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Ethylenediaminetetraacetate-Extractable Protein-Lipopolysaccharide Complex of *Pseudomonas aeruginosa*: Characterization of Protein Components

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Five major outer membrane proteins (D1, D2, E, G, and H1) of *Pseudomonas aeruginosa*, but not proteins F (porin), I (lipoprotein), and H2, were detected in high-molecular-weight protein-lipopolysaccharide complex(es) solubilized from sucrose-stabilized cells on exposure to ethylenediaminetetraacetate and tris(hydroxymethyl)aminomethane.

Exposure of *Pseudomonas aeruginosa* to EDTA causes the lysis of whole cells (3, 8, 18) and the release of a protein-lipopolysaccharide complex(es) (PrLPS) from isolated cell envelopes of this organism (6, 19). Extraction with *N,N'*-dimethylformamide of cell envelopes, outer membranes (OM), and the PrLPS isolated from *P. aeruginosa* shows that major proteins from the OM were present in the PrLPS (22, 24). Because protein-lipopolysaccharide interactions are considered to be important requirements for OM assembly and function (1, 2, 7, 13, 20, 21, 25, 26), we undertook the present study to confirm and extend our previous work by characterizing the protein components of the PrLPS released from *P. aeruginosa* on exposure to EDTA, using present-day extraction and gel electrophoresis techniques and protein classifications.

P. aeruginosa PAO1 was grown at 37°C in a rotary incubator shaker (250 rpm) in the basal salts medium previously described (4) supplemented with 14 mM glucose (final concentration) until the cells reached late exponential growth. The cells were harvested by centrifugation at ambient temperature and washed twice with fresh basal salts medium.

The PrLPS was released from these cells in a manner similar to that described previously (24). Briefly, the cell pellet was suspended to the original culture volume in 1 mM EDTA in 33 mM Tris-hydrochloride buffer, pH 8, containing in final concentration 0.55 M sucrose. The suspension was stirred gently for 30 min at ambient temperature. This cell suspension was monitored spectrophotometrically at 660 nm throughout the extraction procedure to determine that no lysis had occurred. The treated cells were removed by centrifuging at 16,000 × *g* for 15 min at ambient temperature. The supernatant containing the PrLPS was concentrated to 1/100th the original culture volume by ultrafiltration (XM300 membrane filter; Amicon Corp., Lexington, Mass.) at 4°C. After concentration, the supernatant was extensively diafiltered against 10 mM Tris buffer, pH 8, over the same XM300 membrane filter. This solution was then centrifuged at 100,000 × *g* for 1 h at 4°C. The resultant supernatant was concentrated by lyophilization and then suspended in deionized water to a final concentration of 1 mg of protein per ml.

The PrLPS was further purified by gel filtration, using Sepharose CL-4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The material was applied to the column (1.5 by 85 cm), which was equilibrated with 10 mM Tris buffer, pH 8, and the PrLPS was eluted in the void volume with the same buffer. The PrLPS was then concentrated by lyophilization.

Isolated OM were prepared from *P. aeruginosa* by fractionation of cell envelopes by sucrose density gradient centrifugation according to the procedure of Hancock and Nikaido (11). Proteins were extracted from OM and from the PrLPS with 2% sodium dodecyl sulfate (SDS) at 100°C for 5 min and analyzed by SDS-polyacrylamide gel electrophoresis as described by Hancock and Carey (9).

Analysis of the proteins of the PrLPS by SDS-polyacrylamide gel electrophoresis (Fig. 1) revealed that the predominant proteins comigrated with the major OM proteins D1, D2, E, G, and H1 (nomenclature of major OM proteins of *P. aeruginosa* as proposed by Hancock and Carey [9]). It should be noted that protein G is considered by other workers to be a major OM protein in *P. aeruginosa* (9); in our hands, how-

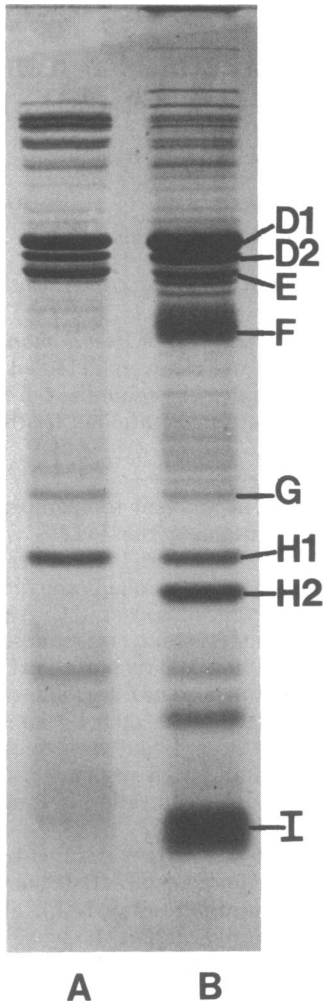

FIG. 1. *SDS-polyacrylamide gel electrophoresis of proteins extracted from the PrLPS liberated by EDTA from sucrose-stabilized cells of P. aeruginosa (lane A) and from isolated OM (lane B). Electrophoresis was carried out at a constant current of 20 mA until the tracking dye just exited the gel bottom.*

ever, it has consistently been detected in lesser amounts than the other major OM proteins. Proteins F (porin), H2, and I (lipoprotein) were not detected in the PrLPS. The latter proteins have been identified as peptidoglycan-associated OM proteins of *P. aeruginosa* (10, 15, 16). Finally (Fig. 1), there were also a few unclassified minor OM proteins in the PrLPS preparation in addition to the major OM proteins.

SDS-polyacrylamide gel electrophoresis was also done on proteins extracted from: (i) material retained by the Amicon XM300 membrane ultrafilter and (ii) material eluted from the Sepharose CL-4B column immediately after the void volume. Because higher amounts of minor OM proteins were detected in the ultrafiltered sample as compared with the PrLPS material in the Sepharose CL-4B void volume, we used the latter material to obtain the data shown in Fig. 1.

The data in Table 1 show 3-deoxy-D-mannooctulosonic acid (i.e., 2-keto-3-deoxyoctonate [KDO]) to protein in: (i) intact cells; (ii) isolated OM; (iii) PrLPS-containing material released from cells on exposure to EDTA-Tris and concentrated by membrane ultrafiltration; and (iv) Sepharose CL-4B void volume eluate. That each of these steps led to increasing purification of the PrLPS is evidenced by the parallel increase in the KDO-to-protein ratio. Thus, the ratio of KDO to protein was greater in isolated OM than in intact cells. The ratio was still higher in the PrLPS-containing material retained by the Amicon PM10 ultrafilter membrane (cut-off, M_w 10,000), and the ratio increased even more in the PrLPS material retained by the Amicon XM300 ultrafilter membrane (cut-off, M_w 300,000). Finally, the greatest KDO-to-protein ratio was observed in the Sepharose CL-4B void volume eluate. From these data, as well as from gel electrophoresis data, we concluded that the PrLPS was most likely to be in its purest state in the Sepharose CL-4B void volume eluate; this is what we used to obtain the data shown in Fig. 1.

It should be noted, too, that the KDO-to-protein ratio in isolated OM of *P. aeruginosa* (Table 1), although lower, is similar to that reported for *Salmonella typhimurium* (for review, see ref. 17).

Our present data confirm and extend previous data that a high-molecular-weight complex(es) composed of major OM proteins and noncovalently linked lipopolysaccharide is released from *P. aeruginosa* upon exposure to EDTA-Tris (5, 6, 19, 22, 24). Moreover, electron microscopic

TABLE 1. *Ratio of KDO to protein in various components of P. aeruginosa*[a]

Component	μmol of KDO per mg of protein
Intact cells	0.053
Isolated OM	0.17
Material retained by:	
PM10 ultrafilter	0.23
XM300 ultrafilter	0.36
Sepharose CL-4B void volume eluate	0.85

[a] KDO was estimated by the procedure recommended by Keleti and Lederer (12). Protein was determined by the Lowry procedure as modified by Markwell et al. (14).

data revealed that the PrLPS had an ultrastructural appearance of spherical units 7 ± 1 nm in diameter and that these spherical units were located in the OM (5, 6, 19, 23). Evidence strongly suggestive that the PrLPS may play a role in maintaining the structural integrity of the cell envelope of *P. aeruginosa* was also advanced (5, 23, 24).

It was reported recently by Hancock et al. (10) that the EDTA-Tris soluble fraction of sucrose-stabilized cells of *P. aeruginosa* contained little of any major OM proteins except protein E. Our data, both present and past, clearly show otherwise.

It is difficult to explain the discrepancy between our findings and those of Hancock et al. (10). The latter authors, however, used a 10-fold-higher concentration of EDTA for their studies than that used in our past and present studies. We suspect that use of the higher concentration of EDTA causes extensive cellular lysis, liberating cell membrane and cytoplasmic proteins, so that the OM proteins became obscured in the massive background of cell membrane and cytoplasmic proteins.

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