Novel O-polysaccharide expression, as a lipid A-core-free form, in a lipopolysaccharide-core-defective mutant of *Pseudomonas aeruginosa*

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***Pseudomonas aeruginosa* PML14e is a mutant strain, isolated from strain PML14 (Homma serotype I), that is resistant to all types of R-pyocins. PML14e completely lacked glucose and rhamnose as components of the lipopolysaccharide (LPS) outer core region. Whereas the O-polysaccharide attachment site on the LPS core was considered to be absent, PML14e was agglutinable with anti-serotype-I antibodies. The O-polysaccharide of PML14e was recovered in the supernatant after ultracentrifugation of the aqueous layer from a hot phenol/water extraction. Chromatographic behaviour and chemical analysis indicated that the PML14e O-polysaccharide was not linked to the lipid A. ¹H-NMR spectroscopy indicated that the structure of the PML14e O-polysaccharide was the same as that of the O-polysaccharide from PML14. The above evidence indicated that the O-polysaccharide is expressed on the cell surface of the mutant strain PML14e as the lipid A-free form. To examine the nature of the cell surface, the accessibility of monoclonal antibodies (mAbs) against cell surface antigens was tested by enzyme-linked immunosorbent assay. An anti-lipid A mAb and an anti-outer-membrane protein mAb, the epitopes for which are considered to be exposed on rough strains, bound to a greater extent to the PML14e cells than to two other LPS-core-defective rough mutants, PML14b and PML14d. Whereas these mutants appeared to have lesser defects in the LPS core, they expressed less O-polysaccharide than PML14e. The results indicated that the epitopes exposed on rough strains, such as lipid A and outer-membrane proteins, were mainly hindered by covalently linked core oligosaccharide rather than by the O-polysaccharide chain.**

Keywords: *Pseudomonas aeruginosa*, lipopolysaccharide, O-antigen, LPS-core-defective mutant, R-pyocin

INTRODUCTION

Lipopolysaccharide (LPS) is a major component of the Gram-negative bacterial outer membrane. LPS from wild strains of *Pseudomonas aeruginosa* has a typical structure comprising O-antigenic polysaccharide, core oligosaccharide and lipid A, like members of the *Enterobacteriaceae* (Knirel, 1990; Wilkinson, 1983). To study LPS structure and biosynthesis, mutants with various defects

in LPS would be good tools, so the phenotypes of these mutants require detailed characterization. Among several means of generating LPS-core-defective mutants, the isolation of mutants resistant to phages and R-pyocins is one of the most useful. Some phages and R-pyocins recognize a specific structure of the LPS core as a receptor (Jarrell & Kropinski, 1981a, b, c; Meadow & Wells, 1978; Temple *et al.*, 1986; Yokota *et al.*, 1994). Most LPS-core-defective mutants have the rough phenotype with none, or markedly decreased levels, of the O-polysaccharide (Berry & Kropinski, 1986; Rowe & Meadow, 1983).

Yokota *et al.* (1994) found that an R-pyocin-resistant mutant derived from *P. aeruginosa* PML14 [Homma serotype I; corresponding to International Antigenic

Abbreviations: D-FucNAc, 2-acetamido-2,6-dideoxy-D-galactose; D-GalNAc, 2-acetamido-2-deoxy-D-galactose; D-Glc (NAc)₂A, 2,3-diacetamido-2,3-dideoxy-D-glucuronic acid; KDO, 3-deoxy-D-manno-octulosonic acid; OMP, outer membrane protein; D-QuiNAc, 2-acetamido-2,6-dideoxy-D-glucose; rLPS, rough LPS; sPS, soluble polysaccharide; TPS, total polysaccharide.

Typing Scheme O1 (Liu *et al.*, 1983)] had an unusual O-polysaccharide. Whereas the mutant, PML14e, completely lacked the neutral sugar residues, D-glucose and L-rhamnose, as components of the LPS outer core region (Yokota *et al.*, 1994) and was resistant to all types of R-pyocins (Ito & Kageyama, 1970; Shinomiya & Shiga, 1979), it was clearly typed as Homma serotype I. Furthermore, the O-polysaccharide was not found in the LPS of PML14e prepared by the method of Uchida & Mizushima (1987), which included ultracentrifugation.

In this study, the O-polysaccharide prepared from PML14e was purified and structurally characterized, and its properties were compared with those of the O-polysaccharide from the parent strain PML14 and the mutant strains PML14b and PML14d. Furthermore, the cell surface nature of these strains was examined using monoclonal antibodies (mAbs) against cell-surface anti-

METHODS

Bacterial strains. *P. aeruginosa* PML14 (formerly P14) and its mutants (Ito & Kageyama, 1970; Shinomiya & Shiga, 1979), which have different degrees of sensitivity to various R-pyocins (summarized in Table 1), were kindly donated by M. Kageyama, Mitsubishi Kagaku Institute of Life Science, Tokyo, Japan.

Preparation of LPS. *P. aeruginosa* cells were cultured in heart infusion broth to the late exponential growth phase. LPS was prepared by the phenol/water method basically according to Westphal & Jann (1965). Briefly, the cells were agitated with 45% (v/v) phenol at 70 °C for 15 min. After cooling and centrifugation, the aqueous phase was collected. After exhaustive dialysis against deionized water, the aqueous phase was digested with 50 µg DNase I ml⁻¹ and 10 µg RNase ml⁻¹ (Boehringer Mannheim) in 10 mM Tris/HCl containing 2 mM MgCl₂ (pH 6·8) at 37 °C for 6 h. After exhaustive dialysis against deionized water and lyophilization, the material was used as total polysaccharide (TPS) preparation. Aliquots of TPS were centrifuged at 100 000 *g* for 90 min at 15 °C in 50 mM Tris/HCl containing 2 mM MgCl₂ (pH 6·8), and the resulting pellet was used as LPS preparation. The supernatant and pellet derived from TPS of strain PML14e were named soluble polysaccharide (sPS) and rough LPS (rLPS), respectively. The

sPS was further purified by successive Superose 6 chromatography and DEAE-Sephacel chromatography as described below.

Chromatographic procedures. Anion-exchange column chromatography was performed using a DEAE-Sephacel (Pharmacia) column equilibrated with 5 mM ammonium acetate (pH 7·4). The column was eluted with a linear gradient of NaCl (0–0·5 M) in 5 mM ammonium acetate (pH 7·4). Superose 6 (Pharmacia) column chromatography was carried out in 50 mM (NH₄)₂CO₃ without or with 0·1% sodium deoxycholate.

mAbs. The anti-*P. aeruginosa* mAbs are presented in Table 2. The hybridoma cell lines producing the human mAbs were generated by cell fusion of pokeweed-mitogen-stimulated human peripheral blood lymphocytes derived from healthy adult volunteers, with the human-mouse heteromyeloma SHM-D33 or the mouse myeloma P3x63-Ag.8.653 (only for YK-1H5) using polyethylene glycol as described by Yokota *et al.* (1989). The murine mAb specific for serotype I (Mei-assay I; IgM) was one of the mAbs in the Mei-assay serotyping kit (Meiji Seika Kaisha). Hybridomas were cultured in Iscove's-modified minimum essential medium (GIBCO) containing 10% (v/v) foetal bovine serum (HyClone), and the resulting culture supernatants were used for further studies. The mAb concentration in the culture supernatant was determined by means of a sandwich ELISA consisting of goat anti-human IgM or IgG antibodies (KPL) as the coated antigen and alkaline-phosphatase-conjugated goat anti-human IgM or IgG antibodies (KPL) as the second antibody. Standard human IgM and IgG prepared from human plasma were purchased from Cosmo Bio.

Preparation of saccharide fraction of LPS. Saccharide fractions derived from LPS or TPS were prepared basically according to Koval & Meadow (1977). Briefly, TPS or LPS were hydrolysed with 2% (v/v) acetic acid at 100 °C for 90 min. After neutralization with NaOH and centrifugation at 10 000 *g*, the supernatant was applied to a Biogel P6 (Bio-Rad) column (1 × 50 cm) in 50 mM pyridine/acetate (pH 5·0). The O-polysaccharide used for further examination was purified by successive DEAE-Sephacel and Superose 6 column chromatography.

Miscellaneous materials and methods. ELISA was carried out in microtitre plates coated with the whole cells fixed with 1% (v/v) glutaraldehyde as described by Yokota *et al.* (1989). Alkaline-phosphatase-conjugated anti-IgG or IgM antibodies (KPL) and sodium *p*-nitrophenyl phosphate were the second

Table 1. Summary of phenotypes of *P. aeruginosa* PML14 and its mutants

Strain	Agglutination with anti-serotype I antiserum*	Agglutination with acriflavine†	R-pyocin sensitivity‡					Composition of R core§				
			R1	R2	R3	R4	R5	Glc	Rha	GalN	Ala	Hep
PML14	+++	—	S	S	S	S	S	3·14	1·10	1·00	1·02	2·05
PML14b	++	+++	S	S	R	S	S	2·95	0·82	1·00	1·00	2·12
PML14d	+	+++	S	R	R	R	S	2·04	0·88	1·00	0·78	2·09
PML14e	+++	+	R	R	R	R	R	—	—	1·00	0·43	2·17

* Similar results were obtained for both the antiserum and the mAb. + + +, Strong; + +, medium; +, weak.

† Data from Yokota *et al.* (1994). + + +, Strong; +, weak; —, no agglutination.

‡ Data from Shinomiya & Shiga (1979). s, Sensitive; R, resistant.

§ Data from Yokota *et al.* (1994). Values are molar ratios relative to galactosamine, which was set as 1·00. —, Not detectable.

Table 2. Anti-*P. aeruginosa* mAbs used in this study

Clone	Species/isotype	Recognition site	Specificity	Characteristics*	Reference
HY-1F10	Human IgM (λ)	LPS; O-polysaccharide	Serotype I specific	Reacts to semirough LPS	This study
Mei-assay I	Murine IgM (κ)	LPS; O-polysaccharide	Serotype I specific	Does not react to semirough LPS	Commercial
FK-2E7	Human IgM (κ)	LPS; outer core	Various serotype strains of <i>P. aeruginosa</i> (especially serotype E and I)		Yokota <i>et al.</i> (1992)
NM-3D7	Human IgM (λ)	LPS; lipid A	Lipid A derived from <i>E. coli</i> , <i>S. minnesota</i> and <i>P. aeruginosa</i>	Antigen binding inhibited by polymyxin B (ID ₅₀ 6 μ g ml ⁻¹ to <i>E. coli</i> J5 cells in ELISA)	Yokota <i>et al.</i> (1992)
YK-1H5	Human IgG3 (κ)	Outer-membrane protein G	All <i>P. aeruginosa</i> strains		This study

* These characteristics were first presented in this paper.

antibody and substrate, respectively. SDS-PAGE and Western blotting were carried out as described previously (Yokota *et al.*, 1989, 1992). Serotype was determined by the slide agglutination method using a kit containing rabbit antisera (Denka Seiken) or murine mAbs (Mei-assay). ¹H-NMR spectroscopy was performed in 99.96% D₂O at 60 °C with a Jeol FX-400 spectrometer. Chemical shifts are given with sodium 3-trimethylsilylpropane sulfonate as an internal standard (δ = 0.00 p.p.m.). The rough nature of *P. aeruginosa* strains was estimated using the acriflavine agglutination test as described by Yokota *et al.* (1994). Neutral sugars were determined using the phenol/sulfuric acid method (Dubois *et al.*, 1956) with D-glucose as a standard. Amino sugars were determined by the method of Tsuji *et al.* (1969), using glucosamine as a standard after N-deacylation with 2 M HCl at 100 °C for 2 h. 3-Deoxy-D-manno-octulosonic acid (KDO) was determined using the thio-barbituric acid method (Karkhanis *et al.*, 1978) after hydrolysis with 10 mM H₂SO₄ at 100 °C for 30 min. Standard KDO was purchased from Sigma. Lipid A was determined by an inhibition ELISA using human anti-lipid A mAb NM-3D7. Briefly, the sample was hydrolysed with 2% (v/v) acetic acid for 90 min, then lyophilized. The resulting material was dissolved in phosphate-buffered saline containing 1% (w/v) bovine serum albumin by exhaustive sonication. Serially diluted samples and 100-fold-diluted culture supernatant of human anti-lipid A mAb NM-3D7 (500–800 ng IgM ml⁻¹) were mixed and applied to microtitre plates coated with lipid A derived from *Salmonella minnesota* R595 (Ribi Immunochemical). Standard lipid A was prepared from *P. aeruginosa* IID1001 according to the method of Bhat *et al.* (1990). Endotoxicity was estimated by a colorimetric limulus test using ENDOSPECY: ES Test kit (Seikagaku Kogyo). *Escherichia coli* O111:B4 LPS was used as a standard.

RESULTS

Phenotypes of *P. aeruginosa* PML14 and its mutants

Strain PML14 has been used as the indicator strain for R-pyocins (Ito & Kageyama, 1970; Shinomiya & Shiga, 1979). Kageyama and his collaborators have isolated various types of resistant strains, shown in Table 1. We determined the chemical composition of the core oligosaccharide of LPS derived from these mutants (Yokota *et al.*, 1994). PML14e completely lacked D-glucose and L-

rhamnose residues as outer core components and was resistant to all types of R-pyocins. Whereas PML14e lacked most of the outer core region, which included the attachment site of the O-polysaccharide (Rowe & Meadow, 1983; Knirel, 1990), both its viable and heat-killed cells clearly agglutinated with anti-serotype I antisera and mAb. Because PML14e weakly agglutinated with acriflavine, it was considered to have a much less rough phenotype than PML14b and PML14d. Data from the whole-cell ELISA (Fig. 1a) also indicated that PML14e expressed more type I O-polysaccharide on the cell surface than the other rough mutants PML14b and PML14d. The chemical composition of the core oligosaccharide and R-pyocin resistance indicated that PML14b and PML14d had lesser defects in the LPS core region than PML14e.

Isolation and characterization of the sPS derived from strain PML14e

TPS was prepared from PML14 and its mutants by the hot phenol/water extraction method (Westphal & Jann, 1965). The preparations were analysed by Western blotting with two anti-serotype I mAbs (Fig. 2). One of these, HY-1F10, was able to recognize one repeating unit of O-polysaccharide, namely it bound to semirough LPS. The other, Mei-assay I, did not bind to semirough LPS. The mAb Mei-assay I seemed to recognize a structure of the O-polysaccharide formed by the polymerization of the O-polysaccharide repeating unit. PML14 expressed high levels of serotype I O-polysaccharide, and PML14b and PML14d expressed less than the parent strain when evaluated by both HY-1F10 and Mei-assay I. Semirough LPS was also detected in the TPS from these strains by the mAb HY-1F10 (Fig. 2b, lanes 1–3). In contrast, PML14e expressed moderate levels of long O-polysaccharide, but semirough LPS was undetectable in the TPS (Fig. 2b, lane 4).

To prepare LPS, aliquots of TPS were centrifuged at 100 000 g at 15 °C for 90 min in the presence of Mg²⁺. LPS

Fig. 1. Reactivity of anti-*P. aeruginosa* mAbs, anti-serotype I O-polysaccharide mAb Mei-assay I (a), anti-LPS outer core mAb FK-2E7 (b), anti-lipid A mAb NM-3D7 (c) and anti-outer-membrane protein G mAb YK-1H5 (d), to *P. aeruginosa* PML14 and its mutants in ELISA. Strains were PML14 (○), PML14b (△), PML14d (▲) and PML14e (●). *P. aeruginosa* cells were fixed to the microplate with glutaraldehyde.

Fig. 2. Western blotting of TPS derived from *P. aeruginosa* PML14 and its mutants. TPS (1 µg per lane) was applied to SDS-PAGE on a 15% (w/v) polyacrylamide gel. The transferred filter was blotted with Mei-assay I (a) and HY-1F10 (b). TPS fractions were prepared from *P. aeruginosa* PML14 (lanes 1), PML14b (lanes 2), PML14d (lanes 3) and PML14e (lanes 4) by hot phenol/water extraction. Arrows indicate the elution positions of rough LPS (R) and semi-rough LPS (SR) of PML14.

is insoluble in the presence of divalent cations (Coughlin *et al.*, 1983; Galanos & Luderitz, 1975), thus LPS and capsular polysaccharide were separated by this procedure (Jann & Jann, 1983). The polysaccharide portions of TPS and LPS were characterized by gel filtration chromato-

graphy on Biogel P6 after removing lipid A by mild acid hydrolysis. The typical chromatogram of wild strains, such as PML14 (Fig. 3a), contained three peaks. Analytical data of sugar composition (not shown) suggested that the peaks were (i) polysaccharide containing O-polysaccharide and a common polysaccharide antigen (D-rhamnan), (ii) semirough-type R-core oligosaccharide and (iii) rough-type R-core oligosaccharide, in order of elution from the column. The distribution of the saccharide fractions was similar between TPS and LPS derived from PML14, PML14b and PML14d (Fig. 3). PML14b and PML14d had much less polysaccharide fraction than PML14, consistent with the results of whole-cell ELISA (Fig. 1a) and Western blotting (Fig. 2). The supernatant fractions of these strains contained mostly nucleic acids, together with small amounts (less than 1% of TPS) of O-polysaccharide component (data not shown). The results indicated that almost all the O-polysaccharides were sedimented by the ultracentrifugation. In contrast, the polysaccharide fraction was not detected in the LPS preparation derived from PML14e (rLPS), and it was recovered in the supernatant after centrifugation at 100 000 g (Fig. 3d). PML14e had lower-molecular-mass R-core oligosaccharide than the other strains, because it lacked most of the outer core (Table 1). Also PML14e did not have semi-rough core oligosaccharide. In addition, data of ELISA and Western blotting indicated that PML14e lacked D-rhamnan.

The O-polysaccharide of PML14e was recovered in the supernatant after ultracentrifugation (sPS fraction), in contrast to other strains. To characterize the sPS, it was subjected to gel filtration chromatography on a Superose 6 column without detergent. The sPS was eluted from the column as a single peak with an apparent molecular mass

Fig. 3. Gel filtration of the saccharide fraction derived from acetic-acid-treated TPS (upper panels) and LPS (lower panels) derived from *P. aeruginosa* PML14 (a), PML14b (b), PML14d (c) and PML14e (d) on a Biogel P6 column. After acetic acid treatment of TPS or LPS, the soluble material was applied to a Biogel P6 column (1 × 50 cm). Fractions (0.6 ml) were collected and assayed for amino sugars (●) and neutral sugars (○).

of about 30 kDa (Fig. 4a). Furthermore, the sPS was eluted from the DEAE-Sephacel column with about 0.3 M NaCl (Fig. 5a). This chromatographic behaviour was closely similar to that of the O-polysaccharide preparation from which lipid A had been removed, derived from PML14 (Figs 4d and 5c). In contrast, intact LPS derived from PML14 was eluted in the void volume from the Superose 6 column (Fig. 4b), and in the unadsorbed fraction from the DEAE-Sephacel column (Fig. 5b). In buffer containing deoxycholate, intact PML14 LPS was eluted from the Superose 6 column as a single broad peak with a mean molecular mass of about 30 kDa, which was similar to those of PML14 O-polysaccharide and PML14e sPS (Fig. 4c).

The behaviour of PML14 LPS in the presence of deoxycholate resulted from micelle formation by intact LPS. These results indicated that the intact O-polysaccharide of PML14 was included in an LPS micelle structure, whereas PML14e O-polysaccharide (sPS) was not in the LPS micelle. The KDO and lipid A contents of each preparation were determined by colorimetric assay and inhibition ELISA, respectively (Table 3). In PML14e, both KDO and lipid A were recovered in the rLPS, namely the pellet fraction from ultracentrifugation of the TPS. The sPS of PML14e contained neither KDO nor lipid A. The endotoxicity estimated by the colorimetric limulus assay also indicated the sPS did not contain lipid A.

These polysaccharide preparations were subjected to Western blotting analysis (Fig. 6) with anti-serotype I mAb HY-1F10. Whereas intact PML14 LPS showed ladder-like bands together with semirough LPS, mild-acid-treated PML14 LPS did not show any bands with HY-1F10 (lane 2). Similarly, Seid *et al.* (1984) reported

that an O-polysaccharide preparation removed from lipid A was not amenable to Western blotting analysis and a lipid carrier should be required. In contrast to PML14, sPS derived from PML14e migrated as ladder bands similar to the LPS of PML14, but it lacked semirough LPS. Mild acid-treated sPS also possessed ladder bands (lane 4), but the alkali-treated sPS did not (lane 5). In the rLPS fraction derived from PML14e, O-polysaccharide-related materials were not detected (data not shown). The results suggested that the O-polysaccharide (sPS) and lipid A-core (rLPS) existed as separate molecules in PML14e, and that the O-polysaccharide was linked to an anchor (probably lipid). The mode of junction to the anchor seemed to be more acid-stable than the KDO linkage of the lipid A anchor.

The chemical structure of the O-antigenic sPS of PML14e was compared with that of the O-polysaccharide derived from PML14 by $^1\text{H-NMR}$ spectroscopy. The structure of the O-polysaccharide of PML14 has a repeating unit comprised of $\rightarrow 4)\text{D-Glc}(\text{NAc})_2\text{A}(\beta 1 \rightarrow 3)\text{D-FucNAc}(\alpha \rightarrow 3)\text{D-QuiNAc}(\alpha 1 \rightarrow 4)\text{D-GalNAc}(\alpha 1 \rightarrow$ (Suzuki, 1988), which is the same as that of the standard strains of the corresponding serotypes (Dmitriev *et al.*, 1982). The $^1\text{H-NMR}$ spectra (Fig. 7) indicated that the O-polysaccharide derived from PML14 and sPS derived from PML14e had closely similar repeating units as the main polysaccharide chain. Both spectra contained three α -anomeric protons (5.22 p.p.m., 3.8 Hz; 5.19 p.p.m., 3.2 Hz; 4.86 p.p.m., 3.6 Hz), one β -anomeric proton (4.71 p.p.m., 8.3 Hz), and exhibited signals ascribable to two methyl groups (between 1.19 and 1.22 p.p.m.) and five acetamido groups (between 1.90 and 2.07 p.p.m.). There was no signal between 2.1 and 2.2 p.p.m. ascribable to the methyl group of an O-acetyl ester in the repeating unit.

Fig. 4. Gel filtration on a Superose 6 column (1×30 cm) of: (a) sPS derived from *P. aeruginosa* PML14e; (b, c) LPS derived from PML14 without (b) or with (c) 0.5% deoxycholate; and (d) O-polysaccharide derived from *P. aeruginosa* PML14 LPS. Fractions (0.6 ml) were collected and assayed for amino sugars. Arrows V_0 , T_{500} , T_{40} , T_{10} and V_i indicate the elution positions of blue dextran, dextran T-500, T-40 and T-10, and glucose, respectively.

Reactivity of anti-*P. aeruginosa* cell-surface antigen mAbs

PML14e appeared to express a unique O-polysaccharide, so the nature of its cell surface was examined using mAbs against various *P. aeruginosa* cell surface components by means of ELISA using whole cells as the coated antigen (Fig. 1). O-polysaccharide expression was detected by the mAb Mei-assay I. The order of O-polysaccharide expression seemed to be PML14 > PML14e > PML14b > PML14d. This was consistent with the Western blots of TPS (Fig. 2). Anti-LPS outer core mAb FK-2E7 bound to PML14b and PML14d cells to a greater extent than to PML14 cells. These results indicated that the mutants had markedly decreased levels of O-polysaccharide expression so the mAb FK-2E7 had better access to the cell surface. The epitope for FK-2E7 was completely absent from PML14e, so FK-2E7 did not bind to PML14e cells at all.

Outer membrane protein G (OMP G) and lipid A were common cell surface antigens in PML14 and its mutants. The amounts of OMP G in the outer-membrane fraction

Fig. 5. DEAE-Sephacel chromatography of sPS derived from *P. aeruginosa* PML14e (a), and of LPS (b) and O-polysaccharide (c) derived from *P. aeruginosa* PML14. The samples were eluted through a DEAE-Sephacel column (1×2 cm) equilibrated in 5 mM ammonium acetate (pH 7.4), with the same buffer, then with a linear gradient of NaCl (-----) in the same buffer. Fractions (1 ml) were collected and assayed for amino sugars (●).

Table 3. KDO and lipid A content of polysaccharide fractions

Sample	KDO* (nmol mg ⁻¹)	Lipid A† (μg mg ⁻¹)	Endotoxicity‡ (EU mg ⁻¹)
PML14 LPS	114	220	1.1×10^6
PML14 O-PS	22	ND	8.8
PML14e sPS	< 5	< 10	1.4×10^2
PML14e rLPS	226	380	4.2×10^6

* KDO was determined by a colorimetric assay using a thio-barbituric acid method (Karkhanis *et al.*, 1978).

† Lipid A content was estimated by an inhibition ELISA. ND, Not determined.

‡ Endotoxicity was estimated by the colorimetric limulus test using *E. coli* O111:B4 LPS as a standard.

of these strains were similar according to SDS-PAGE and Western blotting (data not shown). These epitopes were expected to be exposed on the cell surface of the rough strain, because of the lack of steric hindrance by the O-polysaccharide. However, PML14e expressed more of the O-polysaccharide than PML14b and PML14d. The anti-

Fig. 6. Western blotting, with the anti-serotype I O-polysaccharide mAb HY-1F10, of native LPS (lane 1) and mild-acid-treated (1% acetic acid, 100 °C, 30 min) LPS (lane 2) derived from *P. aeruginosa* PML14, and native (lane 3), mild acid-treated (1% acetic acid, 100 °C, 30 min) (lane 4) and alkali-treated (0.1 M NaOH, 37 °C, 18 h) (lane 5) sPS derived from *P. aeruginosa* PML14e. Each sample was neutralized with NaOH or HCl, then treated with the SDS-PAGE sample buffer. The arrow indicates the elution position of rough LPS.

Fig. 7. $^1\text{H-NMR}$ spectra of sPS derived from *P. aeruginosa* PML14e (a) and the O-polysaccharide from *P. aeruginosa* PML14 (b). The structure of the repeating unit of PML14 O-polysaccharide was reported as $\rightarrow 4)\text{-D-Glc}(\text{NAc})_2\text{A}(\beta 1 \rightarrow 3)\text{-D-FucNAc}(\alpha 1 \rightarrow 3)\text{-D-QuiNAc}(\alpha 1 \rightarrow 4)\text{-D-GalNAc}(\alpha 1 \rightarrow 4)$ (Suzuki, 1988).

OMP G mAb YK-1H5 showed most binding to PML14e cells, and the least to PML14 (Fig. 1d). The anti-lipid A mAb NM-3D7 bound significantly only to PML14e (Fig. 1c). The binding activity seemed to correlate with the degree of the LPS core defects, and not with the amount of O-polysaccharide expressed.

DISCUSSION

The present study revealed unusual O-polysaccharide expression in mutant PML14e, which was resistant to all types of R-pyocins. PML14e lacked most of the LPS outer core region. Only D-galactosamine and L-alanine were found as outer core components; D-glucose and L-rhamnose were undetectable. The markedly defective lipid A-core region of PML14e was not likely to act as the acceptor for the O-polysaccharide chain. However, cell agglutination and an ELISA using whole cells as the plate-coated antigen, using the serotype I-specific antibodies, indicated that PML14e expressed serotype I O-polysaccharide on the cell surface in an antigenic form. The O-polysaccharide of PML14e was isolated by a conventional method, namely phenol extraction (Westphal & Jann, 1965). However, it was not sedimented by ultracentrifugation, so it was referred to as soluble polysaccharide (sPS). The chromatographic behaviours in gel filtration and anion-exchange chromatography of the sPS derived from PML14e were similar to those of the lipid A-removed O-polysaccharide that was derived from the parent strain PML14. The results suggested that the O-polysaccharide of PML14e was not linked to a lipid A anchor, so it did not form a micelle structure with rough LPS molecules that consisted of lipid A and markedly defective core oligosaccharide. In fact, neither lipid A nor KDO was detected in purified intact sPS. PML14e O-polysaccharide was not likely to have structural modifications in the repeating unit of the main chain compared with the parent strain PML14 as judged by $^1\text{H-NMR}$ spectroscopy.

Lipid A-free O-polysaccharide expression has been reported previously, and may be classified into two categories. The first category was a capsular polysaccharide with the same structure as the lipid A-core-bound O-polysaccharide chain in some *E. coli* strains (Goldman *et al.*, 1982; Krallmann-Wenzel & Schmidt, 1994; MacLachlan *et al.*, 1993; Peterson & McGroarty, 1985). Furthermore, a recent report showed that rough mutants (*rfaL*-defective) derived from *E. coli* O111:K58(B4) still produced the O-antigen capsule but not the O-antigenic LPS (Krallmann-Wenzel & Schmidt, 1994). However, the capsular polysaccharide expressed on the cell surface had some properties that differed (Goldman *et al.*, 1982; Peterson & McGroarty, 1985) from the PML14e O-polysaccharide-like material: (i) the mean molecular masses of the capsular polysaccharides were nearly twice as large as that of lipid A-carrying O-polysaccharide; (ii) the capsular polysaccharides were released by heat treatment; (iii) the capsular polysaccharides were observed in both smooth and rough strains of *E. coli*, whereas in *P. aeruginosa* the sPS was not detected in the parent strain and other rough mutants (PML14b and PML14d) (Fig. 3). The other category of lipid A-free O-polysaccharide expression was the O-polysaccharide that accumulated probably as the precursor linked to an acyl carrier lipid in the intracellular space, such as *Salmonella rfa* mutants (Beckmann *et al.*, 1964; Nikaido, 1969) and *Brucella melitensis* rough strain B115 (Cloeckert *et al.*, 1992). This phenomenon was thought to arise because synthesized O-

polysaccharides could not transfer to the lipid A-core anchor due to a defect in part of the LPS core structure or O-polysaccharide/LPS core transferase(s). The example most similar to the present study is recombinant *S. typhi* oral vaccine strain 5076-1C, which carries *Shigella sonnei* genes encoding form I O-specific antigen (Seid *et al.*, 1984). This strain expresses the form I antigen on the cell surface without lipid A-core. It seemed that *S. sonnei* form I O-specific polysaccharide could not transfer to the *S. typhi* LPS core because the LPS core structure of *S. typhi* was distinct from that of *S. sonnei*. The enterobacterial common antigen (ECA) is another interesting example. Three types of the ECA molecules have been reported, lipid A-core-linked form, L-glycerophosphatide form and cyclic polysaccharide form (Dell *et al.*, 1984; Kuhn *et al.*, 1984). The lipid A-core-linked form occurred in strains having specific LPS core structures, namely R1 and R4 types (Kuhn *et al.*, 1984).

In this study, O-polysaccharide expression was analysed by using serotype-I specific mAb HY-1F10, which binds to one repeating unit of O-polysaccharide, i.e. semirough LPS (Fig. 2). This mAb detected O-polysaccharide distribution with high sensitivity and specificity in Western blotting. In PML14e, LPS carrying one or a few repeating units of O-polysaccharide was not detected, in contrast to PML14, PML14b and PML14d. The synthesized O-polysaccharide did not transfer to the lipid A-core carrier because of the absence of the outer core region containing the attachment site of the O-polysaccharide. The resulting lipid A-free O-polysaccharide was translocated to the cell surface. The O-polysaccharide having a high molecular mass was present on the cell surface, but that with a low molecular mass, such as semirough LPS, was not. There may be an alternative translocation mechanism specific for the mature molecular size of the O-polysaccharide, which was lipid A-free. However, the mechanism(s) of the translocation remains to be investigated, because little information is available about the genetic and enzymic background of pseudomonal LPS biosynthesis. Recently, Dasgupta *et al.* (1994) have reported that some rough mutants of *P. aeruginosa* expressed O-polysaccharide in a leaky manner, as detected by Western blotting. Such O-polysaccharide expression in LPS-core-defective mutants may be frequently found. In fact, we examined polysaccharide antigen expression in another series of LPS-core-defective mutants, which will be described elsewhere. The PML14e sPS seemed to link to an acyl carrier in a more acid-stable manner than the KDO linkage. The carrier has not been identified at the present time. Preliminary fatty acid analysis of the sPS indicated that the preparation contained small portions of hexadecanoic acid, tetradecanoic acid and 3-hydroxydecanoic acid in addition to other minor fatty acids; however, characteristic components of lipid A such as 2-hydroxy- and 3-hydroxydodecanoic acids were not detected (data not shown). It was not determined if these were contaminants or were covalently linked to the sPS. Further work is needed to determine the anchor structure of the sPS.

The cell surface nature of a series of mutants derived from

PML14 was also characterized by examining the reactivity of the mAbs against cell-surface antigens. The predicted binding activities of the anti-OMP mAb and the anti-lipid A mAb were influenced by the rough nature of the strains. Such mAbs bound more to the rough than to the smooth strains, because of the lack of hindrance by the O-polysaccharide (Yokota *et al.*, 1992; Yokota, 1995). However, PML14e expressed the lipid A-free O-polysaccharide to a relatively higher extent (Figs 1a and 2), and the OMP G and lipid A epitopes were much more exposed compared with those of the other rough mutants, PML14b and PML14d. Especially, the lipid A epitope recognized by mAb NM-3D7 seemed to be masked by D-glucose and L-rhamnose residues of the outer core region for the greater part. Considering that PML14e expressed more O-polysaccharide on the cell surface than the other mutants, the core defect contributed more to the exposure of the rough-characteristic epitopes than the O-polysaccharide defect. The neutral sugar residues of the LPS outer core region were likely to greatly hinder the lipid A epitope, which was recognized by the mAb NM-3D7.

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