JOURNAL OF VIROLOGY, Oct. 1974, p. 904-909
Vol. 14, No. 4

Printed in U.S.A.

Studies on the Bacteriophage 2 Receptors of *Pseudomonas aeruginosa*

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Received for publication 7 June 1974

The lysogenization of *Pseudomonas aeruginosa* strain BI with phage 2 resulted in the loss of the capacity to adsorb the same phage. The absence of phage 2 receptors on the surface of the lysogenized strain BI(2)$_8$ was confirmed by the failure of purified slime polysaccharide (SPB) or lipopolysaccharide (LPS) to inactivate phage 2. SPB and LPS from a phage 2-resistant strain also failed to inactivate phage 2 in contrast to the phage inactivation exhibited by the SPB and LPS obtained from the wild-type strain BI. Chemically, quantitative differences were apparent when the SPB and LPS of strains BI(2)$_8$ and BI/2S$_2$ were compared with those of the wild-type strain BI. The most striking difference noted was the absence of amino sugars in the SPB of strain BI/2S$_2$. The SPB of strain BI(2)$_8$ also contained a lower percentage of amino sugars compared with the SPB of the wild-type strain BI.

After collision, the first step in the infection of a susceptible cell involves the attachment of phage to specific receptors on the bacterial surface. Various structural components present on the surface of gram-negative bacteria have been shown to possess receptor activity (16, 21). The purified slime polysaccharide (SPB) and lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* strain BI have been shown to exhibit receptor activity as demonstrated by the ability to specifically inactivate phage 2. Lipoprotein and glycopeptide fractions from the same strain were devoid of receptor activity (3). The chemical moieties responsible for the inactivation of phage 2 have not been identified. It has also been suggested that interaction between the phage 2-associated SPB depolymerase and its specific substrate in the slime layer may play a role in the adsorptive process (22).

The data presented in this report show that lysogenization of *P. aeruginosa* strain BI with phage 2 is accompanied by certain chemical changes in both the SPB and LPS fractions, resulting in a loss of the ability to adsorb phage 2. The study further attempts to identify those moieties which may be involved in the inactivation of phage.

(This investigation is taken in part from a dissertation submitted by F. J. C. to the Graduate School of Biomedical Sciences, College of Medicine and Dentistry of New Jersey, Newark, in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Microorganisms. P. aeruginosa strain BI and *Pseudomonas* phage 2 have been previously described (P. F. Bartell, T. Orr, D. Shima, and I. S. Thind, Fed. Proc. **22**:324, 1963; 2). Phage 2-lysogenized and -resistant cells were isolated after spotting drops of a high-titered phage suspension on freshly prepared lawns of strain BI. After incubation at 37 C for 18 h, colonies growing within the lytic area were picked and streaked on Trypticase soy agar (TSA) (BBL). The isolated colonies were further tested for sensitivity to phage 2 by spotting phage on lawns prepared with the respective colonies. Initial screening to differentiate between lysogenic, BI (2), and resistant, BI/2, colonies was performed by spotting broth suspensions of the isolated colonies on lawns prepared with the wild-type strain BI. Those colonies surrounded by a halo, indicating the activity of phage 2-induced polysaccharide depolymerase, were tentatively identified as lysogens, and those without a halo were labeled as resistants. After repeated subculturing, to eliminate the possibility of a carrier state, direct proof of lysogeny was obtained by the induced release of phage 2 after exposure to UV irradiation (20 μW/cm^2 for 15 s). Resistant colonies did not release phage 2 when exposed to the same dose of UV irradiation. Resistant colonies were labeled as previously described (22) and lysogenic colonies were numbered according to their order of isolation. Indistinguishable by colonial morphology, the relationship between wild-type strain BI, phage 2-resistant mutants of strain BI, and phage 2-lysogenized BI was demonstrated by the induction of prophage 29 present in all three strains. Phage 29 was characterized by the ability to infect strain C, but not strain BI, and the failure to induce the synthesis of polysaccharide depolymerase. Phage 2 was characterized by the

ability to infect strain BI, but not strain C, while inducing the synthesis of polysaccharide depolymerase. In this study, the resistant strain BI/2S_2 and the lysogenic strain BI(2)$_8$ were utilized.

Trypticase soy broth (TSB) and TSA were used to grow all bacterial strains and to propagate phage. Stocks of phage 2 were produced by infecting log-phase cultures of strain BI, containing approximately 10^8 cells/ml, at a multiplicity of infection of 1. After 18 h of incubation at 37 C, chloroform was added to a final concentration of 1.5%. The suspension was centrifuged at 6,000 × *g* for 15 min to sediment whole cells and bacterial debris. A second method of propagation consisted of layering TSA plates with soft agar (0.7% agar in TSB) containing a mixture of strain BI and phage 2 at concentrations known to produce confluent lysis. After incubation (37 C for 18 h), the soft-agar layer was scraped off and resuspended in 0.2 M sodium phosphate buffer, pH 7.5, containing 1.5% chloroform. The mixture was blended for 1 min and centrifuged at 6,000 × *g* for 15 min to sediment cells, debris, and agar. The centrifugation procedure was repeated with the supernatant fluids, which were finally stored at 4 C. Further purification of phage 2 was accomplished by centrifugation at 70,000 × *g* at 4 C for 1 h. The pellets were washed in 0.01 M Tris buffer, pH 7.5, centrifuged again, and then resuspended in the same buffer and placed at 4 C.

SPB and LPS. Cultures of *P. aeruginosa* strains BI, BI(2)$_8$, and BI/2S_2 were used for the extraction of SPB and LPS. The procedures used have been previously described (1-3).

DNA extraction. A 24-h culture of strain BI was sedimented at 4,000 × *g* for 10 min and resuspended in a mixture of 0.15 N sodium chloride and 0.015 N sodium citrate (SSC) containing lysozyme (200 μg/ml). The suspension was incubated at 37 C for 2 h. An equal volume of phenol (90%) was added, mixed for 10 min, and then centrifuged at 4,000 × *g* for 5 min to separate the phases. The aqueous phase was decanted and an equal volume of chilled ethanol (95%) was added. The DNA was collected with a glass rod, dried in cold ethanol (95%), and finally dissolved in SSC.

UV absorption spectrum. SPB and LPS were suspended at a concentration of 500 μg/ml and DNA at a concentration of 12 μg/ml in 5 mM Tris buffer, pH 7.5, supplemented with 0.1 M NaCl and 0.01 M $MgSO_4$. UV absorption curves were studied between 210 and 300 nm in a Beckman DB-G spectrophotometer.

Phage adsorption. Log-phase cultures were sedimented at 6,000 × *g* for 10 min and resuspended in fresh TSB at a concentration of approximately 10^7 cells/ml. The cellular suspension was mixed with an equal volume of phage containing 10^5 PFU/ml at 37 C. Portions were removed at various time intervals, diluted 1:10 in cold TSB, and immediately centrifuged at 6,000 × *g*, for 10 min to sediment cells and adsorbed phage particles. The supernatant fluids were diluted and plated on wild-type strain BI to determine the number of unadsorbed or free phage particles. An adsorption medium composed of 5 mM Tris buffer, pH 7.5, supplemented with 0.1 M NaCl and 0.01 M $MgSO_4$ was also used in place of TSB (22).

Phage inactivation. Various concentrations of SPB or LPS were prepared in 5 mM Tris buffer, pH 7.5, supplemented with 0.1 M NaCl and 0.01 M $MgSO_4$. Phage 2 suspensions (approximately 10^5 PFU/ml) were mixed with SPB or LPS and incubated at 37 C for 15 min. The mixtures were then diluted in cold buffer and titrated on wild-type strain BI cells to determine the number of infective phage particles.

Analytical methods. Neutral sugars were released from SPB or LPS by hydrolysis in 2 N HCl at 100 C, for 4 h. Amino sugars were released by two methods of hydrolysis: (i) 4 N HCl at 100 C for 8 h, or (ii) 6 N HCl at 100 C for 24 h, and for amino acid analyses, 6 N HCl at 100 C for 24 h. Total hexoses were determined by the method of Scott and Melvin (24), and pentoses as described by Fernell and King (8). In addition, quantitative and qualitative analyses of total sugars were performed with the Technicon autoanalyzer by the method of Kesler (12). Amino sugars were determined by the method of Belcher (4), on the autoanalyzer as described by Lee et al. (13), and by descending paper chromatography on Whatman no. 1 paper developed with butanol-pyridine-water (6:4:3). Spots of amino sugars were visualized by the method of Partridge (20). Total amino acids were determined by the method of Yemm and Cocking (27), heptoses by the method of Dische (7) as modified by Osborn (19), thiobarbituric acid reactive material as described by Osborn (19), uronic acids by the method of Bitter and Miur (5), and phosphorus by the method of Chen et al. (6).

Electron microscopy. One-half milliliter of a log-phase suspension of strain BI was mixed with phage 2 at an input multiplicity of 300 to 500. After allowing 10 min at 37 C for adsorption, a drop of the mixture was placed on a grid for 1 min and the excess material was blotted off. The samples were examined after negative staining with 2% sodium phosphotungstate (pH 7.6) in a Hitachi HU-12 electron microscope at 75 kV.

RESULTS

The attachment of phage 2 to whole cells of the wild-type *P. aeruginosa* strain BI (85 to 92% within 10 min) has been previously reported (3). Electron micrographs show that phage 2 particles attach to the cell surface in the characteristic tail-first manner. However, after the lysogenization of strain BI with phage 2, it was observed that strain BI(2)$_8$ could not adsorb phage 2. These results were identical to those observed with a phage 2-resistant strain, BI/2S_2, which was also observed not to adsorb phage 2. The failure of strains BI(2)$_8$ and BI/2S_2 to adsorb phage 2 was observed even when the input multiplicity was increased to 1,000. Thus, strain BI appears to lose its capacity to adsorb phage 2 after lysogenization with phage 2.

The absence of phage 2 receptors on the surface of strain BI(2)$_8$ was further examined by comparing phage 2 inactivation by purified

SPB or LPS. The failure of SPB, derived from strain BI(2)$_8$, to inactivate phage 2 is illustrated in Fig. 1. BI(2)$_8$ SPB, in concentrations as high as 500 μg/ml, had no inactivating activity when mixed with phage 2. These results were identical to those observed with SPB from the phage 2-resistant strain BI/2S$_2$, and in sharp contrast to the phage inactivation exhibited by SPB from wild-type strain BI in which 4 μg of SPB per ml inactivated 50% of the phage suspension within 10 min.

When LPS was examined, it was also clear that strain BI(2)$_8$ did not have the capacity to inactivate phage 2. Concentrations as high as 500 μg/ml failed to inactivate the phage (Fig. 2). This was identical to the results observed with strain BI/2S$_2$ LPS. In comparison, 8 μg of LPS per ml from wild-type strain BI inactivated approximately 50% of the phage 2 suspension within 10 min.

These results suggested that the failure of strain BI(2)$_8$ to adsorb phage 2 was related to some change(s) involving the phage 2 receptor sites of the lysogenized cells.

It was then of interest to analyze and compare the chemical compositions of the SPB and LPS of the wild-type strain BI, BI(2)$_8$, and BI/2S$_2$ in an attempt to gain information regarding the nature of the specific chemical moieties responsible for phage 2 inactivation.

The UV absorption spectra of SPB and LPS obtained from strains BI, BI(2)$_8$, and BI/2S$_2$ were examined and compared with the spectrum obtained with DNA extracted from strain BI. As expected, DNA from strain BI showed an absorption peak at 260 nm, but no absorption peak was observed at the same wavelength with SPB or LPS, indicating that these preparations were free of contaminating nucleic acids.

A summary of the partial chemical composition of SPB obtained from strains BI, BI(2)$_8$, and BI/2S$_2$ is presented in Table 1. When compared with the wild-type strain BI, the SPB of the lysogenized strain BI(2)$_8$ was found to contain lesser amounts of rhamnose, mannose, glucose, thiobarbituric reactive material, heptoses, amino acids, phosphorous and amino sugars, but increased amounts of galactose and uronic acids. Likewise, when the SPB of the resistant strain BI/2S$_2$ was compared with that of the wild-type strain BI, lesser amounts of most constituents were found with a notable increase in the uronic acid content. In addition, amino sugars were undetectable in SPB obtained from strain BI/2S$_2$.

A chemical comparison of LPS from strains

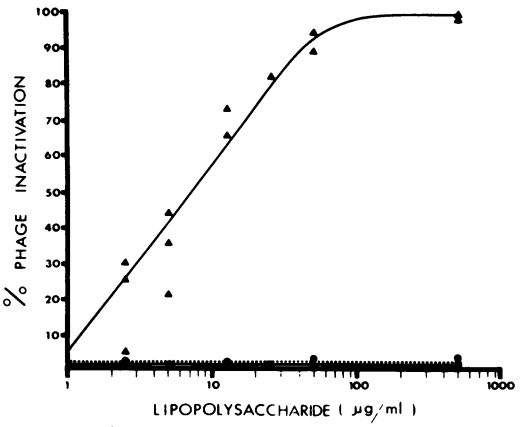

FIG. 2. *Interaction of Pseudomonas phage 2 with purified LPS. Inactivation of phage determined after mixing with LPS from P. aeruginosa strains BI (▲), BI(2)$_8$ (●), and BI/2S$_2$ (■).*

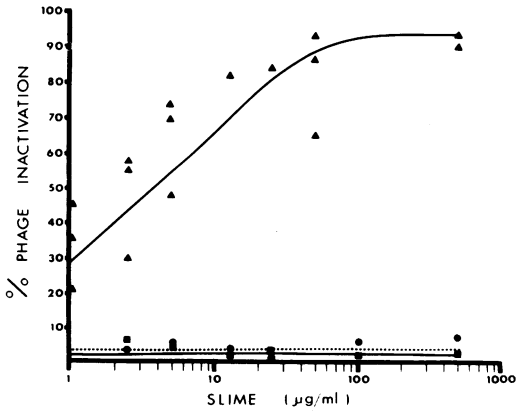

FIG. 1. *Interaction of Pseudomonas phage 2 with purified SPB. Inactivation of phage determined after mixing with SPB from P. aeruginosa strains BI (▲), BI(2)$_8$ (●), and BI/2S$_2$ (■).*

TABLE 1. *Partial chemical composition of components in the SPB extracted from Pseudomonas aeruginosa strains BI, BI(2)$_8$ and BI/2S$_2$*

Substances	Slime polysaccharides (% dry wt)		
	BI	BI(2)$_8$	BI/2S$_2$
Pentoses	0.0	0.0	0.0
Rhamnose	5.2	2.9	0.6
Mannose	0.82	0.32	1.4
Galactose	2.0	9.1	4.8
Glucose	3.6	1.1	1.8
TBRM[a]	1.7	0.2	0.1
Uronic acids	4.0	6.0	12.5
Heptoses	2.0	0.8	0.8
Amino acids	10.0	7.0	9.0
Phosphorus	2.0	0.3	0.1
Amino sugars	20.0	12.0	0.0

[a] TBRM, Thiobarbituric reactive material.

BI, BI(2)$_8$, and BI/2S$_2$ is presented in Table 2. In general, only slight differences were noted when the LPS of strain BI(2)$_8$ was compared with the LPS of strain BI. The greatest differences involved an increased content of amino acids and amino sugars. With respect to the LPS from strain BI/2S$_2$, decreases in the content of rhamnose, thiobarbituric acid reactive material, and amino sugars were evident with a concomitant increase in heptoses, amino acids, and phosphorous.

The amino sugar composition of SPB and LPS of the various strains was examined in greater detail. After hydrolysis for 8 h and analysis in the Technicon autoanalyzer, the SPB from strain BI was found to contain four major amino sugar peaks. Glucosamine and galactosamine appeared at 55 and 60 min, respectively, whereas an unknown peak appeared at 66 min and another, broader peak, at 79 min. In contrast, the SPB from strain BI(2)$_8$ showed only traces of a glucosamine peak and one unknown broad peak at 79 min. As expected, from the results presented above, the SPB from strain BI/2S$_2$ was found free of amino sugars. After more extensive hydrolysis (24 h), five amino sugar peaks were observed in the hydrolyzed SPB of strain BI. These appeared at 40, 55, 60, 66, and 79 min. Three peaks appeared in the hydrolyzed SPB of strain BI(2)$_8$ at 40, 66, and 79 min. Examination of the LPS of strain BI/2S$_2$ after 24 h of hydrolysis indicated the presence of only glucosamine (55 min) and galactosamine (60 min). The LPS of strain BI and BI(2)$_8$ produced peaks at 40, 55, 60, and 65 min.

Paper chromatographic analyses of hydrolyzates (24 h) of the SPB and LPS of strains BI and BI(2)$_8$ revealed the presence of six amino sugars in each. These were identified as *N*-acetylglucosamine, glucosamine, galactosamine, glucosamine-uronic acid, and galactosamine-uronic acid. The chromatogram spotted with a hydrolysate of the LPS from strain BI/2S$_2$ contained five spots identified as glucosamine, galactosamine, glucosamine-uronic acid, and galactosamine-uronic acid. The SPB of strain BI/2S$_2$ showed a very faint, questionable spot with the same R glucosamine as glucosamine-uronic acid. In all of the materials examined, with the exception of SPB of strain BI/2S$_2$, one spot with an R glucosamine of 0.57 remained unidentified.

TABLE 2. *Partial chemical composition of components found in the LPS extracted from P. aeruginosa strains BI, BI(2)$_8$, and BI/2S$_2$*

Substances	Lipopolysaccharide (% dry wt)		
	BI	BI(2)$_8$	BI/2S$_2$
Pentoses	0.0	0.0	0.0
Rhamnose	3.0	3.0	1.0
Mannose	0.0	0.0	0.0
Galactose	0.0	0.0	0.0
Glucose	3.2	3.2	3.3
TBRM[a]	1.7	1.2	0.7
Uronic acids	1.9	2.0	1.4
Heptoses	2.65	2.0	3.75
Amino acids	4.0	6.0	10.0
Phosphorus	4.0	4.5	7.0
Amino sugars	15.0	19.0	6.0

[a] TBRM, Thiobarbituric reactive material.

DISCUSSION

The factors influencing the adsorption of phage 2 to cells of *P. aeruginosa* have been described (22). Phage 2 was found to adsorb to strain BI in 5 mM Tris buffer, providing that cations like Na^+, Mg^{2+}, and Ca^{2+} were present. Adsorption was observed over a broad pH range, reaching a maximal level around pH 7.5. Several experiments (3, 22) have established the receptor-like activity or purified SPB, which contains the specific substrate for the phage 2-associated depolymerase, by its ability to inactivate phage 2. Receptor-like activity or phage inactivation was not observed with SPA or SPB preparations that were devoid of the depolymerase substrate (3, 22). Thus, it was suggested that the depolymerase-substrate interaction may play a major role in the adsorptive process. LPS preparations derived from strain BI also have been shown to inactivate phage 2 (3).

Phage conversion in P. *aeruginosa* was first demonstrated by the experiments of Holloway (10). Lysogenization of strain I with temperate phage D3 was observed to result in a loss of the ability to adsorb the same phage. More recently, Liu (17) has reported changes in the somatic antigens of *P. aeruginosa* after phage lysogenization.

The data presented in this report show that the lysogenization of strain BI by phage 2 results in a loss of the capacity to adsorb phage 2. The absence of phage 2 receptors, in both SPB and LPS, was further confirmed by the failure to inactivate phage 2 in concentrations as high as 500 μg/ml. In contrast, SPB from the nonlysogenized, wild-type strain BI was observed to inactivate 50% of the phage 2 suspension in a concentration as low as 4 μg/ml. The absence of phage 2 receptor sites was also demonstrated by using the SPB and LPS of

resistant mutants of strain BI (BI/2S_2) which failed to adsorb phage 2.

Phage and bacteriocin-resistant bacterial mutants have proven to be very useful tools in the study and identification of specific receptors. Comparison of phage-inactivating activities of various Ra *Salmonella* mutants led to the identification of *N*-acetylglucosamine as essential for the attachment and inactivation of the Felix 0-1 phage (14, 15). Smit et al. (26) studied different mutants resistant to a *Proteus vulgaris* bacteriocin and, by comparing the LPS of the mutants with that of the wild-type sensitive strain, they concluded that glucuronic acid provided the receptor specificity. The receptors for pyocin R in *P. aeruginosa* strains PII and P_4 were studied by using two resistant strains, PI and PIIR^r. These studies (11) revealed the pyocin R receptors to reside in the LPS fraction.

The present study has examined the chemical compositions of SPB and LPS of a phage 2 lysogenized strain, BI(2)$_8$, and a phage 2-resistant strain, BI/2S_2, comparing these with the wild-type strain BI in an attempt to identify common moieties which might act as phage 2 receptors. It must be emphasized that SPB and LPS of *P. aeruginosa* strain BI have been shown to be distinct entities based on immunological, chemical, and physical properties, and by differences in biological activity (25). The UV absorption spectra of SPB and LPS, while demonstrating the absence of contaminating nucleic acids, further showed distinct patterns for SPB and LPS.

Chemically, quantitative differences were apparent when the SPB and LPS of strains BI(2)$_8$ and BI/2S_2 were compared with those of the wild-type strain BI. Perhaps the most striking difference noted was the absence of amino sugars in the SPB of strain BI/2S_2. The SPB of strain BI(2)$_8$ also contained a lower percent of amino sugars compared with the SPB of the wild-type strain BI.

The observed chemical changes are not to be considered unique, as a review of the literature shows. For example, phage-resistant colonies originally isolated by Goebel and Jesaitis (9), called chemotype Re mutants, have been found to contain only lipid A, 2-keto-3-deoxyoctonate, and ethanolamine in the lipopolysaccharide. These mutants lacked heptose, galactosamine, glucosamine, and neutral sugars (18). The defect in the chemotype R mutants has been suggested to be due to a lesion in the synthesis or activity of a transferase which in the wild type incorporates the next sugar in the chain. Rosen et al. (23) showed that a phosphomannose isomeraseless mutant of *S. typhimurium*, having no O-antigenic side chains in its lipopolysaccharide, was resistant to a virulent derivative of phage P22. When this mutant was grown in the presence of mannose, it became sensitive to this phage as a result of formation of a complete lipopolysaccharide.

The possible association of neutral sugars and amino sugars as active moieties of the phage receptor sites is also suggested by the results of other experiments. The interaction of phage 2 depolymerase and its substrate, contained in SPB, has been observed to result in the release of neutral sugars and amino sugars (2). The depolymerase-SPB substrate interaction appears to play a major role in the adsorption of phage 2 (22). The receptor activity of SPB (and LPS) is lost after treatment with the phage 2 depolymerase (F. J. Castillo, and P. F. Bartell, Abstr. Annu. Meet Amer. Soc. Microbiol., 1974, V268, p. 243). However, the exact chemical and structural nature of the phage 2 receptors cannot be precisely defined at this time. The functional relationship of the receptor-like activity of SPB and LPS also remains to be determined.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant AI-08504 from the National Institute of Allergy and Infectious Diseases. F. J. Castillo was supported by a predoctoral scholarship from the Instituto Venezolano de Investigaciones Cientificas.

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