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## LPS integration into outer membrane structures

### Robert E.W. Hancock and D. Nedra Karunaratne

Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1W5

#### Introduction

It is now well established that the outer membranes of at least some, and perhaps all, Gram-negative bacteria are asymmetric bilayers [1,2]. The major lipidic component of the outer or surface monolayer of the outer membrane is LPS, whilst this component is replaced by phospholipids in the inner monolayer. The early studies of Loretta Leive [3], using the divalent cation chelator EDTA as a tool, firmly established the importance of divalent metal cation bridging between adjacent LPS molecules in anchoring the LPS in the outer membrane, and in the barrier properties of this membrane. Subsequently the interaction of LPS with outer membrane proteins was also demonstrated [2,4]. This short paper will review studies from my laboratory that have examined, in both *Escherichia coli* and *Pseudomonas aeruginosa*, the way in which LPS is integrated into outer membrane structures from both physical and functional points of view.

## LPS bridging by divalent cations and its role in self promoted uptake

As stated above, divalent cations crossbridge adjacent LPS molecules in the outer membrane. This stabilization, through divalent cations, of outer membranes results in many of their important barrier properties, including exclusion by many (but not all) bacteria of hydrophobic compounds and exclusion of potential harmful enzymes, like lysozyme and proteases [5]. Not all divalent cations can occupy these sites.  $Ca^{2+}$  and  $Mg^{2+}$  are usually preferred, although both  $Sr^{2+}$  and  $Mn^{2+}$  are permitted. Removal of these stabilizing divalent cations either by chelation with a compound like EDTA or by competitive displacement with a larger organic cation (see for example Table 1) results in increased outer membrane permeability to a variety of probe compounds (see for example [6]). Thus we have termed such compounds 'permeabilizers'. Cationic or polycationic permeabilizers interact directly with the LPS as determined by their abilities to competitively displace cationic probes like the cationic spin label probe  $CAT^{12}$  [7] or the fluorescent probe dansyl polymyxin [8].

Mutant studies have suggested that these interactions are physiologically relevant. Outer membrane mutants with either enhanced or reduced ability to interact with specific permeabilizers are correspondingly supersusceptible or resistant to

#### Table 1. Self promoted uptake

Molecules that utilize as part of their uptake pathway, or interact with, sites at which divalent cations bridge adjacent LPS molecules

Molecule	Nature	Evidence for inter-action with divalent cation sites on LPS <sup>a</sup>	Bacteria <sup>b</sup>	Reference
Polymyxins	Polycationic cyclic peptide with fatty acyl tail	M,P,I,D	E.c., P.a., etc.	5,6,7,8,9
Aminoglycosides	Polycationic tri- or tetrasaccharides	M,P,I,D	E.c., P.a., etc.	6,7,8
Chelators (e.g. EDTA)	Non-cationic, divalent cation chelators	M,P,D	E.c., P.a., etc.	2,3,10
Tris	Monovalent organic cation	P,D	P.a., etc.	10
Defensins	Polycationic bactericidal peptides from neutrophils	P,I,D	P.a.	11
Bactericidal/permeability increasing protein	Polycationic protein from neutrophils	P,D	E.c.	12
Gramicidin S	Organic cationic antibiotic	P,D	P.a.	6,13
Azithromycin	Divalent cationic macrolide antibiotic	P,D	E.c.	с
Hexametaphosphate	Non-organic chelator	Р	P.a., E.c.,	14
Cecropins	Polycationic bactericidal peptides from cecropia moths	ND	etc. -	15
Magainins	Polycationic bactericidal proteins from frog skin	ND	-	16

 $^{a}M$  = Mutant evidence (i.e., mutants affecting LPS lead to resistance or supersusceptibility); P = permeabilization of outer membranes to hydrophobic probes, lysozyme and/or  $\beta$ -lactams; I = direct measurements of interaction of molecule with LPS using the spin label probe CAT<sub>12</sub> or dansyl polymyxin etc., as a probe; D = divalent cations block action of the compound in killing cells; ND = no direct evidence, interaction inferred from nature of molecule and literature data.

<sup>b</sup>E.c. = Escherichia coli; P.a. = Pseudomonas aeruginosa; etc. = other bacteria were also shown to interact.

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these compounds [2,7,9,10]. This suggests that interaction at the outer membrane is part of an uptake process resulting in eventual death of the bacterium. Since the outer membrane becomes permeabilized to a variety of probe compounds when it interacts with permeabilizers, we have proposed that it also becomes permeable to the permeabilizer itself. Thus we have termed this uptake mechanism 'selfpromoted uptake'. Among the permeabilizer compounds described in Table 1 are four classes of antibiotics (originating from various microorganisms) and four antimicrobial proteins from different eukaryotes (including moths, frogs and rabbits). Thus use of polycations to kill Gram-negative bacteria after interaction with, or self-promoted uptake across, the outer membrane, appears to be a conserved evolutionary theme. I have previously suggested [10] that DNA during transformation of cells, and several other cations, are also taken up by a similar mechanism.

#### Interaction of LPS with major outer membrane proteins

When specific membrane proteins are purified free of other proteins, using standard chromatographic methods, they are often associated with a molar excess of LPS molecules [17]. Direct evidence of association with LPS in the native state (as opposed to copurification with proteins) has been harder to obtain (for example see [2,4]). Two pieces of evidence from our laboratory that have indicated that such associations do exist, are crossed immunoelectrophoresis studies showing that LPS and porin OprF from *P. aeruginosa* form fused rockets in crossed immunoelectrophoresis experiments [18], and chemical crosslinking of OprF to LPS [19].

Porins, the channel-forming outer membrane proteins of Gram-negative bacteria, are apparently associated with LPS [4,17]. Indeed we were able to isolate a monoclonal antibody, (MA3-6), after fusion of a myeloma cell with the  $\beta$ -lymphocytes of mice immunized with purified *E. coli* OmpF porin-LPS complexes [17]. This antibody reacted strongly in ELISA with both outer membranes and OmpF porin-LPS complexes but extremely weakly with purified LPS and not at all with LPS-free OmpF porin (Table 2). Moreover, when the OmpF porin and LPS were separated on SDS-PAGE and Western blotted, the monoclonal antibody proved to be LPS-specific. This indicated that the antibody recognized a configuration of LPS that resulted from interaction of rough LPS (from *E. coli* K-12) with OmpF porin (or as determined in other experiments with OmpC porin). Since the monoclonal antibody reacted well with outer membranes, this implies that porins can influence the presentation of LPS molecules in the outer membrane.

We then asked the inverse question. Does LPS influence the configuration or function of porins? Our data implied that it did not in the cases of the OmpF porin of *E. coli* and protein P (OprP) of *P. aeruginosa*. These proteins were freed of LPS (to less than 0.4 mole percent) by SDS-PAGE followed by excision of the trimer bands and elution [17]. The resultant LPS-free porins were functionally identical,

Antigen <sup>a</sup>	ELISA reading <sup>b</sup>		Western	
	1 hour	24 hours	immunoblotting	
OmpF/LPS	++	++++	Not done with rough LPS	
OmpC/LPS LPS Outer membranes OmpF	++ +/- ++ 0	++++ ++ ++++ 0	not done reaction with rough LPS not done no reaction	

Table 2. Interaction of monoclonal antibody MA3-6 with various antigens

<sup>a</sup>Antigens OmpF/LPS, OmpC/LPS – conventionally purified porin:LPS complexes; OmpF-electroeluted porin with less than 0.4 mol % LPS contamination; <sup>b</sup>++++ = Very strong reaction, ELISA titre  $\ge 1.0$ ; ++ = intermediate reaction, ELISA titre 0.3-0.5; +/- = marginal reaction, ELISA titre  $\le 0.15$ ; 0 = no reaction above background.

as assessed by channel formation in planar bilayers, to their LPS-associated counterparts.

# Outer membrane stabilization under conditions of divalent cation deficiency

Brown and Melling [20] showed that *Pseudomonas aeruginosa*, grown in media with reduced levels of divalent cations (e.g.,  $0.02 \text{ mM Mg}^{2+}$ ), became resistant to polymyxin B and EDTA. We subsequently isolated mutants resistant to these same agents under condition of divalent cation (e.g.  $0.5 \text{ mM Mg}^{2+}$ ) sufficiency [9]. Both the mutants and adaptively resistant cells were cross resistant to aminoglycosides and had dramatic increases in the level of an outer membrane protein H1 (now called OprH). There was a correlation in these strains between the level of protein H1 and the amount of Mg<sup>2+</sup> in the cell envelope (when cells were grown such that Mg<sup>2+</sup> was the sole divalent cation in the cell envelope) [9]. Thus we hypothesized that the function of protein OprH was to replace divalent cations in the outer membrane under conditions of divalent cation deficiency, and that this loss of divalent cation crossbridging sites resulted in resistance to the polycationic antibiotics and EDTA (by blocking self-promoted uptake (see above)).

The proposed interaction of OprH with LPS was consistent with the observation that LPS remained associated with OprH through two cycles of ion exchange chromatography in detergent solution [21]. In addition, the nucleotide sequence of *OprH* gene revealed that this protein was relatively basic, a situation that would favor interaction with negatively charged LPS molecules [21]. Recently, we placed the cloned oprH gene behind the *tol* promoter in a broad-host range expression vector. Introduction of this recombinant plasmid into *P. aeruginosa* wild-type strains and consequent overproduction of OprH protein caused cells to become EDTA resistant under Mg<sup>2+</sup> sufficient conditions (A. Bell, Ph.D. Thesis, U.B.C., Vancouver, 1988). This data is therefore consistent with a role for OprH in displacing divalent cations in the outer membrane by interaction with LPS. Interestingly, however, the cells overexpressing OprH from the cloned gene displayed an incomplete phenotype in that such cells were less resistant to EDTA, than were the polymyxin resistant mutants described above, and were completely sensitive to polymyxin B. Thus the complete phenotype of the resistant mutants and  $Mg^{2+}$ -deficient medium grown wild type cells cannot be explained solely by OprH overproduction and these cells must contain another alteration. Since rough mutants of these strains did not express the resistance phenotypes under conditions where OprH is fully derepressed, it seems likely that this other alteration affects the LPS.

#### Acknowledgements

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