. Phages P4, P9c, and G-C1 were all specific for somatic antigen 7 and were serologically very similar, whereas M3 was the only phage specific for somatic antigen 8. Thus, those phages specific for the same somatic antigen were all very closely related serologically.

Kinetics of reaction with heterologous antisera.

A series of antiserum neutralization-type experiments was done between a phage and a heterologous antiserum to which it showed no detectable neutralization in the first series of tests, in which samples to be plated out for plaque-forming units were removed at 5-min intervals. There was invariably, depending on the strength of the antiserum and the dilution at which the test was being carried out, a response which we have called the "inhibition response." There appeared to be, for the first 5 or 10 min, apparent neutralization of the phage in the sense that there was a reduction in the numbers of plaque-forming units which paralleled the neutralization curve of that phage by its homologous antiserum. This response was a transient one, however, since by 15 to 20 min, when the neutralization determinations had previously been made, the numbers of plaque-forming units were either the same as the beginning or, on occasion, greater than the original number. Thereafter, no reduction in plaque-forming units occurred, and the numbers of plaques eventually stabilized at about 25 min. Thus, for example, phage P4 showed an inhibition response with antisera against phages B1 and P22, and phage G-C1 showed an inhibition response with antiserum against phage B1. Results of an experiment in which P4 was reacted both with its homologous antiserum and antiserum against B1 are shown in Fig. 1.

FIG. 1. Reaction of *Salmonella blegdam* phage B1 against (A) B1 antiserum, showing a typical neutralization, and (B) antiserum against *S. potsdam* phage P4, showing a typical inhibition response.

Plaque-forming units in excess of the number originally assayed probably resulted from the dispersion of individual plaque-forming units from clumps. Thus, it appears that the heterologous antiserum had interacted with the phages to cause them to separate but that the initial response which inhibited their plaque-forming ability was only transient. Control phage preparations, placed at the same temperature at which the phage/antiserum reactions were being tested, did not show the significant increases in plaque-forming units as shown when mixed with heterologous antiserum, nor was an inhibition response given with normal serum.

Kinetics of reaction with a mixture of homologous and heterologous antisera. When a phage was exposed to a mixture of two antisera, one of which was the homologous antiserum with which it was neutralized in the regular way and the other was a heterologous antiserum with which it gave the inhibition response, the rate of neutralization was significantly increased. Thus, the rate of neutralization of P4 in a mixture of antisera against phages P4 and B1 was significantly greater than with the P4 antiserum alone. This result could indicate that the sites of reaction of the antisera with the phage particle might be the same. In the presence of the homologous antiserum, the inhibition effect was not reversible but actually appeared to be additive to the true neutralization response. If the heterologous antiserum was added during the course of neutralization of a phage by its homologous antiserum, there was an increase in the rate of neutralization. This result would suggest that

the reaction sites of the heterologous antiserum with the phage were still available even after exposure to the homologous antiserum.

Reaction with two mixed heterologous antisera. When a phage was reacted with a mixture of two different heterologous antisera, the resulting inhibition response did not appear to be additive but was similar to that given by the antiserum which gave the most marked inhibition. The inhibition response which resulted from reaction with the antiserum mixture was similar to this response except that it was of slightly longer duration. This result would appear to support the idea that the reaction sites in the phage were the same with the different heterologous antisera so that a mixture of these two antisera would give an inhibition response which was maximal.

Reaction with two heterologous antisera added at different times. A phage that had been mixed with one heterologous antiserum to which it responded with an inhibition response was able to give a second inhibition response when it was exposed to a second, different heterologous antiserum. Thus, when *S. muenchen* phage M3 was exposed first to antiserum against *S. blegdam* phage B1 and subsequently to antiserum against *S. potsdam* phage P4, it gave an inhibition response with both antisera. This result indicates that the reaction sites on the phage were available again for reaction with the second heterologous antiserum. It also appears to indicate that the antibody molecules in the first reacting antiserum were modified by their reaction with the phage antigenic sites in such a way that they were now unable to react in the original way.

Reaction with antibacterial antiserum. Phage antiserum always had some bacterial antibodies in it, because of the presence of bacterial cell debris in the lysate used for injection. It was considered possible that the inhibition response might be due to the presence of bacterial cell antibodies reacting in some nonspecific way with the phage particle. To test this suggestion, we did a series of neutralization-type tests with antisera produced against those bacteria in different K-W groups which were used as the propagating culture for the production of phage lysates. There was no inhibition response in any of these tests. Thus, for example, *S. potsdam* phage P4 gave no inhibition response when mixed with antisera against its own propagating culture nor when mixed with antiserum against *S. blegdam* SB2, the propagating culture for phage B1. Similarly, phage B1 gave no inhibition effect when mixed with antiserum against its own propagating culture of *S. blegdam* nor with antiserum against *S. potsdam*. Phage antiserum from which the bacterial antibodies had been adsorbed also

continued to give the inhibition response when mixed with heterologous A phages. Thus, the inhibition effect resulted from the reaction of the phage with phage antibodies rather than from a nonspecific reaction with bacterial antibodies.

Reaction with an heterologous antiserum followed by neutralizing antiserum. The rate of normal neutralization of a phage by a homologous antiserum was not affected by prior exposure of the phage to an heterologous antiserum. Thus, for example, the rate of neutralization of phage P4 by P4 antiserum was unchanged after exposure of the phage to the heterologous antiserum against B1. Similarly, the rate of neutralization of phage B1 by its homologous antiserum was not affected by prior exposure of the phage to antiserum against phage P4.

DISCUSSION

The group A *Salmonella* phages form a distinct morphological group. Although the number of phages used here is small, they are undoubtedly representative of a widespread group of phages among many serological groups. Thus, phages having properties of the A phages were described years ago by Lilleengen for the K-W group D (10), by Williams-Smith for groups C₁ and D (14, 15), by Edwards, Davis, and Cherry for groups C₁, C₂, D, E, and G (7), and by Wasserman and Saphra for groups B, C₁, C₂, D, and E (13), each of whom isolated phages with specifications useful in *Salmonella* phage typing. The most distinctive aspect of all these phages was that lysis was restricted to strains of *Salmonella* that possessed a specific somatic O antigen identical to that possessed by the lysogenic culture from which the phage was originally isolated. In addition, most of them were found to be the vectors of transduction. With the exception of phages P4 and P9c isolated from *S. potsdam*, the A phages here have also been shown to be transducing phages.

Adsorption of a phage to a bacterial cell is possible because of the presence, on the bacterial cell wall, of phage-specific receptor sites to which the phage can become associated by reason of attachment organs which are generally understood to be the tail fibers. The phage antigenic specificity, detected by neutralization of plaque-forming units, must therefore reside in the tail fibers rather than in any other part of the phage. Results in this paper indicate that the tail fiber antigens of A phages specific for a particular O antigen are antigenically very similar and are distinctly different antigenically from the tail fiber antigens of A phages specific for different O antigens. Thus, phages P22 and B1, both specific for antigen 12, were neutralized by anti-

serum prepared against P22; phages P4, P9c, and G-C1, each specific for antigen 7, were neutralized by antiserum against P4; and phage M3, specific for antigen 8, was not neutralized by any of the antisera used here.

Explanations for the inhibition effect with heterologous antisera are, however, harder to deduce. Since the effect is seen by a reduction in the number of plaque-forming units, it would appear that it is primarily due to an effect which is also directed against the adsorption organs of the phage. This interpretation is supported by the demonstration of an increase in the rate of the regular neutralization when heterologous and homologous antisera were mixed. It is also supported by the observation that the level of plaque-forming units after reaction with a heterologous antiserum was occasionally greater than the original numbers of plaque-forming units, an effect which we interpreted as being probably related to the breaking up of phage clumps. Electron micrographs of A phages frequently showed the phage particles to be clumped together apparently entangled by their tail fibers. If the heterologous antiserum were to react with the tail fibers, it is not difficult to conceive that many of the phage particles could become detached from the clump and later, after the inhibition effect, be capable of becoming attached to a cell and being a plaque-former. It is also possible that even if the tail fibers were the site of reaction of the heterologous antiserum, there may be a second antigen with which it reacts rather than the O-specific antigen. It is not yet known whether the tail fibers of each of the A phages constitute a single antigenic species or more than one.

If the attachment sites on the phage for the heterologous antibodies were indeed the same sites as the neutralizing antibodies which presumably interfere with its ability to attach to the bacterial cell, why is there a reversion a few minutes later? The O antigens of *Salmonella* consist of linear side chains of complex lipopolysaccharides which project from the cell wall (12). The O antigens that have been the most intensively studied are those of the K-W group E *Salmonella* because of the antigenic conversions brought about by phages ϵ^{15} and ϵ^{34} . Chemical analyses of the different antigens have shown that the immunological specificity of the O antigens is determined by relatively minor structural changes in the side chains of otherwise identical polysaccharides which consist of repeat trisaccharide units of D-galactosyl-D-mannosyl-L-rhamnose sequences. Although it is possible that the chemical modifications may be a little more complicated than those described for the group

E antigens, they probably comprise the same kinds of modifications. These modifications consist of changes in the linkage positions, altered anomeric configurations, attachment of different monosaccharides at certain parts of the repeat trisaccharide sequences, deletion of or substitution for one of the monosaccharides of the sequence, and the presence of acetyl groups on the sugar residues (6). These differences in chemical structure are clearly distinguished by the tail fiber antigens of the A phages which attach to the specific lipopolysaccharide receptor sites possibly by a lock-and-key type of mechanism.

Although neutralization of the infectivity of a phage is generally considered to be nonreversible, reversible neutralizations have been shown to occur with neutralizing antibodies produced early in immunization (8). Antibodies against the different phage tail fibers may be less able to distinguish clearly between the different lipopolysaccharide antigens than the tail fiber antigens themselves. Thus, an antibody against the tail fiber antigens of an A phage specific for a particular O antigen may initially develop a weak association with the tail fibers of an A phage specific for a different O antigen, thus inhibiting cell attachment. This association is, however, of short duration. The disassociation would bring about no significant modifications of the tail fiber antigens which would then be free to react with other tail fiber antibodies, whether heterologous or homologous, to give either another inhibition response reaction or a typical neutralization, respectively. On the other hand, the disassociation of the antigen-antibody complex brings about modifications of the antibody molecules so that they cannot react a second time with the antigen. The addition of a second heterologous antiserum after a primary inhibition response will therefore result in a second inhibition response.

If these interpretations are correct, they would indicate that the A phage tail fiber antigens actually display a finer specificity than the antibodies against them, further evidence that the chemical differences between the antigens both on the *Salmonella* of the different K-W groups and on the A phage tail fibers are probably minor.

The *Salmonella* A phages may thus be clearly divided into subgroups which are related to their antigenic specificity. A phages specific for somatic

antigen 12 possessed by *Salmonella* in K-W groups A, B, and D and which are serologically similar consist of one subgroup which we call subgroup 1, represented here by phages P22 and B1. Phages specific for antigen 7 and serologically similar, represented here by phages P4, P9c, and G·Cl, represent subgroup 2, and phages specific for antigen 8 and serologically similar, represented by phage M3, are included in subgroup 3. Upon the discovery of other *Salmonella* A phages with other specificities, new subgroups may easily be assigned.

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