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Identification of the Lipopolysaccharide Core Region as the Receptor Site for a Cytotoxin-Converting Phage, φCTX, of *Pseudomonas aeruginosa*

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A temperate phage, φCTX, is a cytotoxin-converting phage of *Pseudomonas aeruginosa*. In this study, we characterized the lipopolysaccharide (LPS) structures of φCTX-resistant mutants derived from φCTX-sensitive strains. φCTX infectivity was neutralized by LPS preparations derived from sensitive strains but not by those from resistant strains. φCTX-resistant mutants had lower-molecular-weight rough (R)-type LPS than the parental strains and lacked the reactivity of some anti-LPS core monoclonal antibodies. Some LPS core components were lacking or significantly decreased in the resistant mutants. These results suggested that a receptor site of the cytotoxin-converting phage φCTX was the LPS core region and that especially L-rhamnose and D-glucose residues in the outer core were involved in phage binding. The host range of φCTX was nearly O-serotype dependent, probably because of the diversity of the LPS core structure among *P. aeruginosa* strains. φCTX bound to most strains of Homma serotypes A, G, and I but not to strains of serotypes B and E. Furthermore, we found that a genetic locus specifying φCTX sensitivity (and consequently participating in the biosynthesis of part of the LPS core) existed in or near the locus participating in the determination of O-serotype specificity (*somA*), which has been mapped between *leu-10* and *eda-9001*. φCTX, as well as anti-LPS core monoclonal antibodies, will be a good tool for structural characterization of the *P. aeruginosa* LPS core region.

Pseudomonas aeruginosa cytotoxin shows a wide range of cytotoxic activity against eukaryotic cells, especially leukocytes (9, 22, 31). Unlike other toxins and enzymes produced by *P. aeruginosa*, cytotoxin is not secreted into the culture medium. It is produced as a cell-associated procytotoxin, released into the medium during cell lysis, and then converted to an active cytotoxin by C-terminal processing (8, 9, 31).

In previous studies (6, 7, 11), it was found that the gene encoding cytotoxin, *ctx*, existed only in two of about 400 *P. aeruginosa* strains from various sources and that in both strains the genes existed on the genomes of the prophages. Furthermore, both of the bacteriophages isolated, which were phylogenetically closely related to each other (11), actually converted *P. aeruginosa* strains to cytotoxin producers by their lysogenization (7, 11). φCTX is one of the cytotoxin-converting phages isolated from strain PA158, a strong cytotoxin producer which has been widely used for the study of cytotoxin.

Since φCTX could infect only some but not all *P. aeruginosa* strains (reference 6 and this study), the host range of φCTX seemed to depend on the presence of a specific receptor on the bacterial cell surface. However, the receptor site for φCTX has not been revealed yet. Recently, we and others found that bacteriophage φCTX is related to the R-type pyocins (11). The finding suggested that, as has been proposed for the R-type pyocins (25), lipopolysaccharide (LPS), not outer membrane protein, could be a receptor site for φCTX. The present study examined whether LPS could be the receptor for φCTX. We characterized the structures of LPSs derived from φCTX-sensitive strains and mutant strains with altered φCTX sensitivity. Furthermore, we also examined the host range of φCTX in *P. aeruginosa* and a role for a genetic locus (or loci) specifying the φCTX sensitivity.

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MATERIALS AND METHODS

φCTX and its clear-plaque mutant, φCTX-c. φCTX was prepared as described previously (7). φCTX-c is a clear-plaque mutant spontaneously derived from φCTX. It forms a clear plaque with no centered colony on sensitive cells but does not form any plaque in their φCTX lysogens. The size and shape of the phage particle and the restriction endonuclease map of the phage genome were the same as those for the wild-type phage (7, 10). We have not observed any difference in their host ranges, either.

Bacterial strains. *P. aeruginosa* PML14 and its mutants which showed altered R-pyocin sensitivity (15, 33) were kindly donated by M. Kageyama, Mitsubishi Kasei Institute of Life Science, Tokyo, Japan. *P. aeruginosa* PAC1 and its LPS-defective mutants (16, 28) were donated by P. M. Meadow, University College of London, London, United Kingdom. The sources of the other strains are described in Table 1. In this paper, the *P. aeruginosa* serotype scheme is that of Homma (13), and the correspondence between Homma serotypes and the International Antigenic Typing Scheme (IATS) is as described by Liu et al. (21). Serotypes were determined by the slide agglutination method with a grouping kit, Mei-assay *Pseudomonas aeruginosa* (Meiji Seika Kaisha, Tokyo, Japan).

Isolation of φCTX-resistant mutants. φCTX-resistant mutants were isolated from *P. aeruginosa* 58-F and PAS10 by using φCTX-c as follows. Approximately 10^7 cells were incubated with 10^5 to 10^6 PFU of φCTX-c for 20 min at room temperature and plated on an L plate with 0.65% soft Luria

TABLE 1. Bacterial strains

Bacterial strain	Serotype		Characteristics	Reference
	Homma	IATS		
PA158	G	6	Lysogenic for ϕCTX	31
PML14	I	1	Indicator strain for ϕCTX	14
PAC1	A	3	Indicator strain for ϕCTX	1
PAS10	M	NT[a]	Indicator strain for ϕCTX	7
58-F	A	3	Indicator strain for ϕCTX	39
58-F-c	A	3	*thr-1 leu-1 met-1* derivative of 58-F	This study
58-F-7	A	3	Hybrid strain obtained by mating 58-F-c with PTO66 (R68-45$^+$), inheriting *rec-102*, *somA* (O-5), *leu-10*$^+$, and *thr*$^+$ of PTO66	This study
PTO66 (R68-45$^+$)	B	5	Hybrid of PAO220 and PAT2228 carrying plasmid R68-45, which mediates the conjugal chromosome transfer; *his-4 lys-12 ilv-1118 trp-6 pro-82 rec-102*	3
PAO1840	B	5	Derivative of PAO1 with mutations *met-9020* (*9011*), *eda-9001*, and *leu-9005* (*10*)	11; this study
5933	I	1	Standard strain for Habs serotype O1	5
PAO2103	I	1	*eda*$^+$ *somA* (O1)$^+$ transductant of PAO1840 obtained by infecting F116 propagated on 5933	11; this study

[a] NT, nontypeable.

agar. After 24 h of incubation at 37°C, several colonies with various sizes were observed. Each colony was picked and subjected to single-colony isolation twice. Finally, the sensitivities of the colonies to the phage were checked by the standard soft-agar overlay method and used for further analyses. By this method, mutants with reduced sensitivity to the phage as well as completely resistant mutants could be obtained (see Results).

Construction of hybrid strains. As summarized in Table 1, strain PAO1840, a *leu-9005* (*leu-10*) mutant, was derived from PAO1 through two intermediates, PAO1834 [*met-9020* (*met-9011*)] (29) and PAO1838 (*met-9020 eda-9001*) (27), with the intention of constructing recombinant strains for the O serotypes. This construction was based on the fact that genes participating in the determination of the O-serotype specificity (*som*, now renamed *somA*) and the smooth-rough conversion (*somB*) are located between *leu-10* and *eda-9001* (12, 23, 24). When phage F116 was employed for the transduction, both *somA* and *somB* were cotransducible with *leu-10* and *eda-9001* at frequencies ranging from 10 to 16%, while *leu-10* and *eda-9001* alone were not cotransducible (23). PAO1840 was infected with phage F116 propagated on strain 5399, with selection for *eda*$^+$, and an *eda*$^+$ transductant, designated PAO2103, was chosen for further experiments. PAO2103 had serotype I antigen and no longer reacted with anti-serotype B antibodies.

By analogy, 58-F-c was derived from 58-F via 58-F-1 (*leu-1*, corresponding to *leu-10* of PAO) and 58-F-b (*leu-1 thr-1*; *thr-1* corresponds to *thr-66*). Strain 58-F-c was mated with PTO66 (R68-45$^+$), with selection for *thr*$^+$. A transconjugant, 58-F-7, which inherited the *thr*$^+$ and *leu*$^+$ alleles of the donor and agglutinated with anti-serotype B antibodies but not with anti-serotype A antibodies, was chosen. The recombinant inherited the *rec-102* mutation from the donor as well. R-pyocin sensitivities of the strains were determined as described previously (11), using R-pyocin-producing strains as described elsewhere (34).

Phage adsorption assay. An overnight culture of each strain was diluted 1/100 into fresh Luria broth containing 10 mM $MgSO_4$ (LB-Mg) and grown with shaking at 37°C to the mid-log phase (2.5 to 3 h). Each culture was centrifuged at 10,000 × *g* for 5 min at room temperature. Cells were washed with LB-Mg and suspended in the same medium to an A_{550} of 2.0. Cell suspension (0.1 ml) was mixed with 0.9 ml of LB-Mg containing ϕCTX (3×10^4 PFU ml^{-1}) propagated on PAS10. Following incubation for 25 min at 37°C with gentle shaking, the cells were removed by centrifugation at 15,000 × *g* for 5 min at 4°C and the supernatant was treated with chloroform. The plaque-forming titer of the supernatant was determined by the agar overlay method with PAS10 as an indicator. The percentage of phage bound to the cells was calculated as [(titer of added phage − titer of unbound phage)/(titer of added phage)] × 100.

Preparation of LPS. *P. aeruginosa* cells were cultured in heart infusion broth to the late exponential phase. LPS was extracted by the method of Uchida and Mizushima (38) with some modifications (43). Briefly, the bacterial cells were boiled for 10 min in 10 mM Tris-HCl–10 mM $MgCl_2$–2% Triton X-100 (pH 8.0). After centrifugation at 15,000 × *g*, the pellet was washed once with 10 mM Tris-HCl–10 mM $MgCl_2$ (pH 8.0). The resulting pellet was suspended with 50 mM EDTA–0.5 M NaCl–2% Triton X-100 (pH 8.0), incubated at 37°C for 60 min with shaking, and then centrifuged at 15,000 × *g*. To the resulting supernatant, $MgCl_2$ was added at a final concentration of 0.1 M, and the mixture was incubated at 37°C for 60 min. The resulting opaque solution was immediately centrifuged at 100,000 × *g* for 90 min at 15°C. The pellet was treated with proteinase K (20 μg/ml) at 60°C for 60 min and washed twice with 10 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$ (pH 8.0). The pellet was suspended and dialyzed with deionized water and used as an LPS preparation. Protein and nucleic acid contents were less than 0.5% in the preparation.

Assay of neutralization of ϕCTX with LPS. Neutralization of the phage infectivity by LPS was determined by a modification of the procedure of Lindberg (20). Briefly, 0.8 ml of LB-Mg and 0.1 ml of serially diluted LPS solution (dissolved in distilled water) were mixed and incubated at 37°C. After 5 min, 0.1 ml of phage propagated on PAS10 (3×10^4 PFU ml^{-1}, suspended in LB-Mg) was added to the mixture, and the mixture was incubated at 37°C with gentle shaking. After 60 min, a 0.1-ml sample was plated by the agar overlay method with PAS10 as an indicator strain. Plaques were counted after 12 to 16 h of incubation at 37°C. ID_{50} is the concentration of LPS required for 50% inhibition of phage infectivity.

MAbs. Human monoclonal antibodies (MAbs) (immunoglobulin M [IgM]) FK-2E7, MH-4H7, OM-1D6, and NM-3G8 against the outer core region of *P. aeruginosa* LPS were described previously (43). Serotype-specific murine MAbs were purchased from Meiji Seika Kaisha. A human MAb (TS-1B2; IgM) against *P. aeruginosa* common polysaccharide

TABLE 2. ϕCTX sensitivity of *P. aeruginosa* PAC1R and its LPS-defective mutants and binding of anti-LPS core MAbs

Strain	Chemotype[a]	Composition of LPS[b]	ϕCTX sensitivity[c]	Binding of ϕCTX		Binding of anti-LPS core MAb (A_{405})[d]	
				Adsorption to cells (%)	ID_{50} of LPS (μg/ml)	FK-2E7	MH-4H7
PAC1R	S	$(O\text{-}Ag)_n Rha_1 Glc_3 GalN_1 Ala_1 Hep_1 KDO_2$	+	100	0.22	0.15	0.25
PAC609	S	$(O\text{-}Ag)_n Rha_1 Glc_2 GalN_1 Ala_1 Hep_1 KDO_2$	+	100	0.14	0.18	0.28
PAC608	SR	$(O\text{-}Ag)_1 Rha_1 Glc_3 GalN_1 Ala_1 Hep_1 KDO_2$	+	56	2.3	1.48	1.11
PAC557	R	$Rha_1 Glc_3 GalN_1 Ala_1 Hep_1 KDO_2$	+	66	2.6	1.62	1.13
PAC556	R	$Glc_3 GalN_1 Ala_1 Hep_1 KDO_2$	−	25	20	0.38	0.02
PAC611	R	$Glc_1 GalN_1 Ala_1 Hep_1 KDO_2$	−	7	>200	0.20	0.04
PAC605	R	$GalN_1 Ala_1 Hep_1 KDO_2$	−	4	>200	0.02	0

[a] S, smooth; SR, semirough; R, rough.
[b] Data are taken from reference 28. (O-Ag), repeating unit in the O-polysaccharide chain; Glc, D-glucose; Rha, L-rhamnose; GalN, D-galactosamine; Hep, heptose; KDO, 3-deoxyoctulosonic acid.
[c] +, sensitive; −, resistant. The efficiency of plaque formation of four ϕCTX-sensitive strains was nearly the same as that of PAC1R.
[d] Binding activity was determined by ELISA with plates coated with whole cells of each strain and was expressed as A_{405}.

antigen E87 (D-rhamnan) (30, 41) was prepared by cell fusion of pokeweed mitogen-activated human peripheral blood lymphocytes from a healthy adult volunteer and human-mouse heteromyeloma SHM-D33 (ATCC CRL 1668) as described previously (42).

ELISA. The enzyme-linked immunosorbent assay (ELISA) using glutaraldehyde-fixed bacterial cells as a coated antigen was described previously (42). Alkaline phosphatase-conjugated anti-human IgM or anti-mouse IgM antibodies (Kirkegaard & Perry Laboratories, Inc.) and sodium *p*-nitrophenylphosphate were used as the secondary antibody and substrate, respectively.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (18). LPS on the gel was developed by the silver staining method (35).

Agglutination with acriflavine. To estimate the rough nature of each strain, the activity of agglutination with acriflavine was tested (17). One hundred microliters of the bacterial cell suspension in 0.85% NaCl (A_{550} of 0.2) and 50 μl of 0.2% acriflavine were mixed and incubated at 4°C overnight, and then agglutination was observed.

Preparation of saccharide fractions of LPS. Saccharide fractions of LPS were prepared by the method of Koval and Meadow (16). Briefly, the LPS preparation was hydrolyzed with 1% acetic acid at 100°C for 90 min. After removal of lipid A by centrifugation, the resulting water-soluble materials were fractionated by gel filtration chromatography on a Bio-Gel P-6 (Bio-Rad) column (1 by 80 cm) in 50 mM pyridine-acetate buffer (pH 5.0). Fractions (0.5 ml) were collected and assayed for neutral sugars by the phenol-sulfuric acid method (2), and amino sugars were assayed by the method of Tsuji et al. (36).

Carbohydrate analysis. Neutral sugars were analyzed with a SUMIPAX PG-amino column (Sumika Chemical Analysis Service Ltd.) after acid hydrolysis with 1 M HCl at 100°C for 1 h. Amino sugars and amino acids were analyzed with an Amino Acid Autoanalyzer L-8500 (Hitachi) after acid hydrolysis with 4 M HCl at 100°C for 4 h. Heptose was determined by the cysteine-sulfuric acid method (40).

RESULTS

Characterization of ϕCTX-sensitive *P. aeruginosa* strains and their derivatives. To determine the receptor site for ϕCTX, strains PAC1R, 58-F, PAS10, and PML14 and their derivatives were analyzed.

(i) Strain PAC1R and its derivatives. The series of PAC1R-derived mutants used have various defects in the LPS outer core region, and the partial structures of their LPS cores had been reported previously (28). PAC1R and its parent strain, PAC1, were sensitive to ϕCTX and reactive to two anti-LPS core MAbs (MH-4H7 and FK-2E7). As previously reported (43) and as shown in Table 2, the LPS-defective mutants showed variously altered sensitivities to ϕCTX and reactivities to anti-LPS core MAbs. LPS preparation derived from PAC1 and PAC1R clearly inhibited the infectivity of ϕCTX (ID_{50}, ca. 0.2 μg/ml). PAC609, which is reported to lack only one glucose residue in the outer core region but has a complete O-polysaccharide chain, was sensitive to ϕCTX. The ID_{50} of its LPS and adsorption of ϕCTX to the cells were similar to those for the parent strains. PAC608 (semirough mutant) and PAC557 (rough mutant), which have defects in the O-polysaccharide chain but contain complete cores, were still sensitive to ϕCTX. LPS preparations derived from these two mutants also inhibited the infectivity of ϕCTX. However, higher ID_{50}s (ca. 2.5 μg/ml) of the LPS preparations, which corresponded to the lowered efficacy of ϕCTX adsorption to the cells, were observed for PAC608 and PAC557. PAC611 and PAC605, which have defects extending to the outer core, were completely resistant to ϕCTX. Their LPS preparations did not show any inhibitory effect on ϕCTX infectivity up to the concentration of 200 μg/ml, in agreement with the fact that ϕCTX was not absorbed by cells of either strain. PAC556, which was reported to have a defect in the rhamnose residue in the outer core, was apparently resistant to ϕCTX in the plaque-forming assay. However, its LPS preparation showed weak inhibition (2 orders of magnitude lower than that of PAC1R) of ϕCTX infectivity. This finding agreed with the weak adsorption of ϕCTX to the PAC556 cells.

(ii) Strain 58-F and its derivatives. Strain 58-F is a smooth strain belonging to Homma serotype A (IATS O-3) and is sensitive to ϕCTX. The rough (R) core of 58-F contains galactosamine, alanine, glucose, and rhamnose in a molar ratio of about 1:1:4:1 (Table 3). Five mutants with altered ϕCTX sensitivities were obtained from the strain. Among them, 58-F-r3, 58-F-r4, and 58-F-r9 were still sensitive to ϕCTX, but the phage bound to the cells at a slightly reduced efficiency (Table 4) and the neutralizing activities of their LPS preparations were also slightly reduced (Fig. 1). 58-F-r3 and 58-F-r4 were rough-natured strains, as determined by their agglutinability with acriflavine, and no O polysaccharide or semirough (SR) core was detected in their LPS preparations (Table 3 and Fig. 2). The sugar compositions of their R cores (Table 3), the

TABLE 3. Analysis of saccharide fractions from LPSs of φCTX-sensitive strains and their mutants

Strain	Saccharide fraction[a]			Composition of R core[b]			
	O-PS	SR core	R core	Ala	Rha	Glc	Hep
58-F	+	+	H	0.87	1.03	3.82	2.09
58-F-r1	−	−	L	0.41	0.32	0.97	2.16
58-F-r2	−	−	L	0.49	—[c]	—	2.26
58-F-r3	−	−	H	1.05	0.92	4.16	2.21
58-F-r4	−	−	H	1.06	0.86	3.89	2.19
58-F-r9	+	+	H	1.03	0.88	3.19	2.16
PAS10	−	−	H	1.07	1.01	2.87	2.22
PAS10-r1	−	−	L	0.47	—	—	2.14
PAS10-r20	−	−	L	0.41	—	—	2.33
PML14	++	+	H	1.02	1.10	3.14	2.05
PML14e	−	−	L	0.43	—	—	2.17
PML14b	+	+	H	1.00	0.82	2.95	2.12
PML14d	+	+	H	0.78	0.88	2.04	2.09

[a] Saccharide fractions were analyzed by gel filtration on a Bio-Gel P-6 column after removal of lipid A by acid hydrolysis. O-PS, O polysaccharide; +, present; −, defective; ++, present in abundance; H and L, high- and low-molecular-weight R cores in comparison with that of the parent strain, respectively. Chromatograms for 58-F and its mutants are presented in Fig. 2 as an example.
[b] Values are molar ratios relative to galactosamine, which was set as 1.00. Abbreviations are as defined in Table 2, footnote *b*.
[c] —, not detected.

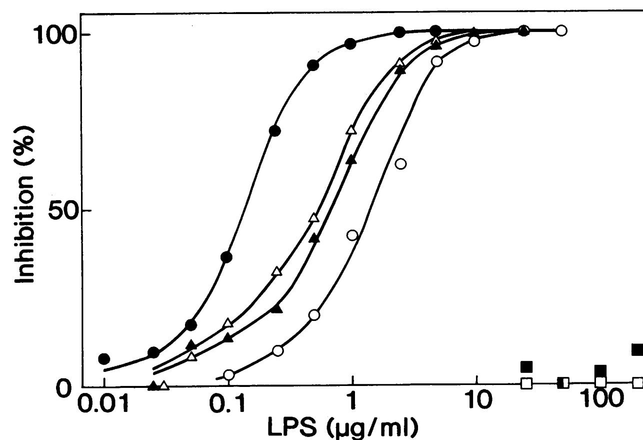

FIG. 1. Neutralization of φCTX infectivity with LPS preparations derived from *P. aeruginosa* 58-F (●) and its mutants 58-F-r1 (■), 58-F-r2 (□), 58-F-r3 (▲), 58-F-r4 (△), and 58-F-r9 (○). φCTX-c was incubated with various concentrations of LPS at 37°C for 1 h, and then residual phage titers were measured, with PAS10 as an indicator strain.

molecular sizes of the R-type LPS as determined by SDS-PAGE (Fig. 3A) and gel filtration analysis (Fig. 2), and the reactivities with anti-LPS core MAbs (Table 4) were similar to those of the parent strain. The results for 58-F-r3 and 58-F-r4 indicated that the two strains had a nearly intact core structure. On the other hand, 58-F-r9 was a smooth strain expressing O polysaccharide but the amount of glucose in its R core was significantly reduced. φCTX bound to this strain with more reduced efficiency than to 58-F-r3 and 58-F-r4. In agreement with these results, the neutralizing activity of the LPS derived from 58-F-r9 was lower than those for the other two mutants (Fig. 1). The molecular size of the R core and the reactivity of anti-LPS core MAbs to 58-F-r9 did not show any significant difference from those for the parent strain.

By contrast, 58-F-r1 and 58-F-r2 were considered completely resistant to φCTX, from the data on plaque-forming activities and adsorption to the cells (Table 4). The neutralizing activities of their LPS preparations were also undetectable (Fig. 1). In SDS-PAGE (Fig. 3A) and gel filtration analysis (Fig. 2), 58-F-r1 and 58-F-r2 were found to have R-type LPS(s) with significantly lower molecular weight(s) than that of the parent strain. The sugar composition of the R core (Table 3) of 58-F-r1 had markedly decreased amounts of glucose and rhamnose, and 58-F-r2 completely lacked them. These findings indicated that the φCTX-resistant mutants 58-F-r1 and 58-F-r2 lacked a part of the outer core region of LPS. The notion was supported by the reactivity of anti-LPS core MAbs, namely, 58-F-r2 did not binding FK-2E7 or MH-4H7, and 58-F-r1 showed weak binding of FK-2E7 but not of MH-4H7.

(iii) Strain PAS10 and its derivatives. On the basis of the reactivities of serotype-specific MAbs and the activity of agglutination with acriflavine (Table 4), as well as the saccharide fraction analysis of LPS (Table 3), parent strain PAS10 was considered a rough strain. The LPS core of parent strain

TABLE 4. Binding of anti-surface antigen MAb to *P. aeruginosa* 58F, PAS10, and PML14 mutants and sensitivity to φCTX

Strain	Serotype[a]	Agglutination with acriflavine[b]	φCTX sensitivity (EOP)[c]	Adsorption of φCTX to cells (%)	MAb binding (A_{405})			
					Anti-LPS core		Anti-serotype M	Anti-D-rhamnan (TS-1B2)
					FK-2E7	MH-4H7		
58-F	A	−	+ (1)	100	1.21	1.62	0.15	1.06
58-F-r1	NT	+++	− (<5 × 10^{-5})	12	0.54	0	1.42	0
58-F-r2	NT	+++	− (<5 × 10^{-5})	9	0.06	0	1.24	0.02
58-F-r3	A	+++	+ (0.6)	91	1.90	1.34	1.44	1.43
58-F-r4	A	+++	+ (ND)	89	1.98	1.50	1.45	1.41
58-F-r9	A	+	+ (0.18)	60	1.87	1.64	0.76	1.23
PAS10	M	+++	+ (1)	58	0.54	1.00	1.64	0.86
PAS10-r1	M	+++	− (<10^{-6})	5	0	0	1.80	0.72
PAS10-r20	M	++	− (<10^{-6})	3	0	0	1.43	0.14
PML14	I	−	+ (1)	100	0.61	0	0.11	1.16
PML14e	I	+	− (<10^{-5})	6	0	0	0.76	0.01
PML14b	I	+++	+ (ND)	63	2.18	0	0.68	1.70
PML14d	I	+++	+ (ND)	58	2.12	0	0.63	1.69

[a] According to the Homma scheme (13). NT, nontypeable.
[b] +++, strong; ++, medium; +, weak; −, none.
[c] +, sensitive; −, resistant. EOP, efficiency of plaque formation; ND, not determined.

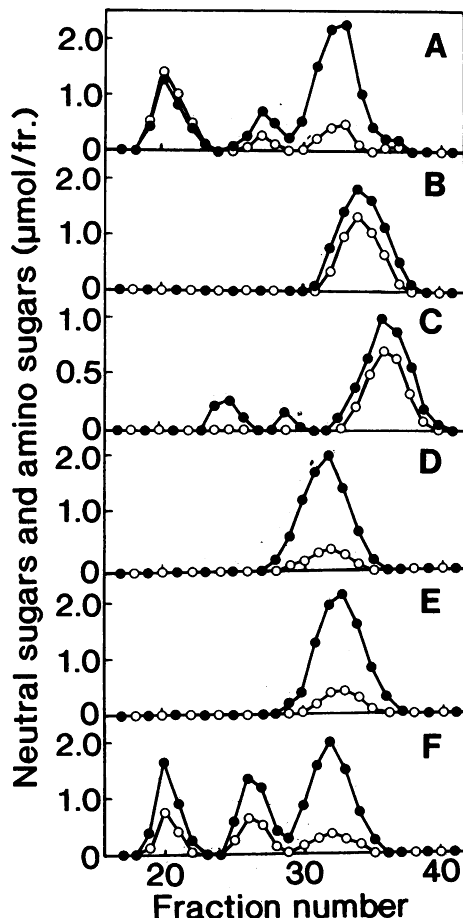

FIG. 2. Analysis of saccharide fractions derived from acetic acid-treated LPSs of *P. aeruginosa* 58-F (A) and its mutants 58-F-r1 (B), 58-F-r2 (C), 58-F-r3 (D), 58-F-r4 (E), and 58-F-r9 (F) by Bio-Gel P-6 chromatography. Each fraction (0.5 ml) was collected and assayed for neutral sugars (●) and amino sugars (○). In the chromatogram of the parent strain 58-F (A), the first peak was the polysaccharide fraction containing O polysaccharide and D-rhamnan as major and minor components, respectively. The second peak was attributable to a semirough core oligosaccharide, which carries one unit or a few repeating units of the O-polysaccharide chain. The third peak was R-core oligosaccharide, which did not contain O-polysaccharide components.

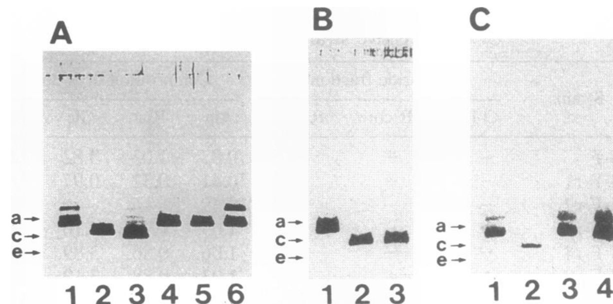

FIG. 3. SDS-PAGE of LPS derived from *P. aeruginosa* PML14, PAS10, and 58-F and their mutants. LPS (1 µg per lane) was applied to an 18% polyacrylamide slab gel. (A) 58-F (lane 1) and mutants 58-F-r1 (lane 2), 58-F-r2 (lane 3), 58-F-r3 (lane 4), 58-F-r4 (lane 5), and 58-F-r9 (lane 6). (B) PAS10 (lane 1) and mutants PAS10-r1 (lane 2) and PAS10-r20 (lane 3). (C) PML14 (lane 1) and mutants PML14e (lane 2), PML14b (lane 3), and PML14d (lane 4). The positions of *Salmonella typhimurium* Ra-, Rc-, and Re-type LPSs (arrows a, c, and e, respectively) are indicated. When 10 µg of LPS per lane was applied to a 15% polyacrylamide gel, ladderlike or smear bands originating from O-polysaccharide-carrying smooth-type LPS were observed for PML14, PML14b, PML14d, 58-F, and 58-F-r9 (data not shown).

PAS10 contained galactosamine, alanine, glucose, and rhamnose in a molar ratio of about 1:1:3:1. Its derivatives, PAS10-r1 and PAS10-r20, which were completely resistant to φCTX, had an R core with a lower molecular weight than that of the parent strain (Fig. 3B and Table 3). Furthermore, the derivatives had undetectable amounts of glucose and rhamnose in the R core and lacked the binding activity of anti-LPS core MAbs FK-2E7, MH-4H7, and OM-1D6 (data not shown).

(iv) Strain PML14 and its derivatives. While PML14 was sensitive to all types of R pyocins (R1 to R5) and φCTX, its derivatives PML14e, PML14b, and PML14d were mutants with altered sensitivities to R pyocins (15, 33) and φCTX (11) (Table 4). Since the proposed receptor site for R-type pyocins was the core region of LPS (25), these mutants should have some defects in their LPS cores. The R core of PML14 contained galactosamine, alanine, glucose, and rhamnose in a molar ratio of about 1:1:3:1 (Table 3). Of the three PML14 derivatives, φCTX bound to PML14b and PML14d at a reduced efficiency. Two strains expressed lesser amounts of O polysaccharide than the parent strain but had R- and SR-core oligosaccharides with molecular weights similar to those of PML14 (Fig. 3C and Table 3). Although PML14b is resistant to pyocin R3, we could not detect a significant difference between the R-core compositions of PML14b and PML14. PML14d, which was resistant to pyocins R2, R3, and R4, contained less glucose in the R core, suggesting a minor defect in the core region.

PML14e was resistant to all types of R pyocins and φCTX. This mutant had an R-type LPS with a markedly lower molecular weight than those of the other strains (Fig. 3C) and completely lacked the rhamnose and glucose residues as core components. A marked defect in its LPS core was also suggested by the lack of binding of the anti-LPS-core MAb FK-2E7 (Table 4). PML14e was interesting in that, although the O-polysaccharide fraction was not detected in its LPS preparation by saccharide fraction analysis (Table 3), it showed agglutination with the anti-serotype I MAb and very weakly agglutinated with acriflavine. The results indicated that PML14e expresses O-polysaccharide antigen on the cell surface, but the O polysaccharide was not recovered in the LPS preparation.

φCTX sensitivities of various serotypes of *P. aeruginosa* wild-type strains. The series of evidence described above indicated that a receptor site of φCTX was LPS and that the existence of a particular LPS core structure was essential for φCTX infection. In a recent study (43), the core structure of *P. aeruginosa* LPS was found to be heterogeneous by examination of strains for their activity of binding to anti-LPS core MAbs. Moreover, this heterogeneity correlated with O serotypes. Therefore, we examined the host range of φCTX among the *P. aeruginosa* wild-type strains from various sources and its relationship to the O serotypes as well as to the binding activities of four anti-LPS core MAbs (43) and an anti-common polysaccharide antigen E87 (D-rhamnan) (30, 41) MAb, TS-1B2. Since we found in the preliminary test that plaque formation assays were significantly affected by the restriction-modification system of the host strains, adsorption of φCTX to the cells was determined instead of the usual plaque formation assay. As

TABLE 5. Host range of φCTX in various serotypes of *P. aeruginosa*

Serotype(s)		No. of strains tested	φCTX adsorption to whole cells (no. of strains)		
Homma	IATS		>90%	30–90%	<30%
A	3	8	8	0	0
K	13, 14	1	1	0	0
I	1	6	5	1	0
D	9	3	0	3	0
M	NT[a]	1	0	1	0
G	6	16	6	6	4
C	7, 8	7	2	2	3
NT[a]	NT	11	0	3	8
B	2, 5, 16	20	0	0	20
E	11	9	0	0	9
F	4	2	0	0	2
H	10	1	0	0	1
L	12	1	0	0	1

[a] NT, nontypeable.

shown in Table 5, the activity of binding (and probably the host range) of φCTX to *P. aeruginosa* strains showed a significant correlation to the O serotypes of the host strains. φCTX bound to most of the strains belonging to serotypes A, I, D, and G but not at all to strains belonging to serotypes B and E. Although the relationship between the O serotype and the binding of anti-LPS core MAbs was similar to that found previously (43), the binding range of φCTX was not identical to any of the binding ranges of four anti-LPS core MAbs; for example, MH-4H7 bound to strains of serotypes A and G with high frequency but not to strains of serotypes B, E, and I, and FK-2E7 bound to most strains of serotype E and I but not to strains of serotype B (43). The binding of anti-D-rhamnan MAb TS-1B2 also did not show any correlation to φCTX sensitivity (data not shown).

Genetic locus specifying φCTX sensitivity. The results described above indicated that φCTX might recognize a specific structure of the outer core distributed among strains of some particular O serotypes, suggesting that not only anti-core MAbs but also φCTX could be used as probes for studying the core structure of *P. aeruginosa* LPS, as shown for several bacteriophages in studies of the LPSs of enteric bacteria (32). Therefore, as the first step to understand the genetic background for the LPS core structure and its biosynthesis, we constructed hybrid strains by interstrain crosses in the region near the *somA* locus which participates in the determination of O-serotype specificity (24, 25), and the structures of the LPS cores were determined by using anti-LPS core MAbs and φCTX (Table 6). R-type pyocins were also used for the probes since it was reported that they bound to the LPS core as well (25). When the *somA* region of PTO66 (resistant to φCTX; serotype B) was conjugally introduced into a 58-F derivative (sensitive to φCTX; serotype A), it changed to serotype B, and this change was accompanied by the loss of φCTX and pyocin R3 sensitivity. The hybrid strain 58-F-7 also lost the binding of an anti-core MAb (MH-4H7). However, the properties of host strain 58-F, such as binding of another anti-core MAb (FK-2E7) and sensitivity to R pyocins (other than pyocin R3), were retained in the hybrid strain. The results suggested that the hybrid strain had a chimeric LPS core structure. We have been unable to introduce the *somA* region of 58-F into either PTO66 or its parental strain PAO1, for unknown reasons.

As previously described (11), PAO2103 is another hybrid with a different combination of serotypes and phage sensitivity, in which the *somA* region of strain 5933 (sensitive to φCTX; serotype I) was introduced into a PAO1 derivative (resistant to φCTX; serotype B) by F116-mediated transduction. In this case, the PAO1 derivative was converted to serotype I and acquired sensitivity to φCTX and R pyocins. However, PAO 2103 did not acquire the reactivity of MAb FK-2E7 and showed less sensitivity to pyocins R3 and R4. These results for 58-F-7 and PAO2103 confirmed our presumption that the *somA* region contains a genetic locus (or loci) responsible for φCTX sensitivity and consequently which participates in the biosynthesis of part of the LPS core. We have constructed another hybrid of PAO, whose *somA* region was replaced with that of a strain of serotype E (resistant to φCTX and all R-type pyocins). This type of hybrid strain remained resistant to φCTX but changed to serotype E and became resistant to all R pyocins, including R5 (data not shown). We have been unable to introduce the *somA* region of PAO into strain 5933.

DISCUSSION

φCTX is a cytotoxin-converting phage of *P. aeruginosa* isolated from strain 158 (7, 11). Recently, we and others showed that φCTX is a bacteriophage phylogenetically and serologically related to the R pyocins (11), whose receptor sites were proposed to be the core region of LPS (25). Furthermore, some LPS-defective mutants of PAC1R (16, 28) and PML14 derivatives with altered R-pyocin sensitivities (15, 33) were found to be resistant to φCTX while their parent strains were

TABLE 6. Sensitivity to φCTX and R pyocins, binding of anti-LPS core MAbs, and serotypes of hybrid strains constructed by interstrain crosses

Strain	Serotype	Sensitivity to φCTX	Adsorption to cells (%)	Binding of anti-LPS core MAb (A_{405})		Sensitivity to R pyocin[a]				
				FK-2E7	MH-4H7	R1	R2	R3	R4	R5
58-F[b]	A	+	100	1.21	1.62	S	S	S	S	S
58-F-7	B	−	3	1.33	0	S	S	R	S	S
PTO66	B	−	9	0	0	R	R	R	R	S
PAO1[c]	B	−	5	0	0	R	R	R	R	S
PAO2103[d]	I	+	100	0	0	S	S	(S)	(S)	S
5933	I	+	100	1.56	0	S	S	S	S	S

[a] S, sensitivity; R, resistance; (S), weak growth inhibition observed (an effect which was completely neutralized by antiserum specific to homologous R pyocin but not by anti-pyocin F3 serum).
[b] 58-F-c showed the same properties as those of 58-F presented here.
[c] PAO1840 showed the same properties as those of PAO1 presented here.
[d] The sugar composition of the O polysaccharide was confirmed to be the same as that of 5933 (34a).

sensitive (11). These findings suggested the possible role of the LPS, especially the core region, as the receptor site for φCTX.

In this study, we isolated several mutants from strain 58-F and PAS10 with altered sensitivities to φCTX and characterized their LPSs, together with those from PAC1R and PML14 derivatives. Our presumption that the receptor for φCTX might be LPS was confirmed by the following findings. (i) LPS preparations derived from φCTX-sensitive wild strains (e.g., PAC1R and 58-F) clearly inhibited the infectivity of φCTX, while those from resistant wild strains (e.g., PAO1) and resistant mutants (e.g., PAC611, PAC605, 58-F-r1, and 58-F-r2) derived from the sensitive strains exhibited no inhibitory effect. (ii) In several mutants showing reduced phage adsorption (PAC608, PAC557, PAC556, 58-F-r3, 58-F-r4, and 58-F-r9), efficiency of phage adsorption to the cells corresponded well to the phage-neutralizing activities of LPS preparations from each strain (Table 4 and Fig. 1). (iii) Changes of LPS structures by genetic recombinations in the *somA* region were accompanied by changes in φCTX sensitivity (Table 6).

Furthermore, an essential role of the LPS outer core region in the binding of φCTX was indicated by the results of structural analysis of LPSs from mutants with resistance or altered sensitivities to φCTX; that is, strains having nearly intact core structures but no O polysaccharide (PAC557 and PAS10) were still sensitive to the phage and φCTX-resistant mutants contained defects in LPS structure extending to the outer core region (Table 3 and Fig. 3). Furthermore, all resistant mutants lacked rhamnose and glucose altogether or contained markedly decreased amounts of these residues as core components. They also lacked binding to anti-LPS core MAbs MH-4H7 and/or FK-2E7. These results indicated that φCTX recognized a particular part of the *P. aeruginosa* LPS outer core region, including L-rhamnose and D-glucose residues. However, it seemed that some D-glucose residues in the outer core region were nonessential for phage binding, because some mutants (PAC609, PML14d, and 58-F-r9) which were still sensitive to φCTX (Table 4) contained significantly reduced amounts of D-glucose residues in their LPS cores. PAC556, which was reported to lack only the L-rhamnose residue in the outer core (28), was φCTX resistant in the plaque-forming assay but still adsorbed φCTX (Table 2). The uninfectivity of φCTX for PAC556 may be due to its inability to functionally bind to the cell surface and/or the presence of an additional mutation(s) in the strain which exerted some effect on the process of phage infection (tolerance).

In contrast to the role of the outer core region, that of the long O-polysaccharide chain has remained ambiguous. It is difficult to deny the possibility that the other sites of LPS might play some role in the binding of φCTX, because most of the φCTX-resistant mutants were defective in not only part of the core region but also the polysaccharide portion, such as the O polysaccharide and the common polysaccharide antigen E87 (D-rhamnan) (Tables 2, 3, and 4). Although the rough mutants (PAC557, 58-F-r3, and 58-F-r4) lacking the O polysaccharides and the semirough mutant (PAC608) which had only one repeating unit of the O polysaccharide but contained a nearly complete core showed reduced binding to φCTX, they were still sensitive to the phage. The results suggested that the long O-polysaccharide chain had some accessory (but not essential) role in the binding of φCTX to the cells or that, in these rough mutants, the core structures had some defect(s) which could not be detected in our study. If the long O-polysaccharide chain had some accessory role, it could be that the reduced binding of φCTX is attributable to the difference in the hydrophobicities of the LPS molecules and cell surfaces, which could affect interaction with the phage. The possibility that surface component(s) other than the LPS molecule, for example, outer membrane proteins, might also be involved in the binding of the phage cannot be excluded.

An interesting finding obtained through the analysis of LPS structures of mutants was the unusual expression of O polysaccharide in PML14e. Although the O-polysaccharide antigen was detected on the cell surface by using anti-serotype I antibodies (Table 4), the O polysaccharide could not be recovered in the LPS preparation (Table 3). This phenomenon may be explained by expression of the O polysaccharide on the cell surface in PML14e. However, the O polysaccharide of this strain did not attach to the core-lipid A anchor and was expressed in a capsularlike manner because a major part of the outer core region was lacking. The O-polysaccharide antigens of 58-F-r3 and 58-F-r4 (Table 3 and Fig. 2) seemed to be expressed in the same manner, because they were agglutinable with anti-serotype A antibodies. These interesting findings will be discussed elsewhere.

Previously, the heterogeneity of the LPS core of *P. aeruginosa*, determined by using human MAbs as probes, was reported (42, 43). The outer core region was heterogeneous among *P. aeruginosa* strains in the properties of binding of a set of anti-LPS core MAbs, and the heterogeneity was serotype dependent. The host range of φCTX also showed a marked correlation to the O serotypes of the host strains (Table 5). The phenomenon also seemed to result from the structural diversity of the outer core of *P. aeruginosa* LPS. However, the host range showed O-serotype dependency different from that of the anti-LPS core MAbs (43), suggesting that the LPS core structures recognized by the MAbs and φCTX differed from each other and that each structure was distributed in a serotype-dependent manner among *P. aeruginosa* strains.

This interesting finding led us to identify a genetic locus (or loci) which participates in the determination of part of the LPS core structure, using φCTX and anti-LPS core MAbs as probes; that is, a genetic locus (or loci) specifying φCTX sensitivity and the structure of part of the outer core exists in or near the *somA* locus. It seems quite reasonable that the genes specifying the O serotype and the core structure are closely linked to each other on the chromosome in *P. aeruginosa*, because of the correlation of the heterogeneity of the core structure with the O serotype (43). The locus described here could be the same as that named *somB*, which has been shown to determine the smooth-rough conversion and mapped to be tightly linked to the *somA* locus (12, 23). Recently, a DNA fragment specifying the production of the O polysaccharide of Fisher immunotype 2 (corresponding to O11 and Homma type E) has been cloned from PA103 (4), and a DNA fragment of PAO1 that complemented the defect of O-polysaccharide production has been cloned (19). The latter fragment was mapped to the region encompassing the three *Spe*I fragments (SpC, SpAI, and SpI) on the PAO1 chromosome by pulsed-field gel electrophoresis. Since *eda-9001* and *leu-10* were mapped to the SpC and SpI fragments, respectively (26, 37), it is likely that the cloned PAO fragment contains *somA* or part of it. However, neither of the previous reports mentioned the possibility that the cloned fragments contain the gene(s) participating in the determination of the core structure.

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