Crystallization of an R-Form Lipopolysaccharide from \textit{Klebsiella pneumoniae}

Nobuo Kato* 1, Michio Ohta1, Yoshichika Arakawa1, Setsuko Naito1, Tsuyoshi Sugiyama1,* 2, Hideo Ito1, 3, Nobuo Kido2,* 3, Kyoyu Sasaki3, and Junpei Asai2

1Department of Bacteriology, 2Department of Pathology, Nagoya University School of Medicine, Nagoya, Aichi 466, Japan, and 3College of Medical Technology, Nagoya University, Nagoya, Aichi 461, Japan

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Abstract: An R-form lipopolysaccharide (LPS) from \textit{Klebsiella pneumoniae} strain LEN-111 (O3⁻:K1⁻) formed crystals, whose shapes were elongated hexagonal plates, trapezoid plates, and rhomboid plates, and whose greatest dimensions were 3.1 × 0.8 µm, when it was suspended in 50 mm Tris buffer at pH 8.5 containing 5 mm MgCl₂ and kept at 4 C for as long as 870 days. \textit{K. pneumoniae} LEN-111 synthesized LPS molecules possessing incomplete repeating units of the O-antigenic polysaccharide portion besides the R-form LPS because of a leaky characteristic, but crystals consisted exclusively of the R-form LPS. Although the size of crystals was not large enough for X-ray analysis and limited crystallographic information was available, it was suggested that the crystals consist of hexagonal lattices with an a axis of 4.62 Å and c axis of 79.8 ± 2.6 Å. The present results showed that R-form LPS lacking the O-antigenic polysaccharide portion tends to form crystals during long-term incubation in Tris buffer at pH 8.5 containing MgCl₂ at 4 C.

Key words: Crystallization, Lipopolysaccharide (LPS), R-form, \textit{Klebsiella pneumoniae}

Bacterial endotoxin (lipopolysaccharide; LPS) is the major constituent of the outer membrane of gram-negative bacteria and LPSs of various bacterial families share a common architecture (15, 20). Wild-type (S-form) LPS of Enterobacteriaceae consists of the O-antigenic polysaccharide, which is linked to the R-core consisting of oligosaccharide, which in turn is linked to the lipid portion termed lipid A. In enterobacterial strains there are mutants lacking the O-antigenic polysaccharide synthesis (rough strains) and LPS lacking the O-antigenic polysaccharide chain is designated as R-form LPS. LPS elicits a variety of host reactivities through interactions with humoral and cellular factors of the host (16). The lipid A portion is most responsible for the biological activities of LPS (1, 2, 19, 21).

We previously showed that R-form LPSs from \textit{Salmonella} spp. and \textit{Escherichia coli} form three-dimensional crystals when they are incubated in 70% ethanol containing 250 mm MgCl₂ at 4 C (4, 5, 7, 8, 11, 14). The LPS crystals are polymorphic but the basic form is considered to be the hexagonal plate form (5, 7, 8). Analyses of crystals suggested that they consist of hexagonal lattices with an a axis of 4.62 Å and c axes of various values depending upon the chemical structures of the R-cores (5, 11), and that hydrocarbon chains of the lipid A portion play the leading part in crystallization (11). Furthermore, we recently revealed that synthetic \textit{E. coli}-type lipid A forms hexagonal plate crystals which consist of hexagonal lattices with the same a-axis value as crystals of R-form LPSs from \textit{Salmonella} spp. and \textit{E. coli} (6).

We isolated \textit{Klebsiella pneumoniae} LEN-111 (O3⁻: K1⁻), which is a mutant lacking the O-antigenic polysaccharide synthesis, from \textit{K. pneumoniae} LEN-1 (O3: K1-) (17). Later we found that LPS from \textit{K. pneumoniae} LEN-111 suspended in 50 mm Tris buffer at pH 8.5 containing 5 mm MgCl₂ had formed crystals when it was kept at 4 C for 870 days. The finding was preliminarily referred in a review of crystallization and electron microscopy of LPS (4). The present paper describes electron microscopic observations and analyses of the crystals of LPS from \textit{K. pneumoniae} LEN-111.

*Address correspondence to Dr. Nobuo Kato, Department of Bacteriology, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466, Japan.

Present addresses: *'Department of Microbiology and Immunology, Aichi Medical University, Nagakute, Aichi 480–11, Japan.
‘2Department of Natural Science Informatics, School of Informatics and Sciences, Nagoya University, Chikusa-ku, Nagoya, Aichi 464–01, Japan.

Abbreviations: LPS, lipopolysaccharide; R-KO3 LPS, R-form LPS from \textit{Klebsiella pneumoniae} LEN-111 (O3⁻: K1⁻); SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.
Materials and Methods

Bacterial strain and LPS. *K. pneumoniae* LEN-111 (O3^-:K1^-), a mutant lacking the O-antigenic polysaccharide synthesis (17), was isolated from *K. pneumoniae* LEN-1 (O3:K1^+) which is a decapsulated strain derived from *K. pneumoniae* strain Kasuya (O3:K1^-) (18). LPS isolated from *K. pneumoniae* LEN-111 by the phenol-water method (25) (designated as R-K03 LPS) was purified by treatment with RNase and by ultracentrifugation at 100,000 X g for 1 hr, as described previously (11).

Experimental conditions to form crystals of R-K03 LPS. R-K03 LPS was further purified by treatment with sodium dodecyl sulfate (SDS) and were finally precipitated by addition of two volumes of 95% ethanol containing 375 mm MgCl₂, as described previously (11). The precipitate was separated by centrifugation at 20 C (to prevent precipitation of SDS) and washed three times with 70% ethanol at 20 C to remove SDS. R-K03 LPS thus treated was suspended in 50 mm Tris buffer at pH 8.5 containing 5 mm MgCl₂ to a concentration of 1 mg/ml and kept at 4 C for 870 days until use in experiments.

Electron microscopy. As described previously (5), an electron microscope (JEM-2000FX, JEOL, Ltd., Akishima, Japan), operating at 200 kV and equipped with a cryo-transfer holder which is cooled with liquid nitrogen was used for observation and analysis of crystals. Specimens to be tested were prepared in the same way as described previously (5). For taking electron diffraction patterns, the length between the camera and the sample was 100 cm. The reflection originating from the plane (111) of gold used for coating the grids, the radius of which corresponds to 2.35 Å, was used as control for calculation of the lattice constant. For electron microscopy of thin sections of crystals, a drop of a suspension of crystals was fixed in 5 ml of 2% OsO₄ in 0.1 M phosphate buffer at pH 7.6 for 30 min at room temperature. The specimens were washed with 0.1 M phosphate buffer by centrifugation at 450 x g for 10 min and fixed with 0.3 ml of 2% agar. The fixed specimens were dehydrated through graded ethanol solutions. Then they were embedded in epoxy resin. Ultrathin sections were cut with an 8800 Ultratome III (LKB, Bromma, Sweden) and stained with uranyl acetate. For negative staining, an air-dried specimen on a carbon-coated grid was stained with 2% ammonium molybdate as described previously (9). The stained ultrathin sections and the negatively stained specimens were examined with an electron microscope (H500, Hitachi) operating at 100 kV.

X-ray diffraction. As in the previous studies (5, 7, 8, 11, 14), X-ray diffraction with synchrotron radiation was carried out in beam line 6A2 of the Photon Factory (National Laboratory for High Energy Physics, Tsukuba, Japan). Diffraction data were collected at 20 C by using a Weissenberg camera, which was specially designed by Sakabe et al (22, 23) for macromolecular crystallography, with an imaging plate (Fuji Film Co., Ltd., Tokyo). The wavelength of the X-ray was set at 1.004 Å. Aggregates of crystals of LPS were mounted in Mark capillaries. Exposure was for 200 s.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out by the method of Tsai and Frasch (24) and the gel was silver-stained by the method of Hitchcock and Brown (3). To prepare the specimens for SDS-PAGE, crystals were disintegrated by boiling in 0.5% SDS and LPS was recovered by the addition of 2 volumes of ethanol containing 10 mm MgCl₂ as described previously (11).

Results

Formation of Crystals of R-K03 LPS

R-K03 LPS, which was prepared as described in “Materials and Methods” and suspended in 50 mm Tris buffer at pH 8.5 with 5 mm MgCl₂, was shown by electron microscopy to form a hexagonal mesh-like arrangement with a lattice constant of 14 nm (Fig. 1a). This was consistent with the finding reported previously (12). When R-K03 LPS suspended in the buffer solution was stored at 4 C for 870 days and examined for electron microscopy, it was found that the LPS preparation mostly formed crystals whose shapes were elongated hexagonal plates, trapezoid plates, and rhomboid plates (Fig. 1b). The greatest dimensions of crystals were 3.1 X 0.8 µm.

Electron Diffraction

Analysis of elongated hexagonal plate crystals by electron diffraction from the direction perpendicular to the basal plate of the crystal showed that the crystals consist of a hexagonal system with a d value of 4.0 Å (the a-axis value is 4.62 Å) (Fig. 2). Analysis by electron diffraction of trapezoid plate crystals or rhomboid plate crystals showed essentially the same results as did elongated hexagonal plate crystals.

X-Ray Diffraction

Aggregates of R-K03 LPS crystals were subjected to X-ray diffraction, since single crystals were not large enough for X-ray diffraction. The X-ray diffraction pattern of the specimens was characteristic of polycrystals and consisted of a series of ring-shaped reflections in the small-angle region (Fig. 3). The series of rings of
Fig. 1. Electron micrographs of R-KO3 LPS suspended in 50 mM Tris buffer at pH 8.5 with 5 mM MgCl₂. (a) R-KO3 LPS just after it was suspended in the buffer. (b) R-KO3 LPS after it was kept at 4°C for 870 days.
reflection proved to be due to a single periodicity of 78.9±2.6 Å.

Cross Sections of Crystals of R-KO3 LPS

Electron microscopy of thin sections of the crystal specimens treated as described in “Materials and Methods” revealed the cross sections of crystals which consisted of lamellar structures with about 6 nm spacing (Fig. 4). The stained zone of the cross section corresponds to the hydrophilic portion of R-KO3 LPS (R-core + disaccharide backbone of lipid A) and the unstained zone corresponds to the hydrophobic portion (fatty acid chains of lipid A).

Electron Microscopy of Crystals of R-KO3 LPS under Degradation Process

When crystals of R-KO3 LPS were negatively stained with a solution of ammonium molybdate and examined for electron microscopy, it was found that the crystals tend to be disintegrated into hexagonal mesh-like structures (Fig. 5a) or onion-like lamellar structures (Fig. 5b). When crystals were suspended in distilled water, allowed to stand for 30 min at room temperature, and stained with ammonium molybdate, the specimens showed essentially the same structures as those of the original LPS specimen shown in Fig. 1a (photograph not shown).

SDS-PAGE of Crystal Specimens

We compared SDS-PAGE profiles of the LPS preparation recovered from crystals of R-KO3 LPS, R-KO3 LPS, and LPS from K. pneumoniae LEN-1 (O3:K1') which possesses the O-antigenic polysaccharide portion (Fig. 6). The SDS-PAGE profile of R-KO3 LPS showed ladder bands of low molecular weights, corre-
sponding to LPS molecules possessing fewer repeating units of the O-antigenic polysaccharide portion, in comparison with the profile of LPS from *K. pneumoniae* LEN-1 (Fig. 6, lane 1). This finding agrees with the previous result that *K. pneumoniae* LEN-111 (O3⁻:K1⁻) is a leaky mutant (17). In contrast, the SDS-PAGE profile

![Electron micrographs of crystals of R-KO3 LPS under degradation process.](image)

Fig. 5. Electron micrographs of crystals of R-KO3 LPS under degradation process.
of the crystal specimen showed no ladder bands corresponding to the O-antigenic polysaccharide, indicating that crystals of R-K03 LPS consist exclusively of an R-form LPS (Fig. 6, lane 2).

Discussion

Although the original preparation of R-K03 LPS contained LPS molecules possessing incomplete repeating units of the O-antigenic polysaccharide portion because of the leaky characteristic of *K. pneumoniae* LEN-111, it was confirmed by SDS-PAGE that crystals examined in the present study contained no repeating units of the O-antigenic polysaccharide portion but consisted exclusively of an R-form LPS (Fig. 6). We previously showed that R-form LPS from various chemotypes of *Salmonella* spp. and *E. coli* formed crystals, the basic form of which was hexagonal plates, when they were precipitated by the addition of two volumes of 95% ethanol containing 375 mM MgCl₂ and kept in 70% ethanol containing 250 mM MgCl₂ at 4°C (5, 7, 8, 11, 14). The results of the present study agree with the previous finding, although the experimental conditions for forming LPS crystals differed in the two experiments. The present study also indicates that long-term incubation in 50 mM Tris buffer at pH 8.5 containing 5 mM MgCl₂ at 4°C can be an experimental condition for forming crystals of R-form LPS in place of treatment with ethanol containing MgCl₂.

Analysis of crystals of R-K03 LPS by electron diffraction from the direction perpendicular to the basal plane showed that they consist of hexagonal lattices with a lattice constant of 4.62 Å. This was consistent with the electron diffraction data of hexagonal plate crystals of R-form LPSS from *Salmonella* spp. and *E. coli* which were formed by treatment with ethanol containing MgCl₂ (5, 7, 8, 11, 14). It is therefore suggested that the hexagonal packing of hydrocarbon chains of the lipid A portion plays the leading part in crystallization of R-K03 LPS in the same way as in crystallization of R-form LPSSs by treatment with ethanol containing MgCl₂ (11).

We previously found electron microscopically that various chemotypes of R-form LPSSs from *Salmonella* spp. and *E. coli* mostly show ribbon-like structures, disc-shaped structures which are stacked in aggregates, smectic concentric lamellar structure (multilayered vesicles, liposomes), or hexagonal mesh-like structures (10, 13). The hexagonal assembly of R-form LPSSs is Mg⁺⁺ dependent and closely related to the chemical structure of their R-cores. It was found that Rb LPS formed the densest hexagonal assembly (lattice constant, 14 nm) when it was suspended in 50 mM Tris buffer at pH 8.5 containing MgCl₂ after removal of basic materials present in the LPS molecules by electrodialysis (10). R-K03 LPS suspended in the same buffer showed a hexagonal mesh-like structure similar to that formed by Rb LPS of *Salmonella* spp. (12). The thickness of a bilayer of R-K03 LPS was calculated from the X-ray diffraction data (78.9±2.6 Å). This value approximates that of Rb LPS of *Salmonella* spp. (4, 5). From these results, it is speculated that R-K03 LPS possesses an R-core whose length is very similar to that of Rb LPS of *Salmonella* spp., although the chemical structure of the R-core of R-K03 LPS has not yet been determined.

The thickness of a bilayer of R-K03 LPS estimated electron microscopically on thin sections of crystals was approximately 6 nm (Fig. 4). This value is smaller than the value calculated from the X-ray diffraction data. This difference possibly results from the fact that thin sections of crystals of R-K03 LPS were prepared after dehydration of crystals.

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Fig. 6. SDS-PAGE of LPS preparations. Lanes: 1, LPS from *K. pneumoniae* LEN-1 (O3:K1⁺); 2, crystals of R-K03 LPS; 3, R-K03 LPS.
References


