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Lipopolysaccharide-Specific Bacteriophage for *Klebsiella pneumoniae* C3

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Bacteriophage FC3-1 is one of several specific bacteriophages of *Klebsiella pneumoniae* C3 isolated in our laboratory. Unlike receptors for other *Klebsiella* phages, the bacteriophage FC3-1 receptor was shown to be lipopolysaccharide, specifically the polysaccharide fraction (O-antigen and core region). We concluded that capsular polysaccharide, outer membrane proteins, and lipid A were not involved in phage binding. Mutants resistant to this phage were isolated and were found to be devoid of lipopolysaccharide O-antigen by several criteria but to contain capsular material serologically identical to that of the wild type. The polysaccharide fraction was concluded to be the primary phage receptor, indicating that it is available to the phage.

Klebsiella pneumoniae C3 is a frequently encountered freshwater isolate, and some of its interesting metabolic properties have been studied in various laboratories (6, 13, 14, 24). Bacteriophage FC3-1, isolated from *K. pneumoniae* C3, has been characterized (20). Antiserum raised against bacteriophage FC3-1 is able to cross-react with other bacteriophages isolated from *K. pneumoniae* (20). Furthermore, there is a high degree of morphological similarity among these phages. Unlike other enterobacteria, this strain contains a large capsular polysaccharide, and it was of interest to determine the nature of the receptor for bacteriophage FC3-1. In this report, we demonstrate that lipopolysaccharide (LPS) is the receptor for phage FC3-1. As far as we are aware, FC3-1 is the only bacteriophage isolated from *Klebsiella* spp. whose receptor is LPS.

MATERIALS AND METHODS

Bacteria, bacteriophages, and media. *K. pneumoniae* C3 was described previously (6, 13), and bacteriophages FC3-1, FC3-2, FC3-3, FC3-4, FC3-6, and FC3-7 were partially characterized previously (20). The basal medium used for bacterial growth and phage propagation was Luria broth (LB) (20) or LB with 1.5% agar (LB-agar). To prepare soft agar, we added 0.6% agar to LB (LB-soft agar). For titration and inactivation assays, phage suspensions were diluted in phage buffer (7).

Bacteriophage FC3-1 was labeled with $^{32}P_i$ as follows. *K. pneumoniae* C3 was grown in minimal medium (phosphate free) containing 33 μM $^{32}P_i$ (26.4 μCi/ml) and 10 mM glucose as a carbon and energy source. Bacteriophage FC3-1 was plated on these cells in LB-soft agar and incubated for 8 h at 30°C. [^{32}P]FC3-1 was recovered from the lysates after centrifugation to remove the cells (10^6 PFU/4,560 cpm).

Spontaneous mutants of *K. pneumoniae* C3 resistant to phage FC3-1 were isolated by spreading a mixture containing ca. 10^8 bacteria and 10^9 phage PFU on LB-agar. After 36 h at 30°C, colonies of phage-resistant mutants were picked and purified by streaking and were cross-streaked against FC3-1 to confirm resistance.

Phage inactivation experiments. Bacteriophages (10^3 PFU) were incubated for 20 min at 30°C with one of the following: 10^7 cells, 200 μg of 1% deoxycholate (DOC)-solubilized outer membrane (OM) (19), 200 μg of phenol-treated, 1% DOC-solubilized OM (LPS removed) (11), 200 μg of purified LPS (unless otherwise indicated), or 50 μg of purified LPS O-antigen or LPS core. Chloroform (3 to 4 drops) was added and mixed for 60 s, and the mixture was centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatants were assayed directly on *K. pneumoniae* C3.

[^{32}P]FC3-1 (2×10^6 PFU) was incubated at 30°C with 2×10^7 *K. pneumoniae* C3 cells. At various times, 100-μl aliquots were either centrifuged at 12,000 × *g* for 10 min or filtered through 0.4-μm filters (Millipore Corp.) and then washed twice with 5 ml of minimal medium. The supernatants or filtered cells were assayed for radioactivity.

Cell surface isolation and analyses. Periplasmic proteins were released by osmotic shock (27). Cell envelopes were prepared by French pressure cell lysis at 16,000 Pa of whole cells followed by the removal of unbroken cells at 10,000 × *g* for 10 min and by the sedimentation of the membrane fraction at 100,000 × *g* for 2 h. Cytoplasmic membranes were solubilized twice with sodium lauryl sarcosinate (10), and the OM fraction was sedimented twice at 100,000 × *g* for 2 h. OM proteins were solubilized in 1% DOC–2 mM EDTA (19). To remove the LPS from the solubilized OM proteins, extracted samples were treated with 88% phenol at 70°C as described by Hancock and Nikaido (11).

Membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by a modification (2) of the Laemmli procedure (15). Protein gels were routinely stained with Coomassie blue. Protein concentrations were determined by the Lowry procedure (16) with bovine serum albumin as the standard.

LPS was purified by the method of Westphal and Jann (26) as modified by Osborn (18). To obtain the O-antigen polysaccharide, core oligosaccharide, and lipid A components, we dissolved the purified LPS in 1% acetic acid and hydrolyzed it at 100°C for 2 h as described by Schmidt et al. (21). The precipitated lipid A was removed by centrifugation and washed with 1% acetic acid. The supernatant and acetic acid washes were freeze-dried and then dissolved in 47 mM pyridinium acetate buffer (pH 4.26). After traces of lipid A were removed by centrifugation, the material (lipid A-free O-antigen polysaccharide) was fractionated on Sephadex G-50 to purify O-antigen polysaccharide and core oligosaccharide components (22).

For chemical analyses, purified LPS was hydrolyzed with 1 N HCl for 2 h at 100°C. Colorimetric analyses of the

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TABLE 1. Inactivation of bacteriophage FC3-1 by whole cells on OM components

K. pneumoniae C3 strain	% Inactivation of bacteriophage FC3-1 by:						
	Whole cells	DOC-solubilized OM[a]			LPS[b]		
		Without phenolysis[c]	With phenolysis[c]	With proteinase K treatment[d]	Complete	Lipid A-free O-antigen polysaccharide[e]	Lipid A[f]
Wild type	90.0	60.2	<1.0	59.8	84.1	57.2	<0.1
FC3-1-resistant mutant	<0.1	<0.1	NT[g]	NT	<0.1	NT	NT

[a] OM solubilized with 1% DOC–1 mM EDTA (19).
[b] Purified LPS (200 μg) (18, 26).
[c] With or without treatment with 88% phenol at 70°C (11).
[d] Treatment with proteinase K (10 μg/ml) for 2 h at 45°C.
[e] LPS fraction free of lipid A, as indicated by the absence of hexosamine (12).
[f] LPS fraction containing lipid A, as indicated by the absence of 2-keto-3-deoxyoctulosonic acid (12).
[g] NT, Not tested.

2-keto-3-deoxyoctulosonic acid and L-glycero-D-manno-heptose (heptose) contents of LPS were performed by the method of Osborn (18). Organic phosphate was assayed by the method of Bartlett (3). Monosaccharides were also analyzed as their alditol acetate derivatives by gas-liquid chromatography on a 3% SP-3840 column (Supelco Inc.) at 225 and 180°C. Alditol acetate monosaccharides were obtained by the following derivatization procedure (bulletin 774A, Supelco Inc., Cras, Switzerland). Samples (50 mg) were neutralized with an equal volume of 0.5 N $NaBH_4$ for 60 min at room temperature and acetylated by the dropwise addition of glacial acetic acid until bubbling stopped. Dried samples were washed twice with 0.1 ml of concentrated HCl per 100 ml of methanol and with ethanol. The supernatant was dried under N_2, suspended in equal volumes of pyridine and acetic anhydride, and heated at 100°C for 15 min. After being cooled, the samples were injected into a chromatograph. Alditol acetate carbohydrate standards were either purchased from Supelco or prepared by us.

Purified LPS was further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver stained by the method of Tsai and Frasch (25).

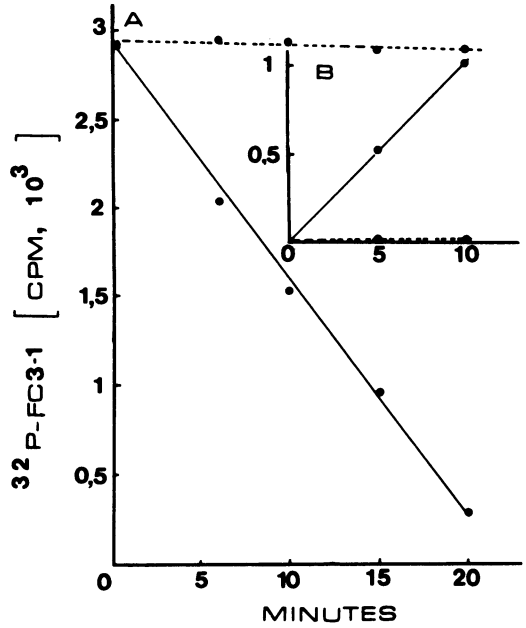

FIG. 1. Binding of ^{32}P-labeled bacteriophage FC3-1 to *K. pneumoniae* C3 strains. Bacteriophage FC3-1 was labeled by lysis of $^{32}P_i$-labeled cells of *K. pneumoniae* (wild type). Phage binding was assayed by the disappearance of ^{32}P-phage (A) and by direct binding to whole cells (B). Background adsorption was determined with *Escherichia coli* C600 as a negative control. ——, *K. pneumoniae* C3 (wild type); – – –, *K. pneumoniae* C3 mutants resistant to bacteriophage FC3-1.

RESULTS

Mutants resistant to bacteriophage FC3-1 occurred at a frequency of ca. 4×10^{-6} and fell into a single class based on their phage sensitivity patterns. The parent strain, *K. pneumoniae* C3, was sensitive to all of the six related phages (FC3-1, FC3-2, FC3-3, FC3-4, FC3-6, and FC3-7). All of the 20 phage FC3-1-resistant mutants tested were also resistant to all the other bacteriophages mentioned above.

Phage FC3-1 adsorbed readily to *K. pneumoniae* C3 but not to the resistant mutants (Table 1), suggesting that phage resistance was due to an altered phage receptor. As a confirmation of this observation, we examined the characteristics of adsorption of ^{32}P-labeled bacteriophage FC3-1 to cells of *K. pneumoniae* C3 (wild type) and the FC3-1-resistant mutants. In a phage inactivation assay (Fig. 1A), only the parent strain caused the loss of ^{32}P-labeled bacteriophage FC3-1 from the incubation mixture; in a filter assay (Fig. 1B), only wild-type cells bound ^{32}P-labeled bacteriophage FC3-1. Under these conditions, the FC3-1-resistant mutants had less than 0.01% of the [^{32}P]FC3-1-binding activity of the wild type.

DOC-solubilized OM from *K. pneumoniae* C3 (wild type) was able to inactivate phage FC3-1, but DOC-solubilized OM from FC3-1-resistant mutants was unable to inactivate the phage (Table 1). DOC-solubilized OM always contained both LPS and OM proteins; to remove the LPS, we used the method of Hancock and Nikaido (11). When LPS was removed by this method, DOC-solubilized OM no longer inactivated bacteriophage FC3-1; however, these conditions also denatured the OM proteins. When we removed the OM proteins by proteinase K digestion, no differences were found in the ability of DOC-solubilized OM from the wild type to inactivate bacteriophage FC3-1 (Table 1).

Purified LPS from *K. pneumoniae* C3 (wild type) was able to inactivate bacteriophage FC3-1, but purified LPS from one of the FC3-1-resistant *K. pneumoniae* mutants was not. The degree of phage inactivation was directly related to the concentration of LPS in the incubation mixture (Fig. 2). Under the conditions of this experiment, about 0.9 μg of LPS was required to reduce the phage titer by 50%. Furthermore, lipid A-free O-antigen polysaccharide was still able to inactivate phage FC3-1 (Table 1), but purified lipid A was

without effect. Neither core oligosaccharide nor O-antigen polysaccharide components, when fractionated, were able to inactivate phage FC3-1 (data not shown).

We also examined the protein composition of the membranes of *K. pneumoniae* C3 (wild type) and the FC3-1-resistant mutants. Some interesting differences appeared in the OM protein composition between the wild-type and the FC3-1-resistant mutants (Fig. 3A). Specifically, the major OM proteins of 35- to 40-kilodaltons (kDa), which are presumably the porins of *K. pneumoniae* C3, were present in the mutants but at a decreased mass of ca. 2 kDa. Also, a small protein of 24 kDa was practically absent in the mutants. We also examined purified LPS from both the wild type and the FC3-1-resistant mutants by the method of Tsai and Frasch (25) (Fig. 3B). There was an apparent complete loss of the O-antigen in the purified LPS from the FC3-1-resistant mutants, confirming the inactivation data of Table 1 and indicating that the O-antigen was lost rather than altered in structure.

Finally, the chemical analyses of purified LPS from *K. pneumoniae* C3 (wild type) and from the FC3-1-resistant mutants are shown in Table 2. These results indicate that ribose and arabinose were lost from the LPS of the FC3-1-resistant mutants and that xylose appeared in the LPS of the FC3-1-resistant mutants, perhaps as a substitution for ribose and arabinose. As expected, the quantities of 2-keto-3-deoxyoctulosonic acid and heptoses (LPS core) per milligram of LPS increased in the LPS of the FC3-1-resistant mutants in comparison with the LPS of the wild type; these data are consistent with the O-antigen-deficient composition of the LPS of the FC3-1-resistant mutants.

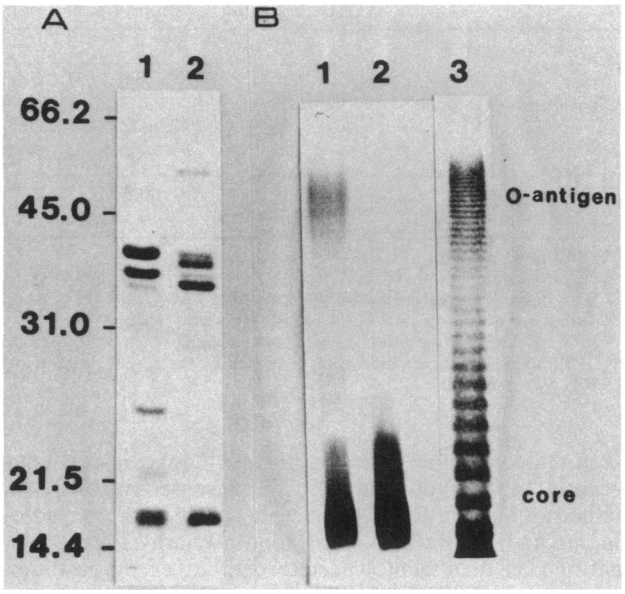

FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of OM proteins and LPS from *K. pneumoniae* C3 strains. (A) OM proteins were obtained as sodium lauryl sarcosinate-insoluble material (10). Molecular size standards (14.4, 21.5, 31.0, 45.0, and 66.2 kDa) from Bio-Rad Laboratories were used. Each lane contained 40 μg of protein. Lanes: 1, *K. pneumoniae* C3 (wild type); 2, a *K. pneumoniae* C3 FC3-1-resistant mutant. (B) Purified LPS was assayed by the method of Tsai and Frasch (25). Lanes: 1, *K. pneumoniae* C3 (wild type); 2, a *K. pneumoniae* C3 FC3-1-resistant mutant; 3, *Salmonella typhimurium* (smooth strain) used as a control for the O-antigen and LPS core.

DISCUSSION

FC3-1 is a bacteriophage classified in the *Myoviridae* family by Matthews (17) and in group A2 by Ackermann (21). *K. pneumoniae* C3 FC3-1-resistant mutants were unable to bind this phage when assayed directly by binding whole cells. Phage inactivation was found in DOC-solubilized OM from wild-type *K. pneumoniae* C3, indicating that the phage receptor is an OM component. To find out which structural components of the OM act as the receptor for bacteriophage FC3-1, we treated DOC-solubilized OM with 88% phenol at 70°C to remove the LPS or with 10 μg of proteinase K per ml for 2 h at 45°C. No differences were found in phage inactivation between the untreated DOC-solubilized OM and the proteinase K-treated DOC-solubilized OM, thus excluding OM proteins as FC3-1 receptors. Also, when LPS was extracted with hot phenol, the DOC-solubilized OM was no longer able to inactivate bacteriophage FC3-1. Furthermore, purified LPS from *K. pneumoniae* C3 (wild type) was fully able to inactivate bacteriophage FC3-1, and the quantity of LPS added to the incubation mixture was directly related to the degree of phage inactivation. Purified LPS from FC3-1-resistant mutants was unable to inactivate bacteriophage FC3-1.

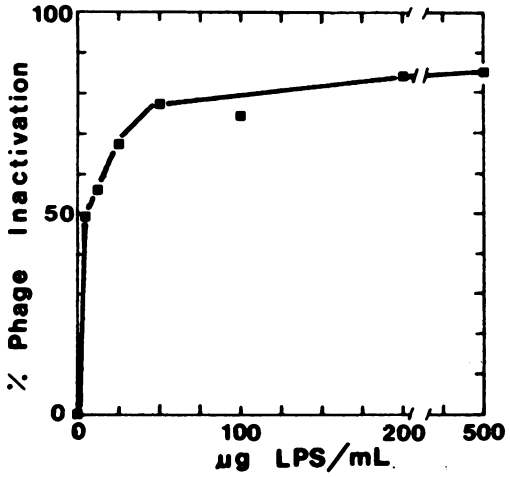

FIG. 2. Inactivation of phage FC3-1 with purified LPS from *K. pneumoniae* C3 (wild type), as determined by the method described in the text.

Thus, it was apparent that LPS alone is the true receptor for bacteriophage FC3-1. To confirm this conclusion, we studied the chemical composition of LPS from the wild type and from the FC3-1-resistant mutants. From these studies and from the lack of a high-molecular-weight carbohydrate in LPS gels, we easily concluded that the LPS from the FC3-1-resistant mutants lacks the O-antigen. Also, O-antigen, after lipid A was removed, was a potent inactivator of bacteriophage FC3-1.

From the chemical composition of LPS from both the wild type and the mutants, we concluded that there was a loss of some hexosamine, ribose, and arabinose, which suggests that these are components of the O-antigen. The mutants appear to have substituted xylose onto their remaining core oligosaccharides. It may be that the insertion of xylose stops the elongation of LPS in the mutants, preventing the insertion of the O-antigen to the inner core. *K. pneumoniae* C3 has been serotyped as K-21 (data not shown), and the FC3-1-resistant mutants belong to the same serotype. For all

TABLE 2. Chemical composition of LPS from *K. pneumoniae* C3 (wild type) and from one of the FC3-1-resistant mutants

LPS from *K. pneumoniae* C3 strain	Amt (μmol/mg of LPS) of:							
	2-Keto-3-deoxy-octulosonic acid[a,b]	Heptoses[a,b]	Hexosamines[b]	Hexoses[b]	Ribose[b]	Arabinose[b]	Xylose[b]	Organic phosphate[c]
Wild type	0.035	0.28	0.52	0.10	0.14	0.13	0	0.72
FC3-1-resistant mutant	0.102	0.71	0.26	0.10	0	0	0.12	0.73

[a] Assayed by colorimetric methods (12).
[b] Assayed by gas-liquid chromatography.
[c] Assayed by the method of Bartlett (3).

bacteriophage FC3-1 and that no capsular polysaccharide is involved.

The changes observed in the OM protein composition between the wild type and the FC3-1-resistant mutants are possibly due to LPS differences. LPS and OM protein interactions have been well documented (2, 4, 23). The change in the LPS composition appears to change the expression of a family of major OM proteins which probably includes porins. Only minor amounts of wild-type porins were present in the mutants, and an enhanced expression of (presumably) porins with lower (ca. 2-kDa) masses was seen. These were only minor bands in the wild type. The reason for this altered expression is presently unknown.

LPS O-antigen is normally a potent endotoxin (5). It will be of interest to obtain FC3-1-resistant mutants of highly pathogenic isolates of *K. pneumoniae* and, by comparing the toxicities of the wild type and such mutants, to find out more about the role of the O-antigen in the infection process and in the development of pathogenesis. Little is known of the molecular basis of pathogenesis of *K. pneumoniae*. We have already successfully tested highly pathogenic isolates, such as *K. pneumoniae* KP1-O (8), for FC3-1 sensitivity and posterior attainment of FC3-1-resistant mutants.

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