Pseudomonas aeruginosa Bacteriophage ϕ PLS27-Lipopolysaccharide Interactions

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Received 19 February 1981/Accepted 2 July 1981

We investigated the ϕ PLS27 receptor in *Pseudomonas aeruginosa* strain PAO lipopolysaccharide (LPS) by analyzing a resistant mutant. This mutant, which was designated AK1282, had the most defective LPS yet reported for a *P. aeruginosa* rough mutant; this LPS contained only lipid A, 2-keto-3-deoxyoctonate, heptose, and alanine as major components. In addition, this LPS lacked galactosamine, which is present in the inner core of the LPS of other rough mutants. The loss of galactosamine but only a small decrease in the alanine content indicated that the core of strain PAO LPS differed from the core structure which has been suggested for the LPS of other well-characterized *P. aeruginosa* strains. Our analysis also indicated that galactosamine residues may be crucial for ϕ PLS27 receptor activity of the LPS. Electrodialysis of LPS and conversion to salt forms (sodium or triethylamine) influenced the phage-inactivating capacity of the LPS, as did the medium in which the inactivation occurred; experiments performed in 1/10-strength broth resulted in much lower PhI_{50} (concentration of LPS causing a 50% decrease in the titer of phage during 1 h of incubation at 37°C) values than experiments performed in regular-strength broth. Sonication of the LPS also increased the phage-inactivating capacities of the LPS preparations.

To try to resolve some of the controversy surrounding the role of *Pseudomonas aeruginosa* lipopolysaccharide (LPS) in pathogenesis, as well as in other properties of this organism, we isolated several LPS-specific phages (22, 23, 25, 28) and used them to enrich for a number of LPS-defective mutants (24, 27, 29). However, the most LPS-defective mutants (strains AK43 and AK1012) that we isolated by phage selection still contained 2-keto-3-deoxyoctonate (KDO), heptose, galactosamine, and alanine in the core polysaccharide (24, 25). This led to the successful search for a rough LPS-specific phage, designated ϕ PLS27, which lysed these mutants and, in addition, was inactivated specifically by the isolated rough LPS (25).

In this work we used ϕ PLS27 to isolate the most defective LPS mutant thus far described for *P. aeruginosa*. The LPS of this mutant lacks galactosamine, and this information is the first indication that the location of alanine in strain PAO LPS is different from the location of alanine in the LPS of other *P. aeruginosa* strains (11, 26). An analysis of the LPS of ϕ PLS27-sensitive and ϕ PLS-resistant cells indicated that

galactosamine may be vital for ϕ PLS27 receptor activity.

In addition, we also examined the effects of different salt forms of electrodialyzed LPS on the capacity of LPS to inactivate ϕ PLS27.

MATERIALS AND METHODS

Bacteria and phage strains. *P. aeruginosa* strain PAO1 was kindly provided by B. W. Holloway. The rough derivatives AK1012 and AK44 (derived from strain PAO307) have been described previously (22, 25). Recently, the rough LPS-specific phage ϕ PLS27 has also been characterized extensively (25). The other phages used were the pilus-specific phages M6, C5 (2), and F116L (33), the smooth LPS-specific phages E79 (23) and PB1 (28), the rough specific phages ϕ PRS43 and ϕ PLS44 (29), and coliphage T7 (from the American Type Culture Collection).

Phage titrations. Phage plaque assays were performed by the agar overlay technique (1). The top and bottom layers consisted of tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) containing 0.6 and 1.5% (wt/vol) agar, respectively.

Isolation of ϕ PLS27-resistant mutants. An overnight culture of strain AK1012 was mixed with 0.1 ml of a ϕ PLS27 lysate (10^9 PFU/ml), and this mixture was spread onto a TSB plate and incubated at either 37 or 30°C. The colonies that appeared after 24 h were purified by two single-colony isolations and then tested for ϕ PLS27 sensitivity. A resistant colony (strain AK1282) was isolated; this strain was also

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resistant to the smooth LPS-specific phages E79 and PB1 and to the core-specific phage ϕ PLS44. Strain AK1282 was identified as *P. aeruginosa* by using API 20E strips (API Laboratories, St. Laurent, Canada); its reactions were identical to the reactions of strain AK1012. Furthermore, strain AK1282 was also sensitive to ϕ PRS43 and to the pilus-specific phages C5, M6, and F116L.

Growth of cells. The bacteria which were used in LPS extractions were grown in 5 liters of TSB by using a 10% (vol/vol) inoculum of overnight cells in an S.M.S. Hi-density fermentor (Lab-Line) at 37°C with an agitation rate of 200 rpm and an aeration rate of 8 to 10 liters/min. The growth of the bacteria was followed by measuring the absorbance at 650 nm with a Beckman model B spectrophotometer. The cells were harvested by centrifugation at 4,200 rpm for 45 min with a Beckman model J6 centrifuge when they reached early stationary phase; then they were washed once and lyophilized.

Isolation of LPS. LPS was extracted from lyophilized cells by using the phenol-chloroform-petroleum ether method (18).

Chemical analysis of LPS. Chemical analyses of purified LPS were performed as described previously (24).

Determination of soluble amines. LPS (~3 mg) was digested with 10 N HCl (0.10 ml) for 5 min at 0°C, diluted with ice-cold EDTA (0.5 ml of a 50- μ g/ml solution), and shaken vigorously with 0.5 ml of chloroform. After centrifugation to separate the phases, 0.4 ml of the aqueous phase was removed and evaporated in a desiccator over KOH. The resulting residue was dissolved in 50 μ l of water, and 10 μ l was used for determination of amino compounds by the method of Dubin (12), using *O*-phosphorylethanolamine as the standard.

Determination of cation concentrations. The concentrations of Na, Ca, and Mg in LPS preparations were determined by atomic absorbance spectroscopy, using an atomic absorption flame emission apparatus (Fisher Scientific Co., Pittsburgh, Pa.). The concentrations of Na, Ca, and Mg were measured by using analytical spectral lines at 589.23, 423.0, and 285.4 nm, respectively.

Thin-layer chromatography. Thin-layer chromatography of amino compounds was performed after hydrolysis of samples at 105°C for 4 h with 6.1 N HCl by using a two-dimensional system (41) on precoated cellulose thin-layer chromatography plates (BDH Chemicals Ltd.). Spots were visualized with ninhydrin. To detect neutral sugars, chromatograms were run and developed by the method of Hansen (21) after samples were hydrolyzed with 1 M HCl for 4 h at 100°C.

Modification of LPS. (i) **Dinitrophenylation.** Dinitrophenylation of strain AK44 LPS was performed exactly as described by Rietschel et al. (38). The product was a light yellow material, was obtained in a 70% yield, and dissolved to give a clear solution in water at a concentration of 1 mg/ml.

(ii) **N-acetylation.** N-acetylation of strain AK44 LPS was performed basically as described by Roseman and Daffner (40), except that after the 10-min reaction at room temperature, the reaction mixture was placed

in dialysis tubing and dialyzed against cold distilled water overnight. Lyophilization produced a white material in an 88% yield, which dissolved easily in water at a concentration of 1 mg/ml. Dinitrophenylation of the treated LPS indicated 60% modification.

(iii) **Deamination.** Deamination of strain AK44 LPS was performed by the method of Dische and Borenfreund (9). The material was then dialyzed overnight and lyophilized; a white material was produced in an 86% yield. This material was somewhat more difficult to dissolve in water than the other products. A total of 60% of the reactive amines were chemically modified, as indicated by dinitrophenylation.

(iv) **Amidase treatment.** In an attempt to remove the alanine from strain AK1012 LPS, this LPS was subjected to treatment with aminopeptidase M (Boehringer Mannheim Corp., New York, N.Y.). Strain AK1012 LPS was dissolved at a concentration of 5 mg/ml in 0.06 M phosphate buffer (pH 7.0) (34) and incubated with 0.35 U of enzyme per ml for 30 min at 37°C. In a control, strain AK1012 LPS was incubated with buffer alone. The reaction mixture and control were centrifuged at 100,000 $\times g$ for 2 h, and the pellets were resuspended and centrifuged again at 100,000 $\times g$ for 2 h. The material in the pellets was lyophilized, electrodialed, and converted to its triethylamine form before it was examined for chemical composition and phage-inactivating properties.

(v) **Electrodialysis of LPS.** Approximately 50 mg of LPS was dissolved in 10 ml of deionized distilled water by successive 30-s rounds of heating and sonication. The LPS was placed in dialysis sacs, which were put into a gel-staining apparatus (Canalco, Rockville, Md.). The apparatus was encased in ice, and electro dialysis was performed for 6 to 7 h at 9 to 10 mA; the water in the chambers was changed every 1.5 h. The pH decreased from a starting value of 6.8 to 7.0 to a value of 3.6 to 3.8 during this time. For strain AK44 LPS, prolonged electro dialysis under the above-described conditions did not reduce the pH below 4.1. Consequently, a current of 50 mA was used with strain AK44 LPS preparations, and the pH under these conditions reached 3.8 after 6 h. At pH 3.8, the LPS preparations formed gels which had to be removed from the dialysis casing. The acidic LPS was neutralized with NaOH or triethylamine and then lyophilized.

(vi) **Effect of sonication on phage neutralization by LPS.** Strain AK44 LPS (1 mg/ml) was sonicated twice (30 s each) with a Sonogen-Z ultrasonic transducer (Branson Instruments Co., Stamford, Conn.). The LPS was then diluted and used in PhI_{50} determinations, as described above.

Inactivation of phage by LPS. The procedure used to inactivate phage with LPS has been described elsewhere (22) but was modified to use diluted TSB (25). Briefly, LPS was dissolved in a solution containing 0.9 ml of distilled water and 0.1 ml of phage in regular-strength TSB, so that the final concentration of TSB in the incubation mixture was $\frac{1}{10}$ strength (diluted TSB). Diluted TSB resulted in much better phage inactivation by LPS (i.e., lower PhI_{50} [concentration of LPS causing a 50% decrease in the titer of phage during 1 h of incubation] values) than regular-strength broth (25).

Competition inactivation studies. Attempts to

compete with the binding of ϕ PLS27 to whole cells and isolated LPS were performed by the method of Dawes (8).

For competition with ϕ PLS27 adsorption to whole cells, strain AK44 cells were grown overnight, subcultured, and grown to log phase. The cells were harvested by centrifugation at $10,000 \times g$ for 10 min, and the resulting pellet was suspended to a concentration of 3×10^8 colony-forming units per ml either in 0.01 M Tris-hydrochloride buffer (pH 7.0) (control) or in a 0.6 M sugar solution in 0.01 M Tris buffer (experimental). To 4.5 ml of the suspended cells we added 0.5 ml of phage (final concentration, 3×10^8 PFU/ml). After 5 min of incubation at 37°C and 150 rpm, the cells were pelleted, and the resulting supernatant was removed and blended in a Vortex mixer with a small amount of CHCl_3 ; then the titer was determined. The percentage of adsorbed phage was determined by comparing the experimental and control values with another control flask, in which the cells were replaced by buffer. The sugars tested were galactose, glucose, xylose, rhamnose, and mannose; alanine was also tested. None of these compounds appeared to be detrimental to ϕ PLS27 infectivity at a concentration of 0.6 M in control experiments.

Competition with ϕ PLS27 inactivation by strain AK44 and AK1012 LPS was also attempted. Phage ϕ PLS27 was mixed with 0.6 M sugar in 0.01 M Tris-hydrochloride (pH 7.0) for 5 min before the LPS was added. The concentration of LPS used in this study was varied to give either 90 or 50% inactivation of phage in 1 h. Controls consisted of ϕ PLS27 plus LPS alone and ϕ PLS27 plus buffer alone. Incubation was for 1 h at 37°C and 150 rpm, and samples were taken at different times during the incubation period. The percentage of inactivated phage was determined by comparison with control tubes in which the sugar competitor was omitted. The compounds tested for competition were glucose, galactose, xylose, mannose, rhamnose, ribose, glucosamine hydrochloride, mannosamine hydrochloride, *N*-acetyl glucosamine, *N*-acetyl galactosamine, *N*-acetyl mannosamine, and alanine; all were tested at a concentration of 0.6 M. When necessary, the pH was adjusted to neutral with NaOH.

Competition with ϕ PLS27 adsorption to whole cells and to LPS was also attempted with column-purified core polysaccharides of strains AK1012 and PAO1, as well as with lipid A of strain AK1012. Concentrations of 1,600 $\mu\text{g}/\text{ml}$ were used in these experiments.

RESULTS

Isolation of ϕ PLS27-resistant mutants. Phage ϕ PLS27 is a recently isolated bacteriophage which lyses strains AK43, AK44, and AK1012 and is specifically neutralized by the purified LPS from these strains (25). Strains AK43 and AK1012 are rough mutants of *P. aeruginosa* strain PAO and possess an LPS which lacks O chain material and contains only heptose, KDO, galactosamine, and alanine as major core constituents (25). Strain AK44 is a rough strain which is believed to possess a com-

plete core polysaccharide (29), as described by Drewry et al. (11).

By using ϕ PLS27 we hoped that even rougher mutants of *P. aeruginosa* (possibly mutants equivalent to the Re chemotypes of the *Enterobacteriaceae*) could be isolated. However, attempts to isolate resistant mutants of ϕ PLS27 were more difficult than anticipated. When selection was performed at 37°C with strain AK1012, only one to two colonies appeared per plate after 24 h of incubation, and these were smooth revertants, as judged by colony morphology and sensitivity to the smooth LPS-specific phages E79 (23) and PB1 (28).

We thought that perhaps mutants rougher than strains AK43 and AK1012 either do not exist or are temperature sensitive. Thus, we repeated the selection procedure at 30°C with strain AK1012. At 30°C , many colonies appeared on the plates after 24 h of incubation, but almost all of these colonies were at least partially sensitive to ϕ PLS27 when they were retested. Of about 40 colonies restreaked from the 30°C plates, one (designated strain AK1282) was totally resistant to ϕ PLS27. Strain AK1282 was also resistant to phages E79 and PB1, indicating that it was not a smooth revertant. The fact that this strain was also resistant to ϕ PLS44, a phage specific for AK44 complete core strains (28), suggested that it had not gained back any core sugars. It was still sensitive to ϕ PLS43, which is a rough specific phage, although apparently not an LPS-specific phage (29). These observations suggested that strain AK1282 was a rougher mutant than strain AK1012, and so we undertook a study of the chemical composition of strain AK1282 LPS.

Analysis of strain AK1282 LPS. We extracted LPS from strain AK1012 and AK1282 lyophilized cells by the phenol-chloroform-petroleum ether method (18), and Table 1 shows the chemical compositions of the LPS preparations. The major difference between the two strains was the lack of galactosamine in strain AK1282, which made strain AK1282 the roughest *P. aeruginosa* mutant thus far described. This was also shown by the very high lipid A content. The other interesting finding concerning strain AK1282 LPS was the alanine content, which was not much different from the strain AK1012 LPS alanine content.

The purity of the LPS was indicated by the low protein contamination and the lack of UV-absorbing material.

Inactivation of ϕ PLS27 by isolated LPS. Phage ϕ PLS27 was inactivated efficiently by strain AK1012 and AK44 LPS and, to a lesser degree, by strain AK43 LPS; phage ϕ PLS27 is

capable of lysing all of these strains (25). Based on the facts that ϕ PLS27 is LPS specific and that strain AK1282 was resistant to ϕ PLS27, we expected that strain AK1282 would contain an LPS that was incapable of inactivating the phage. This was clearly true, as shown in Fig. 1. Even at a concentration of 200 μ g/ml (the highest concentration tested), strain AK1282 LPS did not show any inactivation effect on ϕ PLS27. This was in sharp contrast to the LPS from strains AK1012 and AK44, which had PhI_{50} values (30) of 2.7 and 0.015 μ g of LPS per ml, respectively.

Receptor for ϕ PLS27. Since the only detectable differences between strain AK1282 LPS and strain AK1012 LPS were the lack of galactosamine in strain AK1282 LPS and, possibly, a small difference in alanine content, we assumed that the galactosamine or associated alanine residues or both must be of primary importance in

defining the ϕ PLS27 receptor. When we studied the effects of the various sugars toward ϕ PLS27 adsorption to whole cells, we found no competitive effect. We performed more complete studies with the isolated LPS receptor, but again no competition with ϕ PLS27 inactivation by LPS was found, even with 0.6 M galactosamine, *N*-acetyl galactosamine, or alanine (these compounds were the likely candidates for the receptor). Even when the pH was adjusted, the hydrochloride salts of galactosamine, glucosamine, and mannosamine caused decreases in ϕ PLS27 infectivity and hence could not be used as competitors. This was not a specific effect of these salts on ϕ PLS27, as the same thing occurred with phage T7.

Similar experiments with column-purified cores from strains AK1012 and PAO1, as well as strain AK1012 lipid A, showed that neither the isolated cores nor lipid A could neutralize the phage by themselves at a concentration of 1,600 μ g/ml, nor could any of these substances compete with ϕ PLS27 inactivation by isolated LPS.

Modified LPS as receptor for ϕ PLS27. We attempted to modify the LPS and, more specifically, the ϕ PLS27 receptor region by methods that should have affected the galactosamine and alanine residues. As a control, we used phage T7, which was neutralized well by LPS from rough mutants of strain PAO1 (namely, strains AK44, AK1012, and even AK1282) (Fig. 2) and adsorbed to the rough strains as well. Based on our results, especially the good inactivation of phage T7 by strain AK1282 LPS, it seemed likely that the phage T7 receptor in *P. aeruginosa* LPS is the heptose region, as it is in *Escherichia coli* (35). Using such a system, we felt that we could distinguish a specific effect on the

TABLE 1. Chemical compositions of the LPS of strains AK1012 and AK1282

Component	% (by weight) in:	
	AK1012 LPS	AK1282 LPS
Lipid A	49.5	62
KDO	5.1	4.0
Heptose	9.2	10.9
Glucose	0	0
Rhamnose	0	0
Glucosamine	3.9	6.0
Glucosamine phosphate	2.9	2.6
Galactosamine	2.8	Trace
Alanine	1.7	1.8
Fucosamine	0	0
Phosphorus	7.4	7.3
Protein	2.6	2.9

FIG. 1. Titration of the ϕ PLS27-inactivating activities of LPS extracted from *P. aeruginosa* rough derivatives AK44 ($\bullet$), AK1012 (Δ), and AK1282 ($\square$). Phage ϕ PLS27 was incubated with varying concentrations of LPS for 1 h at 37°C before the titers of residual infectious phage were determined.

FIG. 2. Titration of the T7-inactivating activities of LPS extracted from *P. aeruginosa* rough derivatives AK44 (●), AK1012 (Δ), and AK1282 (□). Phage T7 was incubated with varying concentrations of LPS for 1 h at 37°C before the titers of residual infectious phage were determined.

ϕ PLS27 receptor region from more general damage to the LPS.

Amidase M treatment of strain AK1012 LPS did not cause the release of any alanine, as detected by amino acid analyses of experimental and control LPS, and the PhI_{50} values of the treated and untreated control LPS were identical (data not shown).

For the chemical modification of LPS, we used strain AK44 LPS because it gave the best inactivation of ϕ PLS27 (i.e., lowest PhI_{50} value), and hence we hoped that a more marked difference between the control LPS and the chemically modified LPS would be observed. Table 2 shows the effects of dinitrophenylation, N-acetylation, and deamination of strain AK44 LPS on the inactivating capacity of this LPS toward ϕ PLS27 and T7. This table shows that dinitrophenylation and N-acetylation resulted in 10- to 20-fold increases in the PhI_{50} value for ϕ PLS27 and that deamination caused a 73-fold increase. For phage T7, the dinitrophenylated LPS and N-acetylated LPS also produced 10- to 20-fold higher PhI_{50} values compared with native strain AK44 LPS, and the deaminated material resulted in a PhI_{50} value which was more than 100-fold higher. Thus, there appeared to be no difference in the effects of the modified LPS toward ϕ PLS27 and T7.

Effect of LPS salt form on ϕ PLS27 neutralization. Table 3 shows the effects of electrodialysis and conversion to uniform salts on the ability of strain AK44 and AK1012 LPS to inactivate ϕ PLS27. It is obvious that the inactivation proceeded much better in the diluted broth. Since ϕ PLS27 adsorbed with similar rates in broth and dilute buffer, the broth probably did not affect the adsorption of the phage; it is

TABLE 2. Effects of chemical modifications on the phage-inactivating capacity of AK44 LPS

LPS prepn	PhI_{50} value (μ g of LPS/ml) with:	
	Phage ϕ PLS27	Phage T7
Native	0.015	0.26
Dinitrophenylated	0.25	3.2
N-acetylated	0.16	3.7
Deaminated	1.1	47

TABLE 3. Influence of the salt form of the LPS and effect of the incubation medium on the PhI_{50} values obtained with ϕ PLS27

Strain	LPS prepn	PhI_{50} value (μ g of LPS/ml) in:	
		Regular strength broth	1/10-Strength broth
AK1012	Non-electrodialyzed	150	3.2
	Sodium salt	>200 ^a	11.0
	Triethylamine salt	70	1.6
AK44	Non-electrodialyzed	0.82	0.08
	Sodium salt	57	18.0
	Triethylamine salt	1.1	0.1

^a A preparation containing 200 μ g of AK1012 LPS sodium salt per ml resulted in approximately 30% inactivation of ϕ PLS27. The estimated PhI_{50} was ~800 μ g of LPS/ml.

more likely that the broth affected the presentation of the LPS receptor to the phage. It was in the regular-strength broth that the differences in the inactivating capacities of the different salt forms were most noticeable; the sodium salt was much less effective in inactivating the phage than either the triethylamine salt or the non-electrodialyzed LPS. The salt forms of the LPS varied considerably in their opalescence in water

at concentrations of 1 mg/ml; the triethylamine form gave clear solutions, the non-electrodialyzed LPS gave opalescent solutions, and the sodium salt was rather insoluble. Although we did not calculate particle weights, it seemed that, in accordance with the findings of other workers, the triethylamine salt form had the lowest particle weight, the sodium form had the highest particle weight, and the non-electrodialyzed LPS had an intermediate weight (17).

Effects of sonication of LPS on its phage-inactivating capacity. Sonication of strain AK44 LPS and its salt forms resulted in a lowering of the PhI_{50} values in diluted broth, so that after sonication the PhI_{50} values of the non-electrodialyzed, sodium, and triethylamine forms were all approximately the same (Table 4). There was a dramatic reduction in the PhI_{50} value for the sodium salt, and this represented a vast increase in the phage-inactivating ability of the sodium form after sonication.

Influence of prolonged incubation on the PhI_{50} value. We found that prolonged incubation of ϕPLS27 with strain AK44 in diluted broth could cause a decrease in the PhI_{50} value of five- to sevenfold after 4 h (data not shown).

Cation contents of LPS salt forms. An examination of the non-electrodialyzed LPS preparations and the salt forms indicated that of the ions investigated, sodium was the major ion depleted by the electrodialysis procedure (Table 5). In addition, about one-half of the magnesium ion content of the rough strains was removed, although little change was observed in the smooth LPS. The calcium ion level was not reduced by electrodialysis. Total amines, as measured by the procedure of Dubin (12), were reduced about 50% in the rough strains (0.44 μmol of amine per mg of LPS in non-electrodialyzed strain AK44 LPS, compared with 0.22 $\mu\text{mol}/\text{mg}$ in the sodium form and 0.25 $\mu\text{mol}/\text{mg}$ in the triethylamine form; for strain AK1012, 0.47 $\mu\text{mol}/\text{mg}$ of LPS was found in the non-electrodialyzed LPS, compared with 0.35 and 0.23 $\mu\text{mol}/\text{mg}$ in the sodium and triethylamine forms, respectively). We detected no reduction in the amine content in the smooth LPS, al-

TABLE 5. Concentrations of various cations in non-electrodialyzed and electrodialyzed LPS preparations

Strain	LPS prepn	Concn of ions ($\mu\text{g}/\text{mg}$ of LPS)		
		Na^+	Mg^{2+}	Ca^{2+}
PAO1	Non-electrodialyzed	38.5	2.86	4.00
	Sodium salt	23.0	3.34	5.25
	Triethylamine salt	0 ^a	2.12	4.68
AK44	Non-electrodialyzed	20.0	15.9	3.02
	Sodium salt	22.5	8.5	5.30
	Triethylamine salt	0 ^a	8.7	4.26
AK1012	Non-electrodialyzed	14.5	17.8	4.16
	Sodium salt	21.5	10.2	6.50
	Triethylamine salt	0 ^a	9.8	5.56

^a We detected no increase over the background amount in the deionized water used to resuspend the sample.

though the levels of amines in the non-electrodialyzed smooth LPS were much lower (0.043 μmol of amine per mg of LPS) than the levels in the rough strains.

DISCUSSION

The use of LPS-specific phages to isolate mutants defective in LPS structure is a well-established procedure for enteric bacteria (31, 32, 44, 46). In our efforts to study the LPS of *P. aeruginosa*, we have isolated or identified many smooth LPS-specific phages (22, 23, 28), and we have used these phages to enrich for rough mutants (24, 27, 29). However, we have been unable to isolate any mutants rougher than strains AK43 (24) and AK1012 (25), which contain KDO, heptose, galactosamine, and alanine as major polysaccharide components. No other reports of *P. aeruginosa* mutants rougher than our strain AK43 have been published, although Koval and Meadow (26) have isolated equivalent strain PACI mutants by using pyocin resistance as a selective tool. To isolate mutants in the inner core region, we isolated a phage, ϕPLS27 , which is specific for the LPS of mutants AK1012 and AK43, as well as for AK44 LPS (25).

Selection for ϕPLS27 resistance resulted in the isolation of strain AK1282, a mutant which lacks galactosamine in its LPS. In addition, the 62% lipid A content estimated for strain AK1282 LPS is the highest lipid A content that has been described for a *P. aeruginosa* strain. This is the first *P. aeruginosa* mutant to lack galactosamine in its LPS core structure, and hence it is the roughest mutant yet isolated in this species. The importance of this mutant in elucidating the structure of the *P. aeruginosa* core LPS is immediately obvious. The inner core of *P. aeruginosa* is generally believed to be the same in most strains (5-7, 11) and to consist, in part, of alanine amide linked to galactosamine. Until now, we assumed that this is also true in strain PAC (24,

TABLE 4. Effect of sonication on the phage-inactivating ability of strain AK44 LPS

LPS salt form	PhI_{50} value (μg of LPS/ml) ^a		Increase in ϕPLS27 -inactivating capacity (-fold)
	Before sonication	After sonication	
Non-electrodialyzed	0.08	0.0056	14.3
Triethylamine salt	0.1	0.008	12.5
Sodium salt	18	0.02	900

^a PhI_{50} values were determined in diluted broth.

29). However, our analysis of strain AK1282 LPS has shown that this cannot be the only place that alanine is bound since the loss of galactosamine results in a minimal (15%) decrease in the alanine content. Whether such a situation exists for the alanine residues in other strains is unclear. Using NCTC 1999, Fensom and Gray (13) converted only 15 to 20% of the alanine to a 2,4-dinitrophenol derivative and postulated that the remaining alanine might be present as poly-alanyl chains or might be N-acetylated; 15 to 20% N-terminal alanine corresponds well to the ~15% alanine which is apparently amide linked to galactosamine in strain PAO, judging from the strain AK1012 and AK1282 analyses. Chester et al. (5) also studied strain NCTC 1999 and reported that at least 40% of the alanine was amide linked, although the location of the remainder of the alanine was unexplained; incomplete hydrazinolysis was offered as a possible explanation. Drewry et al. (11) used the same strain and isolated fragments which had ratios of galactosamine to alanine of 1:1 and 1:2. Thus, it is clear that some of the alanine is amide linked to galactosamine in strain NCTC 1999, but whether it is all bound to galactosamine is not clear at present. It is obvious that in strain PAOI a large amount of alanine is not associated with galactosamine.

The alanine that is not galactosamine bound must be located in either the heptose region or the KDO residue that terminates the isolated core polysaccharide. This has to be so since the alanine is not lipid A associated (13, 24) and when Sephadex column chromatography is used, little alanine is found in fraction III, where most of the free KDO is located (25).

The finding that coliphage T7 is inactivated efficiently by the LPS of the rough derivatives AK44, AK1012, and AK1282 indicates that a T7 receptor is located in these *Pseudomonas* LPS preparations. Based on the inactivation of T7 by strain AK1282 LPS, it is likely that the receptor for T7 in *P. aeruginosa* LPS is the heptose region, which has been suggested as the T7 receptor in *E. coli* (35). It should be noted that T7 adsorbs well to the rough strains, although it does not lyse them in spot tests.

FIG. 3. Proposed structure of the core of *P. aeruginosa* strain PAO LPS, indicating the probable sites of the LPS biosynthesis block in strains AK1012 and AK1282.

Figure 3 shows the core structure of strain PAO LPS as we now believe it to be. This structure is based partly on the scheme of Drewry et al. for strain NCTC 1999 and partly on our results. The proposed blocks in the synthesis of LPS by strains AK1012, AK44, and AK1282 are indicated.

Since the major difference between AK1012 LPS and AK1282 LPS is only a galactosamine or galactosamine plus alanine, we felt that the receptor for ϕ PLS27 probably lies in these residues. We were not successful in our attempts to confirm this by studying the effects of high concentrations of sugars and alanine on adsorption of ϕ PLS27 to whole cells and to isolated LPS. The use of an isolated polysaccharide at a high concentration to try to compete with ϕ PLS27 neutralization also failed. This technique has been used previously with success (45), but a much higher concentration of polysaccharide was used. It is also possible that alanylgalactosamine may be necessary for proper competition to occur.

Studies with modified LPS also were inconclusive in defining a receptor region for ϕ PLS27. Treatments which should have affected the galactosamine-alanine region and hence the ϕ PLS27 receptor ability had no greater effect on ϕ PLS27 neutralization than on T7 neutralization, which we used as a control. This could mean a number of things. Either the effect of each of the chemical treatments was a general modification of the LPS so that it affected the two different receptor areas of the LPS to the same degree, or the chemical treatments modified amino groups (e.g., ethanolamine residues) located in or very near the T7 receptor, which is thought to be the heptose region. The higher PhI_{50} values obtained with the modified LPS may also have resulted from changes in the micellar form of the LPS.

The negative or inconclusive results obtained by competition experiments and with modified LPS can be explained in another way. The losses of galactosamine and alanine in strain AK1012 LPS could have caused a change in the three-dimensional appearance of the LPS to the phage receptor organelle. Hence, the receptor may be still present in strain AK1282 LPS, but its conformation may be poorly suited for phage neutralization. Hancock and Reeves (20) have indicated that this may be an important consideration in phage receptors.

Conversion of LPS to uniform salt forms resulted in dramatic changes in the phage-inactivating properties of the LPS. The sodium salts of strains AK44 and AK1012 did not inactivate ϕ PLS27 well and had PhI_{50} values which were up to 180 times greater than the PhI_{50} values of the triethylamine salt forms. A simple explana-

tion for this is that the sodium salt is in the most aggregated state, as judged by its poor solubility and the reported sedimentation values of salt forms in other genera (14-16). This highly aggregated salt may result in phage receptors that are "buried" and, hence, inaccessible to the phage. Thus, although the sodium and triethylamine salt forms would both contain the same physical number of receptors for ϕ PLS27, the number of functionally available sites would be less in the sodium salt. The less-aggregated, lower-molecular-weight triethylamine form would have more sites free to bind phage. This explanation is supported by the results obtained when strain AK44 LPS was sonicated. Sonication resulted in 10- to 15-fold decreases in the PhI_{50} values for the triethylamine and non-electrodialyzed strain AK44 LPS but a 900-fold decrease in the PhI_{50} value with the sodium salt. Sonication does reduce the aggregate particle size of LPS (42), and after sonication it seems probable that the particle sizes of the three forms were quite similar. This is reflected in the PhI_{50} values of the three forms obtained before and after sonication. Before sonication, the sodium salt had a PhI_{50} value approximately 200-fold higher than the PhI_{50} values of the triethylamine and non-electrodialyzed forms, whereas after sonication the differences in PhI_{50} values among the three forms were less than 4-fold. However, there is a limit to the decrease in molecular weight that is allowed in order to still get phage eclipse. Deoxycholate-treated LPS preparations, which reportedly have molecular weights of 12,000 to 16,000 for smooth LPS (37) and 8,000 for rough LPS (43), do not eclipse the phage. In addition, Lindberg (31) has reported that an LPS aggregate having a molecular weight of 9×10^4 is needed to eclipse Felix 0 phage.

Therefore, it seems that phage inactivation can be added to the growing list of biological properties of LPS, including anti-complementary activity (16), binding to erythrocytes (36), pyrogenicity (19), and toxicity (14), which are dependent on the salt form used.

It is interesting that the pH of strain AK44 LPS did not go below 4.1 to 4.2, even after prolonged electrodialysis at 10 mA, whereas the pH's of the rougher strain AK1012 LPS and the smoother strain PAOI LPS preparations dropped to 3.6 to 3.8 under similar conditions. The reason for this difference is not clear, but the final drop in pH is vitally important, both in terms of solubility of the salt and in terms of phage inactivation. When the partially deionized strain AK44 LPS preparation (pH 4.2) was neutralized with triethylamine or sodium hydroxide, solutions of the two salts appeared identical to

a solution of the non-electrodialyzed LPS in opalescence. In addition, the phage-inactivating properties of the sodium, triethylamine, and non-electrodialyzed LPS preparations were indistinguishable (unpublished data).

An examination of the LPS salt forms for specific ions and for polyamines showed that the monovalent sodium ion was most readily and virtually completely lost, whereas the two divalent cations studied (magnesium and calcium) were more variable in their responses to electrodialysis (Table 5). Easy removal of sodium and a less dramatic effect on divalent cations were reported by Galanos and Luderitz (15) in their work on electrodialyzed LPS. The sodium and calcium ion contents of *P. aeruginosa* PAO LPS are comparable to the values found by Galanos and Luderitz (15) in the *Salmonella* strains which they used. However, the magnesium levels of *P. aeruginosa* LPS are much higher than those in the strains tested by Galanos and Luderitz, although they are similar to levels reported in rough strains of *Shigella dysenteriae* (43). This higher magnesium ion level may be a reflection of its key role in the integrity of pseudomonad LPS (3, 4, 39).

Specific polyamines were not determined in the LPS samples, but our results indicated that the removal of contaminating polyamines also occurred during the electrodialysis procedure, especially with the rough LPS. Spermidine, 1,3-diaminopropane, cadaverine, and putrescine have been tentatively identified in *P. aeruginosa* LPS (10), where they occur in trace amounts. Chester et al. (5) also reported small amounts of spermidine and putrescine in the LPS of *P. aeruginosa*. The polyamines and the cations presumably neutralize the negative charges present on the many phosphates and carboxyl groups in the core and lipid A regions (17).

We believe that continued isolation of mutants resistant to ϕ PLS27 or, alternatively, isolation of a rough specific phage for strain AK1282 will lead to the isolation of the first Re chemotypes of *P. aeruginosa*. In addition, in future studies on phage-LPS interactions workers should note our finding that the physical state of the receptor markedly affects its capacity to inactivate.

ACKNOWLEDGMENTS

We thank B. W. Holloway for providing strain PAOI and phages E79 and F116L, D. E. Bradley for phage PB1, and E. Asheshov for phages C5 and M6. We also thank Ludmilla Weir for the amino compound analysis, Scott Meskis for assistance with cation determinations, and G. Gordon Greer for the procedures used for polyamine determination.

This work was supported by grants from the National Research Council of Canada and the Medical Research Coun-

cil of Canada (to A. M. K.) and by a Medical Research Council of Canada Studentship (to K. F. J.).

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