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Distinct Slime Polysaccharide Depolymerases of Bacteriophage-Infected *Pseudomonas aeruginosa*: Evidence of Close Association with the Structured Bacteriophage Particle

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Five new polysaccharide depolymerases were isolated from cultures of *Pseudomonas aeruginosa* infected with phages 6, 7, 8, 9, and 10. The production of enzyme paralleled the release of phage. Depolymerase associated with phage 8 was active on slime polysaccharide A, whereas depolymerases associated with phages 6, 7, 9, and 10, like pseudomonas phage 2, hydrolyzed slime polysaccharide B. None of the depolymerases was active on slime polysaccharide C. Despite exhaustive purification, depolymerase activity was found to band with the phage particles at a density of 1.49 to 1.51 g/ml in a density gradient composed to cesium chloride. These results suggest that the depolymerases are firmly bound to the phage particles.

Infection of *Pseudomonas aeruginosa* with phage 2 has been characterized by the synthesis of a slime polysaccharide depolymerase (6). Biosynthesis of this enzyme was observed to closely parallel the rate of formation of phage-directed virions, and alterations in experimental conditions of infection were reflected by alterations in the production of enzyme. The enzyme was not detectable in uninfected cultures, and experiments with chloramphenicol or an auxotrophic mutant suggested that de novo protein synthesis was essential for synthesis of enzyme. Various mutants of phage 2 have been observed to alter the synthesis of the depolymerase, further supporting the role of the phage genome in the synthesis of this enzyme (5).

After purification, the enzyme behaved as a single entity when tested by electrophoresis in acrylamide gel, and on the basis of gel filtration data the molecular weight was estimated to be 180,000 (4). Exposure of slime polysaccharide obtained from strain BI to the depolymerase resulted in a decreased viscosity and increased levels of hexosamines. However, the enzyme did not react with the slime polysaccharides of certain other strains of *P. aeruginosa*, suggesting a heterogeneity in the slime polysaccharides of this organism (6), which may account for the conflicting reports (8) regarding the composition of the slime polysaccharide of *P. aeruginosa*.

This communication describes the isolation of five new polysaccharide depolymerases from cultures of *P. aeruginosa* infected with phages 6, 7, 8, 9, and 10. The enzyme associated with phage 8 has a substrate specificity different from the enzyme associated with phages 6, 7, 9, and 10, which is similar to the enzyme associated with phage 2 (6). Evidence is presented that these enzymes are firmly bound to the phage particles.

MATERIALS AND METHODS

Microorganisms, cultivation, and assay. *P. aeruginosa* strains BI, CI, DI, EI, FI, and GI were isolated from various human clinical specimens, and the temperate pseudomonas phages 2, 6, 7, 8, 9, and 10 were isolated from lysogenic cells as previously described (6; P. Bartell, T. Orr, D. Shima, and I. S. Thind, Fed. Proc., p. 324, 1963). Trypticase Soy Broth and Agar (BBL) were employed for bacterial cultivation and phage propagation. Bacteriophage titrations were performed by soft agar-layer technique, and other general phage techniques were executed by the method of Adams (1).

Production of enzyme in phage-infected cultures. The elaboration of depolymerase in phage 2-infected bacteria was studied in phage growth experiments. These experiments were constructed as previously described in detail (5). Briefly, 5×10^8 cells/ml in the exponential phase of growth were infected with phage at a multiplicity of infection of 3. Adsorption at 37 C for 5 min with gentle shaking was followed by centrifugation and resuspension of sedimented cells in an original volume of prewarmed broth. Incubation was at 37 C in a shaker bath. Samples were obtained at

various intervals of time, immediately chilled, and centrifuged at 6,000 × *g* for 10 min at 4 C. Supernatant fluids were assayed for free phage and enzyme.

Partial-purification of enzymes. Large quantities of crude enzymes were obtained from soft agar-layer phage-infected cultures. After incubation at 37 C for 18 hr, the agar was scraped into cold 0.1 M sodium phosphate buffer (*p*H 7.5), homogenized, and treated with 10% chloroform. This was followed by centrifugation at 16,300 × *g* at 4 C for 30 min to sediment bacteria, agar, and debris. Supernatant fluids containing enzyme and phage were then centrifuged at 78,480 × *g* at 4 C for 60 min to sediment approximately 99% of the phage particles. Solid ammonium sulfate was added slowly with stirring to the above supernatant fluids to a final concentration of 45% of saturation and allowed to remain at 4 C overnight. The fine precipitate which developed was sedimented and dissolved in 0.1 M sodium phosphate buffer (*p*H 7.5) equal in volume to approximately 4% of the initial crude lysate, and was dialyzed against the same buffer at 4 C. These preparations were stored in the frozen state at −20 C.

Polysaccharide substrates. Polysaccharides were prepared from uninfected cultures of *P. aeruginosa* cultivated on sheets of cellophane overlaying Trypticase Soy Agar as previously described (6). Briefly, the slime polysaccharide was extracted in 0.15 M sodium chloride, and precipitated in three volumes of ethyl alcohol-acetone (equal volumes) at 4 C. The precipitate was sedimented by centrifugation and taken up in distilled water containing 0.1% sodium acetate. After two or three ethyl alcohol-acetone precipitations, the sediment was dissolved in distilled water, passed through several layers of Whatman no. 1 filter paper and dialyzed against distilled water for 20 hr at 4 C. The polysaccharides were then lyophilized and stored at 4 C in glass-sealed ampoules.

Estimation of enzyme activity. Assays were based on the release of hexosamines from polysaccharide substrates as previously described (6). The amount of hexosamine was determined by the method of Rondle and Morgan (10) as modified by Boas (7). Progress curves were constructed and the initial velocity was determined. A unit of enzyme activity was defined as nanomoles of hexosamine released per min per milligram of protein or per milliliter of sample. A standard of D-glucosamine hydrochloride (Eastman Organic Chemicals) was included in each hexosamine determination. A method of assay described by Adams and Park (2) was also useful in estimating enzyme activity. The details, as applied to this study, have been described (6). Briefly, various dilutions of enzyme were placed on mature lawns (24 to 48 hr) of a sensitive indicator strain and incubated at 37 C for 18 hr. The end point was taken as the reciprocal of the highest dilution which produced a perceptible clearing of the lawn. This assay was especially useful when sample sizes were limited.

Purification of phage. Phage lysates were produced in soft agar layer, harvested in cold 0.1 M sodium phosphate buffer (*p*H 7.5), homogenized and treated with 10% chloroform. The homogenate was then centrifuged at 12,000 × *g* at 4 C for 15 min. The supernatant fluid was then brought to 45% of saturation with solid ammonium sulfate, left at 4 C overnight, and centrifuged at 30,819 × *g* at 4 C for 30 min. The sediment was resuspended in 0.1 M sodium phosphate buffer (*p*H 7.5) and dialyzed against the same buffer. After dialysis the suspension was centrifuged at 86,500 × *g* at 4 C for 60 min. The sediment was then soaked in 0.1 M sodium phosphate buffer (*p*H 7.5) overnight, and then resuspended by gentle swirling. Centrifugation at 86,500 × *g* was repeated twice and the phage was finally suspended in 0.1 M sodium phosphate buffer (*p*H 7.5). The phage suspension was then banded by centrifugation in preformed gradients composed of cesium chloride (Matheson, Coleman, and Bell Chemical Co.). Buffered solutions of selected densities were prepared in 0.1 M sodium phosphate buffer (*p*H 7.5) and gradients were prepared (1.30 to 1.63 g/ml) by layering 1-ml quantities in a tube. The phage suspension (0.2 ml) was layered on top of the gradient and mineral oil was employed to bring the volume to the capacity of the tube. The gradients were centrifuged in the Spinco model SW 39 rotor at 86,000 × *g* for 2 hr. Fractions were collected dropwise from the bottom of the tube, and the actual density of each sample was determined by refractive index (Bausch and Lomb, model ABBE-3L refractometer). The material containing the main portions of the bands was pooled, dialyzed against 0.1 M sodium phosphate buffer (*p*H 7.5) to remove the CsCl, and then assayed for phage and depolymerase activity.

RESULTS

Appearance of polysaccharide depolymerases in phage-infected cultures. The infection of various strains of *P. aeruginosa* with pseudomonas phages was found to be characterized by the synthesis of polysaccharide depolymerases. As in the case of the previously described phage 2 system (6), these enzymes were detectable on lawns of phage-infected bacteria by the appearance of a well-defined translucent halo surrounding the plaque, and by the release of hexosamines when reacted with a suitable slime polysaccharide. In addition to the previously described phage 2 system, Table 1 lists five newly isolated pseudomonas phage-host cell systems which were found to produce polysaccharide depolymerases. Thus, pseudomonas phages 2, 6, 7, 8, 9, and 10 produce detectable depolymerases when they infect susceptible host cells as listed in Table 1. As an example, the phage growth curve shown in Fig. 1 depicts the production of depolymerase in cultures of *P. aeruginosa* strain EI after infection with phage 8. In these experiments, the enzyme was first detected, in small amounts, approximately 60 min after infection. This was followed by an accelerated release of enzyme from the infected cells, paralleling the release of phage, until maximal levels were reached. This sequence of events was almost

TABLE 1. *Depolymerases associated with phage-infected cells and the activity of these depolymerases on various slime polysaccharides of P. aeruginosa*

Phage	Host cell	Depolymerase	Depolymerase activity[a] on polysaccharides		
			A	B	C
2	BI	PDB_2	0	+	0
6	CI	PDB_6	0	+	0
7	DI	PDB_7	0	+	0
8	EI	PDA_8	+	0	0
9	FI	PDB_9	0	+	0
10	GI	PDB_{10}	0	+	0

[a] Determined by measuring release of hexosamines and by effect on mature lawns.

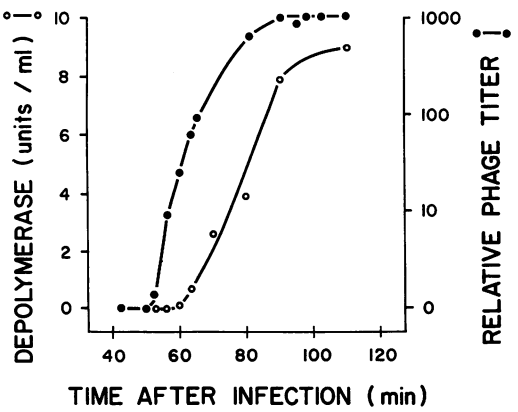

FIG. 1. *Release of phage and polysaccharide depolymerase from phage 8-infected P. aeruginosa strain EI.*

identical to that observed in the case of phage 2-infected *P. aeruginosa* strain BI (Fig. 2). Infection of strain BI with phage 8 was precluded by the failure to adsorb phage, and for the same reason strain EI could not be infected with phage 2.

Specific activity of the depolymerases. Partially purified depolymerases from each of the systems listed in Table 1 were reacted with the slime polysaccharides obtained from three different strains of *P. aeruginosa*. These polysaccharides were arbitrarily designated A, B, and C. As can be seen (Table 1), the depolymerases were active only on certain of the polysaccharides, and extracts prepared from uninfected log-phase cultures exhibited no depolymerase activity when reacted with the polysaccharides. Thus, the depolymerases of phages 2, 6, 7, 9, and 10 showed activity only when mixed with polysaccharide B. No activity was observed with polysaccharides A or C. On the other hand, the depolymerase of phage 8 was reactive with polysaccharide A, but not B or C. Thus, it was evident that the depolymerases produced by phages 2, 6, 7, 9, and 10, on the one hand, and phage 8 on the other, were quite different in regard to their substrate specificities. It should also be noted that slime polysaccharides extracted from other strains of *P. aeruginosa* were hydrolyzed by one or the other of these groups of depolymerases but not both. In addition, still other strains provided slime polysaccharides which were not suitable substrates for any of the depolymerases tested and these were referred to as polysaccharide C (Table 1), indicating an even greater heterogeneity in the slime polysaccharides of *P. aeruginosa*. To facilitate discussion, these depolymerases have been designated according to the reactive slime polysaccharide substrate as well as the phage which produces it. Thus, polysaccharide A depolymerase 8 (PDA_8) refers to the depolymerase synthesized by *P. aeruginosa* infected with phage 8. This depolymerase hydrolyzes slime polysaccharide A. PDB_2 refers to the phage 2-associated depolymerase which hydrolyzes slime polysaccharide B.

Relationship of depolymerases with the infective particle. Although the purification of PDB_2 has been described (4), an experiment was carried out to determine if the depolymerases might also be present in lysates in a form which was firmly bound to the phage particles. For this purpose, phages 2 and 8 were extensively purified as described above and the phage particles were then banded in a density gradient composed of cesium chloride. The results of these experiments are presented in Fig. 3 and 4. It can be seen that

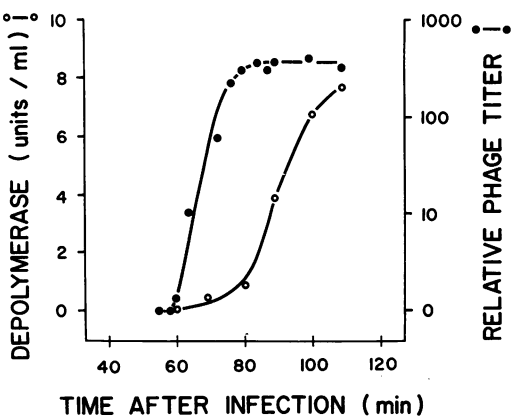

FIG. 2. *Release of phage and polysaccharide depolymerase from phage 2-infected P. aeruginosa strain BI.*

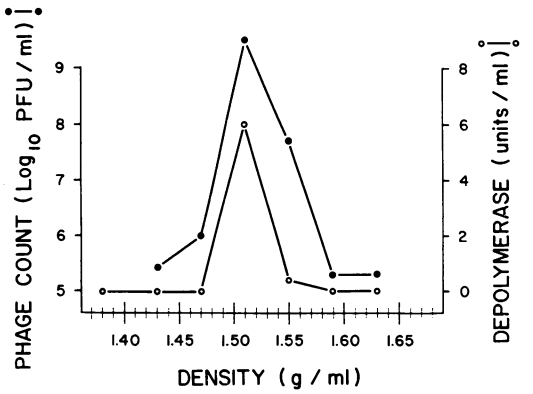

FIG. 3. *Band sedimentation of purified phage 8 in CsCl solutions of different density. Density of the CsCl was determined by measuring refractive index.*

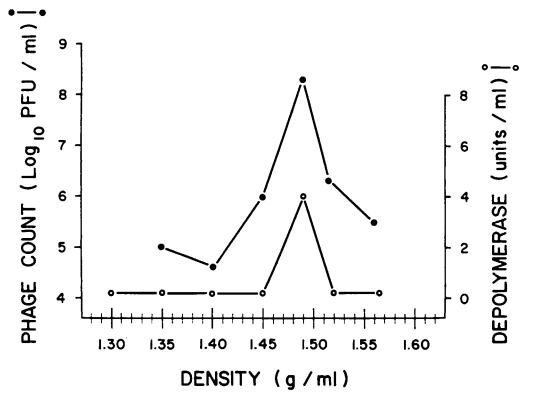

FIG. 4. *Band sedimentation of purified phage 2 in CsCl solutions of different density. Density of CsCl was determined by measuring refractive index.*

despite the exhaustive purification procedure PDA_8 activity was banded with the phage 8 particles, and PDB_2 activity was similarly found to be banded with phage 2 particles at a density of 1.49 to 1.51 g/ml. Thus, it must be concluded that these depolymerases are firmly associated with the phage particles.

DISCUSSION

The observations presented in this paper extend previous findings (4–6) relating to the polysaccharide depolymerase found in lysates of phage-infected *P. aeruginosa* and indicate, for the first time, that distinct phage-related slime polysaccharide depolymerases exist in nature. Five newly isolated depolymerases have been described, bringing the total number of known depolymerases in various phage-infected *P. aeruginosa* systems to six. The depolymerases designated PDB_2, PDB_6, PDB_7, PDB_9, and PDB_{10}, although produced in different host cells infected with various phages, all appear to hydrolyze the same slime polysaccharide B with the release of hexosamines. However, these results do not preclude the possibility that other products might also be released, offering points of difference amongst these enzymes, or that kinetic and physicochemical differences might also exist. In this connection, R. D. Humphrey and O. Wyss (Bacteriol. Proc., p. 174, 1969) demonstrated that the capsule depolymerizing enzymes from lysates of 2 of the 10 phages which attack *Azotobacter vinelandii* "O" (AVO) have different *p*H optima, heat stabilities, and electrophoretic mobilities. Sutherland (11) also was able to detect differences among phage enzymes which hydrolyze the polysaccharide of *Klebsiella aerogenes* A3. These differences were detected by the additional release of reducing substances when a second heterologous depolymerase was added to the same polysaccharide substrate after it had initially been reacted with its homologous depolymerase. In contrast to the above mentioned studies where various enzymes were reacted with the same substrate, the most significant aspect of the present study was the fact that at least two of the pseudomonas depolymerases exhibit distinct substrate specificities. For example, the depolymerase PDB_8 showed no detectable activity when it was mixed with slime polysaccharide B, indicating its specific activity only on polysaccharide A. On the other hand, depolymerase PDB_2 reacted specifically with polysaccharide B and showed no activity in the presence of polysaccharide A. These findings suggest a class of polysaccharide depolymerases which differ in their substrate specificities regarding the various slime polysaccharides of *P. aeruginosa*. Although no phage-induced enzyme reacting with the slime polysaccharide C has yet been recovered, its existence can be expected.

Knowledge regarding the slime polysaccharide of *P. aeruginosa* is at an early stage of development, and the literature regarding its chemical composition is contradictory (8). This, perhaps, can be accounted for by variations in the composition of the slime polysaccharides of different strains of *P. aeruginosa*. In a previous communication (6) attention was drawn to the possible heterogeneity of the slime polysaccharides when it was found that the PDB_2 depolymerase hydrolyzed the slime polysaccharides of some but not all strains of *P. aeruginosa*. The results of the present study have confirmed and extended these observations. Based upon the substrate specificities of these slime polysaccharide depolymerases there appear to be at least three

different types of slime polysaccharides. This heterogeneity of slime polysaccharides also has been suggested by the immunological typing of clinical isolates of *P. aeruginosa* with antisera produced with the slime polysaccharides of various strains (V. M. Young, R. Moody, D. M. Kenton, and G. Vermeulen, Bacteriol. Proc., p. 87, 1969).

The use of hydrolytic enzymes provides an extremely valuable method for the selective removal of capsular, slime, and other layers external to the rigid wall where phage receptor sites exist. In addition to their usefulness for investigating the anatomical relationships of the surface layers of the bacterial cell, the polysaccharide depolymerases are an important adjunct to chemical methods in studying the composition, structure, and biological properties of bacterial polysaccharides (12). The acquisition of these data should clarify the extent of chemical heterogeneity present in the slime polysaccharides of *P. aeruginosa*, which also has a great practical significance, since the slime polysaccharide of *P. aeruginosa* appears to function as a protective antigen in experimental infection (3, 9).

The banding of purified phage in a density gradient composed of cesium chloride indicates that depolymerases PDB_2 and PDA_8 are firmly bound to the phage particles. In conjunction with previous findings (4), this suggests that the depolymerases exist in two forms, one as a freely diffusible enzyme and the other, in a form which is firmly bound to the phage particle. However, since the enzyme may be nonspecifically adsorbed to the phage, it cannot be described as a structural component of the phage particle.

At the present time, it would be inappropriate to indicate a functional role for these enzymes in the phage life cycle. However, during the course of the present study, some observations were made which may well reflect a functional role for these depolymerases. Phage 2, which is associated with PDB_2, clearly did not adsorb to host cell EI which produces slime polysaccharide A. Phage 8, which is associated with PDA_8, did not adsorb to host cell BI which produces slime polysaccharide B. In addition, an enzyme-less mutant of phage 2, pdp_1 (5), was observed to adsorb at a significantly slower rate when compared with the wild-type phage 2. These results imply a selective advantage for those phages which possess a specific depolymerase. Despite these suggestive findings, the functional role of the depolymerases remains to be determined, and one may only speculate on their utility in various stages of the virus life cycle. For example, adsorption, penetration, and release of phage from encapsulated or slime-producing bacteria could be facilitated by the presence of the appropriate hydrolyzing enzyme, acting either alone or in concert with other enzymes.

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LITERATURE CITED

1. Adams, M. H. 1959. Bacteriophages. Interscience Publishers, Inc., New York.
2. Adams, M. H., and B. H. Park. 1956. An enzyme produced by a phage host-cell system. II. The properties of the polysaccharide depolymerase. Virology 2:719–736.
3. Alms, T. H., and J. A. Bass. 1967. Immunization against *Pseudomonas aeruginosa*. I. Induction of protection by an alcohol-precipitated fraction from the slime layer. J. Infec. Dis. 117:249–256.
4. Bartell, P. F., G. K. H. Lam, and T. E. Orr. 1968. Purification and properties of polysaccharide depolymerase associated with phage-infected *Pseudomonas aeruginosa*. J. Biol. Chem. 243:2077–2080.
5. Bartell, P. F., and T. E. Orr. 1969. Origin of polysaccharide depolymerase associated with bacteriophage infection. J. Virol. 3:290–296.
6. Bartell, P. F., T. E. Orr, and G. K. H. Lam. 1966. Polysaccharide depolymerase associated with bacteriophage infection. J. Bacteriol. 92:56–62.
7. Boas, N. F. 1953. Method for the determination of hexosamines in tissues. J. Biol. Chem. 204:553–563.
8. Brown, M. R. W., J. H. Scott Foster, and J. R. Clamp. 1969. Composition of *Pseudomonas aeruginosa* slime. Biochem. J. 112:521–525.
9. Liu, P. V., Y. Abe, and J. L. Bates. 1961. The role of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. J. Infec. Dis. 108:218–228.
10. Rondle, C. J. M., and W. T. J. Morgan. 1955. The determination of glucosamine and galactosamine. Biochem. J. 61: 586–589.
11. Sutherland, I. W. 1967. Phage-induced fucosidases hydrolysing the exopolysaccharide of *Klebsiella aerogenes* type 54 (A3(S1)). Biochem. J. 104:278–285.
12. Sutherland, I. W. and J. F. Wilkinson. 1968. The exopolysaccharide of *Klebsiella aerogenes* A3(S1) (Type 54). The isolation of O-acetylated octasaccharide, tetrasaccharide and trisaccharide. Biochem. J. 110:749–754.