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Differential Activities of Bacteriophage Depolymerase on Bacterial Polysaccharide: Binding Is Essential but Degradation Is Inhibitory in Phage Infection of K1-Defective *Escherichia coli*

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Host range mutants were derived from bacteriophages PK1A and PK1E specific for the K1 polysialic acid capsule of *Escherichia coli*. The mutants were selected for their ability to infect *E. coli* bacteria with a low level of the K1 capsule. A specific loss of the cleaving activity of the phage endosialidase was observed in all the mutants, while the ability to bind specifically to the polysialic acid capsule was retained. The results indicate that the polysaccharide-binding activity of the bacteriophage enzyme is essential for the infection process. The cleaving activity, in contrast, is required for the penetration of the dense polysaccharide of wild-type bacteria but is inhibitory in the infection of bacteria with a sparse capsular polysaccharide.

Capsular polysaccharides of bacteria often interact with bacteriophages, either by blocking access to the phage receptor beneath or by providing a specific adsorption site for a capsule phage. Capsule phages typically carry spikes which function as adsorption organelles and contain a capsule-depolymerizing enzyme (14, 23). The depolymerase is supposed to be required both for the binding of the capsular polysaccharide and for the degradation of the polysaccharide layer to reach the cell surface proper (3, 22, 23). However, the relationships of the binding and cleaving activities are not understood. It is not known to what extent the depolymerase activity is required for the infection process and how possible variations in the amount and density of the polysaccharide influence this process.

For the K1 polysialic acid capsule of *Escherichia coli* several specific bacteriophages have been described (6, 8, 11, 12). The K1 phages recognize the capsular polysialic acid as a primary cell surface receptor by their capsule-degrading endosialidase. It has been shown that several different K1 phages cleave the polysialic acid receptor substrate by a common endomechanism (4, 8, 11, 20).

In this study we have investigated the role of the phage depolymerase in the infection process using K1 phage and *E. coli* K1 as a model system. We have previously isolated mutants of *E. coli* K1 resistant to K1 phages (17, 18). Using mutant bacteria that have a low level of the capsular polysialic acid we have now isolated host range mutants of the K1 phages. This paper shows how these mutants have specifically lost the polysaccharide-cleaving activity of their endosialidase while retaining the binding activity. The results suggest that cleavage of the polysaccharide is not required and is even harmful for the phages that infect bacteria with a low level of polysialic acid, whereas the splitting activity is required for penetration of the dense polysaccharide of wild-type bacteria.

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MATERIALS AND METHODS

Phages and bacterial strains. Bacteriophages PK1A and PK1E have been described by Gross et al. (6). The following wild-type *E. coli* strains were used: IH3036, IH3044, IH3056, IH3080, and IH3088 (18); IH3064 (10); IH11038 (18); and K235 (11). *Pasteurella haemolytica* A2 was our own isolate. K1-defective PK1A-resistant mutant strains derived from *E. coli* IH3080 (EH908 to EH927) and rough *E. coli* IH3088 (EH928 to EH1016) have been described elsewhere (17, 18). *E. coli* mutants resistant to the mutant phages were isolated and characterized as described before (15, 17, 18). Monoclonal anti-polysialic acid antibody (5) was a kind gift of D. Bitter-Suermann, Hannover, Germany. L broth, L-agar plates, and soft agar were used for the cultivation of the organisms, and Penassay broth was used for the dilution of the organisms (20). Bacteria in the exponential growth phase were used in all experiments.

Isolation of mutant phages. Drops of serial dilutions of PK1A and PK1E were spotted to the plates seeded with K1-defective *E. coli* mutants and their parents in soft agar. Plaques appearing on K1-defective strains at a frequency of less than 10^{-4} times the titer of the phage on its host strain were picked and purified by three successive single-plaque isolations. The lysates were stored at 4°C on chloroform. The phages were propagated and purified on a large scale as described elsewhere (13, 15, 20). Phage proteins and DNA were characterized as described before (20).

Determination of host range. Host range was tested by spotting 10 μl of phage lysate diluted 10^{-2} onto freshly poured, seeded soft-agar overlays of different bacterial strains. The results are based on at least three independent assays.

Phage inactivation and adsorption assays. Phage inactivation and adsorption assays were performed as described by Mäkelä (15). The results are based on at least two independent assays. Inactivation of phage infection was studied by mixing 50 μl of phage dilution (5×10^3 PFU/ml) and 5 μl of the substance studied in water. After 30 min of incubation at 37°C, the mixture was poured in soft agar seeded with the

TABLE 1. Phage sensitivity of *E. coli* expressing different amounts of polysialic acid

Capsular phenotype	No. of strains	Sensitivity[a] of:					
		Wild type		Mutant			
		PK1A	PK1E	PK1A2	PK1A5	PK1A8	PK1E3
Wild-type K1 (parent)	1	S	S	R	R	R	S
Decreased K1 (3 ± 4)[b]	4	Si, R	Si, R	S	S	S	S
Decreased K1 (1 ± 1)[b]	18	R	R	S	S	S	S
No K1 polysaccharide	5	R	R	R	R	R	R

[a] Sensitivity of the rough wild-type strain and its isogenic mutants expressing little or no K1 polysaccharide is scored as follows: S, sensitive (lysis); Si, inhibition of bacterial growth (no lysis); R, resistant.
[b] Total cellular polysialic acid (percentage of parental value).

host strain onto an L-agar plate. Controls with no phage or no inactivating substance were included in all assays. Colominic acid (sodium salt) and *N*-acetylneuraminic acid were purchased from Sigma Chemical Co., St. Louis, Mo. The K1 extract was isolated and treated with mild acid as described previously (19).

For the assay of phage adsorption phage-resistant bacteria were washed in saline and suspended in the original volume. The phage dilution (5×10^3 PFU/ml) (50 μl), chloramphenicol (final concentration, 100 μg/ml), and bacteria (50 μl) were mixed. After 30 min of incubation at 37°C, bacteria were pelleted (10,000 × *g*, 3 min) and 50 μl of the supernatant was poured in soft agar seeded with the host strain on an L-agar plate. The host strain served as a positive control, and omission of the test bacteria gave the actual number of PFU in the assay. To study adsorption to bacteria grown at 18°C, the test bacteria were cultured for 18 h and suspended to the same density as the bacteria grown at 37°C. The bacteria were infected with phage at 20°C to prevent capsule synthesis during the assay.

Determination of endosialidase activity. An oligomer substrate of 12 sialyl units was purified from colominic acid by preparative fast protein liquid chromatography (FPLC) on a MonoQ column (Pharmacia) (9), labelled in the reducing end by tritium (19), and incubated with 2 μg (as protein) of purified phage in 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl in a volume of 50 μl at 37°C for the times indicated. The digestion products were analyzed by polyacrylamide gel electrophoresis as described before (19, 20).

RESULTS AND DISCUSSION

Isolation of phage mutants specific for low-polysialic acid *E. coli*. Phages were isolated on PK1A-resistant, K1-defective rough *E. coli* EH954 and EH1008 (17, 18). In both strains synthesis of the K1 polysaccharide is strongly decreased so that the amount of cell surface and total sialic acid is approximately 1% of the parental value. The polysialyl chains are exposed on the cell surface, as indicated by weak, even immunofluorescent staining and weak latex agglutination with the specific anti-polysialic acid antibody. The polysialic acid polymers are similar in length to those of the wild-type bacterial strains (17).

PK1A and PK1E produced plaques on bacterial strains at a frequency of $<10^{-4}$ times the titer obtained on K1-encapsulated *E. coli*, which suggested that the plaques were formed by mutated phages. After initial screening 4 phage mutants out of the 20 isolated were chosen for this study. The phages PK1A2, PK1A5, and PK1A8 (derived from PK1A) and PK1E3 (derived from PK1E) differed slightly in host range, had different plaque morphologies, and gave rise to phage-resistant bacterial mutants at different rates (results not shown). No phenotypic differences were observed after propagation of the phage mutants on either of the host strains, and the titers (about 10^9 to 10^{10} PFU/ml) obtained were as high as for the parental phages.

The mutant phages could specifically infect only bacteria with a low level of polysialic acid, while the wild-type phages could infect only strains with a complete wild-type capsule (Table 1). All the mutant *E. coli* strains with low levels (1 to 3%) of polysialic acid capsule were sensitive to the mutant phages and resistant to the wild-type phages, whereas the parent rough K1 strain was resistant to all phages (except to PK1E3), as were all the mutant strains lacking polysialic acid. Thus, the sensitivity of the bacterial strains to the wild-type and mutant phages correlated with the amount of the capsular polysaccharide present on the bacteria.

Binding of polysialic acid by mutant phages. The polysialic acid-binding property of the mutant phages was determined by assaying their binding to encapsulated wild-type bacteria. All the mutant and wild-type phages adsorbed to all K1-encapsulated *E. coli* strains, whereas no adsorption to an *E. coli* K5 strain was observed (results not shown). All the phages also efficiently adsorbed to a *P. haemolytica* A2 strain which bears a polysialic acid capsule (1). To confirm that the adsorption was based on polysialic acid, the target bacteria were grown at 18°C, at which temperature the K1 polysaccharide is not synthesized (25). Adsorption diminished remarkably when the wild-type bacteria were grown at 18°C, and the reduction was even more significant when bacteria with a reduced amount of capsule (1%) were used as targets (Table 2). These results showed that the mutant phages adsorb to both the wild-type and mutant bacteria and that the adsorption is based on binding to polysialic acid.

The role of polysialic acid in the infection process was also demonstrated by isolating spontaneous bacterial mutants resistant to the mutant phages. All 21 mutants derived from the K1 strains IH3088 and IH3064 had lost their surface polysialic acid because of resistance to the phage mutants (results not shown), as shown by a lack of reactivity with the anti-polysialic acid antibodies in a latex agglutination assay (18). The high yield of acapsular phenotypes among these bacterial mutants, compared with the yield among the mutants selected for resistance to PK1A (reference 15 and our own unpublished results), suggests that the presence of any polysialic acid on the bacteria suffices to control sensitivity to the phage mutants, whereas for the wild-type phages the amount or organization of the capsular polysialyl chains also plays a role.

Inhibition of phage infection by free polysialic acid. The interaction between the phage and its presumptive receptor

TABLE 2. Adsorption of phages to bacteria grown at 18°C

Strain	Growth temp (°C)	% Adsorbed[a]					
		Wild type		Mutant			
		PK1A	PK1E	PK1A2	PK1A5	PK1A8	PK1E3
IH3088 (wild-type K1)	37	91	83	83	67	99	96
	18	15	55	14	8	75	47
EH1008 (1% K1)	37	77	80	90	84	96	96
	18	0	0	0	0	33	0

[a] Percentage of phage particles adsorbed to the parent *E. coli* K1 strain and a mutant strain with a defective K1 capsule.

was studied by incubation of the phage particles with mono- and polysialic acid before infection of bacteria with the phage. All these structures, free sialic acid (*N*-acetylneuraminic acid), colominic acid (mixture of sialic acid oligomers and polymers), and a capsular extract from *E. coli* K1 containing long polysialic acid polymers (19), were effective, but to different degrees (Table 3). An equal amount (≤0.5 mg) of sialic acid was required to inhibit mutant and wild-type phages. As a control, a fivefold amount of *N*-acetylglucosamine had no effect on phage infection.

Incubation of the phage mutants with colominic acid abolished the infection capacity of the phages 100- to 1,000-fold more efficiently than sialic acid (Table 3). The parental phages required, at a minimum, 1,000-fold more colominic acid than the mutants to achieve 50% inhibition of phage infection. A similar rapid consumption of the substrate has been reported for *E. coli* K5 phage, which is only poorly inhibited by the K5 polysaccharide because of the continuous cleavage of the substrate (7). In the case of free sialic acid, which is not hydrolyzed, equal amounts were required for inhibition.

The K1 capsular extract inhibited infection by the mutant phages at a concentration 3 orders of magnitude lower than that of colominic acid (Table 3). The parental phages required 50- to 1,000-fold more K1 extract than the mutants for inhibition. The much higher sensitivity of the mutant and parent phages to the K1 extract than to colominic acid may be influenced by the size of the substrate. Colominic acid has an estimated maximum chain length of about 20 to 40 sialyl units, whereas the sialyl chains of the K1 extract may contain more than 200 sialyl units (16, 19, 21). The phage may bind to longer polysaccharides much more efficiently than to the shorter ones. Also, the degradation of the K1 extract into inefficiently binding sialyl oligomers may occur much more slowly than that of colominic acid. The high inhibitory potential of the K1 extract may also rely on additional bacterial components which interfere with phage infection, since the extract contains all the structures detached from the bacterial surface by incubation of bacteria in a pyridine acetate buffer (19). Such components additional to polysialic acid would be involved in the interaction of both the parent and mutant phages with the K1 extract, but the up to 1,000-fold difference between the phages in the sensitivity to the extract would arise from the differences in the endosialidase activity.

Molecular changes in mutant phage endosialidase. The endosialidase activity of the phages was investigated by incubating them with a radioactively labelled sialic acid oligomer and analyzing the cleavage products by gel electrophoresis. PK1A2 and PK1A5 did not show any cleavage of the polysialic acid substrate during 2 h of incubation (Fig. 1). In PK1A8 and PK1E3 we observed residual enzymic activity which required an incubation time of 2 h to achieve the same degree of cleavage of the substrate that was achieved with the parental phages in 1 min. Thus, the endosialidase activity of PK1A8 and PK1E3 is in the order of 100-fold less than that of the wild-type phages, whereas no enzyme activity could be detected in PK1A2 and PK1A5.

Comparison of the total phage proteins by gel electrophoresis did not show any differences between the mutant and parental phages (results not shown). Of special importance is the observation that neither the bands corresponding to the endosialidase enzyme of PK1E (24) nor the corresponding bands in PK1A were different in the mutants compared with those in the parent phages. The unchanged sizes of the endosialidase bands are in accordance with the persistence of residual enzyme activity in PK1A8 and PK1E3 and with the polysialic acid-binding activity of all the mutant phages.

No restriction fragment length polymorphism was observed between the parent and mutant phage DNA with the restriction enzymes *Hin*dIII, *Pvu*II, *Bgl*II, and *Pst*I. The patterns of DNA fragments of PK1A2, PK1A5, and PK1A8 were the same as those of PK1A, and the fragment patterns of PK1E3 were the same as those of PK1E (20) (results not shown).

In spite of the drastic decrease in the endosialidase activity the phage mutants were still able to bind to the polysialic acid capsule, as indicated by their binding specificity to

TABLE 3. Inhibition of phage infection by polysialic acid

Inhibitor	μg of inhibitor needed[a] for:					
	Wild type		Mutant			
	PK1A	PK1E	PK1A2	PK1A5	PK1A8	PK1E3
Sialic acid	250	250	250	250–500	125	250–500
Colominic acid	2,500	2,500	2.5	2.5	0.1	0.5
K1 extract	0.05–0.1	0.05–0.1	0.001	0.0001	0.0001	0.001

[a] The amounts of the inhibitory substance needed for the inactivation of 50% of 200 phage particles in 50 μl were determined. Each value is based on two independent assays.

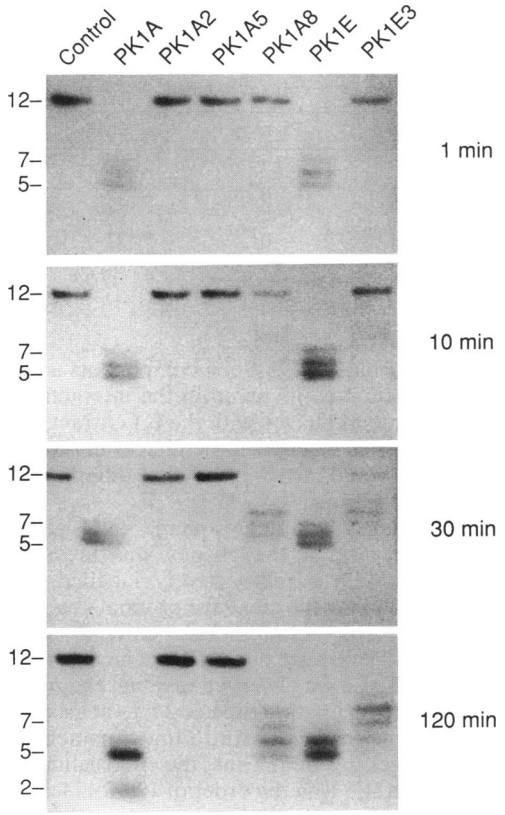

FIG. 1. Endosialidase activity of wild-type and mutant phages. The NaB^3H_4-reduced FPLC-purified substrate consisting of 12 *N*-acetylneuraminic acid units was incubated with the purified phage particles at 37°C. Samples were taken at the given time points and analyzed by gel electrophoresis followed by fluorography. Positions of the oligomers containing 5, 7, and 12 sialyl residues are indicated on the left. The control lane shows the substrate incubated without phage particles.

bacteria differing in polysialic acid expression. The up to 1,000-fold-increased sensitivity to inhibition by polysialic acid compared with that of the parents is consistent with the observations made for the decreased enzyme activity. It may result from the loss of splitting activity alone, or it may in part be enhanced by an increased binding affinity of the endosialidase to polysialic acid. Such an increased affinity could explain why PK1A8 and PK1E3 were highly sensitive to polysialic acid (Table 3) in spite of some residual endosialidase activity. The efficient binding of the mutant phages is also demonstrated by the fact that isolation of new mutant bacteria with the aid of the mutant phages resulted in each case in acapsular mutants. In conclusion, the loss of splitting activity of the endosialidase had occurred in a way that specifically preserved the polysialic acid-binding activity of the molecule and was most likely caused by a point mutation in the endosialidase gene.

Polysaccharide-binding and -cleaving activities in phage infection. Differentiation of the polysaccharide-binding activity from the polysaccharide-cleaving activity in the phage mutants and the availability of the bacteria differing in the amount of the capsular polysaccharide allow conclusions on the roles of these components in phage infection. The polysialic acid-binding property is necessary for the recognition of the receptor and therefore cannot be completely lost. All the wild-type and mutant phages studied had this property. Some capsule must also be present on the bacterial surface, since the acapsular mutant bacteria were completely resistant to all wild-type and mutant phages. The polysialic acid-cleaving activity of the enzyme appears to be necessary for the efficient infection of wild-type *E. coli* that has a complete polysialic acid capsule. The phage mutants had lost the ability to infect efficiently most wild-type *E. coli* K1 strains along with the loss of endosialidase activity. In contrast, the cleaving activity seems to be a disadvantage in the infection of mutant bacteria bearing a sparse capsule.

The enzymic activity may affect the infection of the sparsely encapsulated bacteria in two hypothetical ways. For the first, the sparse polysaccharide chains may rapidly become cleaved by the enzyme, which would result in the elution of the phages from the bacterial surface. In the case of the mutant phages, inactivation of the cleaving activity would prevent the degradation of the polysaccharide and allow the phage to remain adsorbed to bacteria with few surface polysaccharide receptors. The adsorption would be furthermore facilitated by the increased binding affinity of the mutant phage endosialidase. For the second, the endosialidase in its active form may require the presence of a dense capsular layer for the degradation of the capsule and movement of the phage along the capsular strands to the bacterial cell surface. Such a property has been suggested for the K29 phage of *E. coli* (2). In spite of the inactivation of the cleaving activity of the endosialidase, the mutant phages may have kept a capacity to move along polysialic acid chains or acquired a more efficient binding affinity to the few polysialic acid chains present on the bacterial surface.

The observations of this study might apply more generally to the infection process of encapsulated bacteria by bacteriophages. The property to bind to and degrade the capsular polysaccharide layer must have evolved as a vital step for the bacteriophages capable of infecting encapsulated bacteria. But if the capsule layer is sparse, there is no need for the phage to degrade it, and as shown in the present study, a cleaving activity may even be inhibitory for the infection process. This would explain the fact that not all capsule phages show capsule depolymerase activity (22).

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