Lipopolysaccharide Variability in *Pseudomonas aeruginosa*

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Abstract. The lipopolysaccharide (LPS) of *Pseudomonas aeruginosa* (ATCC 9027) exhibited gross changes in both the amount of LPS produced per cell and the composition of the LPS in response to changes in magnesium ion concentrations. Compositional variation in the LPS was detected under both batch and continuous culture conditions, and was particularly apparent as changes in the levels of heptose in the molecule. Compositional changes in the molecule were also reflected as functional alterations in the LPS. However, functionally, these alterations were counterbalanced by increased production of the lipopolysaccharide. We propose that LPS composition changed in accordance with environmental factors, and that cells responded to these changes by increasing or decreasing the amount of LPS produced.

Lipopolysaccharides (LPS) are a heterogeneous collection of molecules unique to the outer cell envelope of Gram-negative bacteria, sharing the same general composition but showing distinct heterogeneity in size [25]. Current models of the Gram-negative outer envelope structure [8] depict the LPS as embedded in a continuum of proteins and phospholipids which form the hydrophobic barrier of the outer envelope. In *Pseudomonas aeruginosa* and in other Gram-negative microorganisms, LPS may be found in association with envelope proteins [22,33]. These associations are noncovalent in nature, essentially involving polar interactions and divalent cation bridges [29]. When LPS is dissociated from its surrounding molecules, there is a disruption of the outer envelope. The end result is a breakdown in the cell permeability barrier, measured by the penetration of antibiotics and other bactericidal agents [16] and changes in the function of some of the LPS associated envelope proteins [11].

The structure and composition of the Gram-negative outer membrane is profoundly influenced by its environment, particularly under conditions of specific nutrient growth limitation [32]. These changes in the composition of the outer cell envelope have been associated with drug resistance by the mechanism of exclusion [2–5,17,20,24,26]. Investigations into changes in the cell envelope have concentrated primarily on protein-composition changes.

However, there is an increasing body of evidence which indicates that the LPS phospholipid composition of the cell envelope also changes in response to nutrient limitation. Studies by others—on EDTA resistance in pseudomonads [37] and on *P. aeruginosa* grown in Mg²⁺-deficient and Mg²⁺-sufficient media [20]—have documented changes in envelope levels of phosphorus, carbohydrate, and keto-3-deoxyoctonate. Changes in the LPS structure in the cell envelope have long been known to be related to permeability changes which are expressed in such widely divergent parameters as competence for nucleic acid transfer [35] and antibiotic sensitivity [36].

We undertook this study of the changes in *P. aeruginosa* LPS composition in response to environmental magnesium concentrations in order to determine the effects of these changes in modulating cell wall permeability of this organism. Changes in LPS were documented both chemically and as functional changes in the molecule and whole cell as measured by their ability to bind the protein lysozyme and the antibiotic gentamicin.

Materials and Methods

Organism and growth conditions. Stock cultures of *Pseudomonas aeruginosa* (ATCC 9027) were maintained on slants of Pseudo-
Pseudomonas P agar (Difco Laboratories, Detroit, Michigan) at room temperature. The organism was grown on tris (hydroxymethyl)aminomethane (Tris)-hydrochloride buffered glucose, ammonium salts, proteose peptone medium at pH 6.8 [7]. Starting levels of MgSO_{4} \cdot 7H_{2}O in the medium were varied from 0.0016 M to 0.05 M. The organism was grown in 1,500 ml volumes in a New Brunswick Scientific (New Brunswick, New Jersey) MultiGen fermentor at 300 rpm agitation and with aeration at 1.5 liters/min. The fermentor was inoculated with a 10% inoculum of an overnight culture grown in 200-ml volumes of the same medium in 500-ml Erlenmeyer flasks. For continuous-culture experiments, the same apparatus was used, set up in a continuous mode. A working volume of 500 ml was maintained with a dilution rate of 0.3.

**Chemicals.** Lysozyme (EC 3.2.1.17) was obtained from the Sigma Chemical Co., St. Louis, Missouri. Radiocarbon-labeled gentamicin sulfate (specific activity 830 μCi/g) was a gift from the Shering-Plough Corp., Bloomington, New Jersey. Pure alkaline phosphatase from *Pseudomonas aeruginosa* was prepared as previously described [10].

**Lipopolysaccharide extraction.** Lipopolysaccharide (LPS) was extracted from 200-ml volumes of culture by the phenol-water extraction procedure [28]. The extracted material was extensively dialyzed, and then concentrated and washed in an Amicon (Danvers, Massachusetts) hollow-fiber device, with a 5,000 MW exclusion limit, to remove any small associated carbohydrates. Before use, the LPS preparations were analyzed for, and found to be free from, any protein contamination.

**Chemical analyses.** Keto-3-deoxyoctonate (KDO) was determined by a thiobarbituric acid method [27], total carbohydrate (CHO) by the phenol-H_{2}SO_{4} method [15], heptose by the method of Dische [13], and organic phosphate by the method of Ames [1].

**Nephelometry.** Nephelometric measurements to follow the interaction of LPS and lysozyme were carried out as described elsewhere [12] using a Coleman (Maywood, Illinois) model 7 nephelometer. A 0.01 M Tris, pH 8.4, buffer system was used.

**Measurement of gentamicin binding.** Radiocarbon-labeled gentamicin sulfate, 100 μg, was mixed with known amounts of LPS suspended in 20-ml volumes of 0.01 M Tris buffer at pH 8.0. This mixture was then centrifuged for four h at 65,900 × g in a Spinco (Palo Alto, California) L2-65B ultracentrifuge. The pellet was resuspended in 1 ml of water, transferred to 15 ml of liquid scintillation counting fluid (AquaSol; New England Nuclear, Boston, Massachusetts) and counted in a Nuclear-Chicago (Des Plaines, Illinois) liquid scintillation counter.

Gentamicin binding to whole cells was measured by monitoring the amount of radioactivity associated with the cells after one-min exposure to medium containing [14C]gentamicin. Harvested, mid-log cells were added to fresh growth medium containing 38 μg/ml [14C]gentamicin sulfate and an appropriate magnesium ion concentration such that the final cell concentration was 0.8 mg/ml dry weight of cells. After one min, a 10-ml portion of cells was filtered through a 0.45-μm membrane filter (Millipore Corporation, Bedford, Massachusetts). The filters were then washed with 10 ml of 3% NaCl, dried, suspended in Aquasol, and the associated radioactivity determined.

**Results**

**Variation in lipopolysaccharide (LPS) composition with growth phase.** *Pseudomonas aeruginosa* (ATCC 9027) was grown as described in Materials and Methods in 0.0016 M Mg^{2+} medium; 100-ml samples were withdrawn over the course of the growth cycle and the LPS extracted by the method of Osborn [28]. A different extraction procedure [18] was found to yield less than 1% of the LPS with this strain of *Pseudomonas* (unpublished data) and hence was not used. The various LPS samples were analyzed for keto-3-deoxyoctonate (KDO) and heptose as LPS markers. Organic phosphate and total carbohydrate were also measured; organic phosphate as an approximation of extracted phospholipid and LPS-associated phosphate, and total carbohydrate as a crude measure of heterogeneity (it was not possible to readily determine the percentage of carbohydrate which was non-LPS polysaccharide due to the nonspecificity of both the phenol-water extraction and the Dubois assay technique). Figure 1 shows the results of these analyses.

When grown in batch culture, the components of the various LPS samples maintained an essentially stable relationship after the mid-log phase of growth (hours three through seven). There was an initial rapid increase in extractable carbohydrate and a rapid decrease in organic phosphate detected as the cell population went from early- to mid-log phase, which may be a reflection of the different physiological states in early-log cells compared to that of mid- and late-log cells.

In an attempt to establish baseline conditions—i.e., in order to avoid changes due to culture variation—parallel experiments were conducted on cells grown in continuous culture at a fixed dilution rate (D) equal to 0.3. Significant changes were found in LPS composition both within and between the samples from batch and continuous-culture cells (Table 1). The batch culture LPS samples showed an approximately equivalent ratio of heptose to phosphate, while the continuous-culture LPS samples exhibited about an 8-fold increase in phosphate over heptose. In both cases, the total carbohydrate extracted was about 5 to 6 times the organic phosphate level. On a dry-weight basis, about twice as much KDO and heptose were extracted from the batch-grown cells, compared to the continuous-culture cells. Conversely, approximately 4 times more phosphate and total carbohydrate were found associated with the continuous-culture fractions compared to the batch-grown extracts.

**Lipopolysaccharide variation with environmental magnesium levels.** The organism was grown both in batch and continuous cultures in the proteose-peptone, glucose, and ammonium salts medium—