The role of lipopolysaccharide in complement-killing of *Aeromonas hydrophila* strains of serotype O:34

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The role of lipopolysaccharide (LPS) in the susceptibility of *Aeromonas hydrophila* strains of serotype O:34 to non-immune human serum was investigated using isogenic mutants (serum-sensitive), previously obtained on the basis of phage resistance, and characterized for their surface components. The classical complement pathway was found to be principally involved in the serum-killing of these sensitive strains. LPS preparations from serum-resistant or serum-sensitive strains, or purified core oligosaccharides (low-molecular-mass LPS) inactivated both bactericidal and complement activity of whole serum, while the O-antigen molecules (high-molecular-mass LPS) did not. The results indicate that LPS core oligosaccharide composition contributes to complement resistance of *A. hydrophila* strains from serotype O:34 with moderate virulence.

Introduction

The bactericidal effect of non-immune serum is important in immediate host defence against bacterial infections. This phenomenon has been widely studied (Morrison & Kline, 1977; Nelson & Roantree, 1967; Taylor, 1983, 1988), and is known to be complement-mediated (Roantree & Pappas, 1960). Although serum resistance in Gram-negative bacteria can be multifactorial *in vivo*, the outer membrane is predominantly important since it is usually the most peripheral component of the bacterial cell envelope. Outer membrane components such as lipopolysaccharide (LPS) (Munn *et al.*, 1982; Mushel & Larsen, 1970; Nelson & Roantree, 1967), and proteins (Guyman *et al.*, 1978; Hildebrandt *et al.*, 1978; Moll *et al.*, 1980), as well as other components of the bacterial cell surface, such as capsular polysaccharides, have been implicated in resistance to the bactericidal activity of serum (Glynn & Howard, 1970; Rice & Kasper, 1977; Sutton *et al.*, 1982).

Complement activation by Gram-negative bacteria can occur via the classical complement pathway (CCP) or the alternative complement pathway (ACP). The CCP can be activated by the interaction of antibody with bacterial surface antigens or sometimes more directly by

the lipid A moiety of LPS (Morrison & Kline, 1977). The ACP can normally be activated by bacterial polysaccharides independently of antibody (Quinn *et al.*, 1977).

Aeromonas hydrophila is both an opportunistic and a primary pathogen of a variety of aquatic and terrestrial animals, including humans (Howard & Buckley, 1985). The clinical manifestation of *A. hydrophila* infection ranges from gastroenteritis to soft tissue infections, septicaemia, and meningitis (Freij, 1984; Ljungh & Wadstrom, 1983). Surface characteristics, such as the presence of an S-layer or the type of LPS, permit classification of *A. hydrophila* into different categories on the basis of their virulence (Janda *et al.*, 1985; Dooley *et al.*, 1985). We recently described a group of *A. hydrophila* strains belonging to serotype O:34 with heterogeneous O-polysaccharide chains in their LPS and without an S-layer, previously reported to be moderate in their virulence for fish and mice (Lallier *et al.*, 1984; Merino & Tomás, 1988; Merino *et al.*, 1989). Also, serotype O:34 has been recently reported as one of the most frequently encountered among mesophilic *Aeromonas* species (Thomas *et al.*, 1990).

In the present study, we investigated the pathway of complement activation by *A. hydrophila* strains of serogroup O:34, and the role of LPS in the susceptibility of these strains to the bactericidal activity of non-immune serum, by using phage-resistant mutants (serum-sensitive) against bacteriophages 18 and PM2, whose bacterial receptor is the LPS-core region (Merino *et al.*, 1990*a, b*). Further, we examined the role

Abbreviations: CCP, classical complement pathway; ACP, alternative complement pathway; HMM-LPS, high-molecular-mass lipopolysaccharide; LMM-LPS, low-molecular-mass lipopolysaccharide; NHS, non-immune human serum; KDO, 2-keto-3-deoxyoctulosonic acid (3-deoxy-D-manno-octulosonic acid).

of high-molecular-mass LPS (HMM-LPS), containing O-antigen repeating units, and low-molecular-mass LPS (LMM-LPS), devoid of O-antigen polysaccharide, in this process.

Methods

Bacterial strains. The strains used are listed in Table 1. Cultures were maintained and grown as previously described (Merino *et al.*, 1989).

Bacterial survival in fresh non-immune serum. The survival of exponential-phase bacteria in non-immune human serum (NHS) was measured at 20 °C and 37 °C as previously described (Tomás *et al.*, 1986). Controls consisting of bacteria in PBS (containing 0.15 M-sodium chloride and 0.15 M-sodium phosphate, pH 7.2) showed no significant changes in viable counts over the incubation period. We used heat-inactivated NHS (56 °C for 30 min) as an additional control. Serum was usually used on the day of the collection or stored at -80 °C, and these sera were unable to agglutinate *A. hydrophila* strains.

Serum-resistant mutants were obtained as survivors after treatment with NHS for 3 h. After 36 h of incubation at 20 °C, colonies of serum-resistant mutants were picked, purified by streaking, and retested with NHS to confirm resistance.

Treatment of serum. CCP activity in whole serum was selectively inhibited by chelation with 20 mM-EGTA plus 2 mM-MgCl₂ as previously described (Fine *et al.*, 1972). ACP activity in serum was selectively inhibited with inulin (2 mg ml⁻¹) by the method of Gotze & Muller-Eberhard (1971), or by heating at 50 °C for 20 min in order to inactivate factor B, which is required for ACP activity (Eidinger *et al.*, 1977). After each serum treatment the decrease in complement activity was assayed by the method of Morrison & Kline (1977), as modified in a microtitre assay by Vukajlovich (1986). Both pathways were inhibited by treatment of serum with 20 mM-EDTA or by heating at 56 °C for 30 min. EGTA, EDTA, and inulin, at the concentrations mentioned above, had no effect on the survival of *A. hydrophila* strains in PBS for up to 3 h of incubation.

LPS isolation and subfractionation. LPS from *A. hydrophila* strains was purified by the method of Westphal & Jann (1965) with the modifications of Osborn (1966). Lyophilized LPS was solubilized at a final concentration of 7.5 mg ml⁻¹ in buffer containing 3% (w/v) sodium deoxycholate and applied to Sephacryl S-300 (Pharmacia) column as previously described (Ciurana & Tomás, 1987). Fractions were analysed by SDS-PAGE or dialysed extensively against distilled water, first at room temperature and then at 4 °C, before chemical analyses or serum inhibition studies were performed.

Electrophoretic techniques. SDS-PAGE was done according to the procedure of Laemmli (1970) as modified by Ames *et al.* (1974). Samples were mixed 1:1 with sample buffer (containing 4% SDS, w/v), and boiled for 5 min; 10 µl portions were then applied to the gel. LPS bands were detected by the method of Tsai & Frasch (1982).

Analytical procedures. Total carbohydrate was measured by the phenol procedure (Hanson & Phillips, 1981) with glucose as standard. 2-keto-3-deoxyoctulosonic acid (KDO) was measured by the thiobarbituric acid method after hydrolysis of samples in 0.05 M sulphuric acid for 30 min (Karkhanis *et al.*, 1978). Monosaccharides were also analysed to their alditol acetate derivatives by GLC on a 3% SP-3840 column (Supelco) as previously described (Tomás & Jofre, 1985).

Inhibition of serum bactericidal activity by complete and fractionated LPS. LPS was suspended in PBS (pH 7.2) to a final concentration of 1–5 mg ml⁻¹ and briefly sonicated at 4 °C until the solution cleared. LPS solution in the concentration range 0.01–0.2 mg ml⁻¹ was added to 0.5 ml serum in a tube. The volume was adjusted to 0.9 ml with PBS, and the solution was incubated at 37 °C with shaking (100 r.p.m.) for 30 min. Then, 0.1 ml of an exponential phase culture (5 × 10⁷ c.f.u.) was added to the tube and incubated at 37 °C for 3 h; samples were taken hourly and bacterial concentrations determined by dilution and plating on agar. Similar experiments were done with LPS fractions which were lyophilized, resuspended in PBS and added to a final concentration of 0.1 mg ml⁻¹ to 0.5 ml serum. Controls without LPS or LPS fractions showed no inhibition of serum bactericidal activity.

Measurement of the anti-complementary activity of LPS. The anti-complementary activity of LPS or LPS fractions was measured

Table 1. *Aeromonas hydrophila* strains

Strains AH-3, Ba5 and AH-101 are wild-types. Strains AH-8, AH-51 and AH-151 are spontaneous mutants resistant to bacteriophage 18, while strains AH-53, AH-54 and AH-159 are spontaneous mutants resistant to bacteriophage PM2. Strains AH-34, AH-301, AH-302, AH-303, AH-304 and AH-305 are isogenic serum-resistant mutants obtained from serum-sensitive strains.

Strain	Relevant characteristics	Origin
AH-3	Serotype O:34, serum resistant	Merino <i>et al.</i> , 1989
AH-8	Rough, isogenic mutant derived from AH-3, serum sensitive	Merino <i>et al.</i> , 1990a
AH-53	Rough, isogenic mutant derived from AH-3, serum sensitive	Merino <i>et al.</i> , 1990b
AH-34	Rough, isogenic mutant derived from AH-8, serum resistant	This work
AH-301	Rough, isogenic mutant derived from AH-53, serum resistant	This work
Ba5	Serotype O:34, serum resistant	Lallier <i>et al.</i> , 1984
AH-51	Rough, isogenic mutant derived from Ba5, serum sensitive	Merino <i>et al.</i> , 1990a
AH-54	Rough, isogenic mutant derived from Ba5, serum sensitive	Merino <i>et al.</i> , 1990b
AH-302	Rough, isogenic mutant derived from AH-51, serum resistant	This work
AH-303	Rough, isogenic mutant derived from AH-54, serum resistant	This work
AH-101	Serotype O:34, serum resistant	Merino <i>et al.</i> , 1989
AH-151	Rough, isogenic mutant derived from AH-101, serum sensitive	Merino <i>et al.</i> , 1990a
AH-159	Rough, isogenic mutant derived from AH-101, serum sensitive	Merino <i>et al.</i> , 1990b
AH-304	Rough, isogenic mutant derived from AH-151, serum resistant	This work
AH-305	Rough, isogenic mutant derived from AH-159, serum resistant	This work

according to Shafer *et al.* (1984), with slight modifications. NHS (0.1 ml) was mixed with LPS (0.01–0.2 mg ml⁻¹) or LPS fractions (0.1 mg ml⁻¹) suspended in PBS, or PBS alone, to a final volume of 0.2 ml, and incubated with shaking at 37 °C for 30 min. Antibody-sensitized sheep erythrocytes in 0.2 ml PBS were added to a four-fold dilution of treated NHS and incubated for an additional 30 min at 37 °C. Ice-cold saline (3 ml) was added to the mixture, the cells were pelleted by centrifugation, and the absorbance of the supernatant was measured at 412 nm. The positive control was sensitized sheep erythrocytes plus NHS without added LPS or LPS fraction, and the negative control was LPS or LPS fraction without added NHS.

Concentration of C1q and C3 complement components were also measured using specific antibodies as described by Theofilopoulos *et al.* (1980). Briefly, specific anti-C1q or anti-C3 sera (Sigma) were coated onto a microtitre plate overnight at 4 °C, washed and incubated for 1 h at 37 °C with 1% human serum albumin. Meanwhile, NHS was treated with *A. hydrophila* whole cells or purified LPS (complete or fractionated) for 30 min at 37 °C. Untreated NHS was used as a standard, using the same incubation period. After washing the plates, the treated or untreated NHS was added and incubated for 90 min at 37 °C. The plates were then washed again and incubated for 1 h at 37 °C with protein-A-alkaline-phosphatase conjugate (Sigma). After washing the plates, the colour reaction was developed with 4-nitrophenyl phosphate (1 mg ml⁻¹) and the A_{405} recorded.

Binding of C3b and C5b-9 to *A. hydrophila* whole cells. The interaction between whole *A. hydrophila* O:34 cells and complement C3b and C5b-9 components was quantified using an enzyme immunoassay. Preopsonized bacteria (10 min) with 90% NHS were washed twice with cold PBS by microcentrifugation, and were incubated with anti-C3b or anti-C5b-9 (Calbiochem) (1:100 in PBS plus 1% BSA), washed intensively by microcentrifugation, followed by protein-A-alkaline-phosphatase incubation at 37 °C for 45 min (1:100 in PBS). After washing, the colour reaction was developed as before and the A_{405} recorded.

Results

Survival in NHS

Mutants resistant to bacteriophages 18 and PM2 from different *A. hydrophila* strains were described previously (Merino *et al.*, 1990a, b). They were devoid of both O-antigen polysaccharide and part of the LPS-core oligosaccharide. Results of NHS survival experiments at 37 °C with these mutants and their respective wild-type strains are shown in Table 2, and no important differences were observed in NHS survival experiments at 20 °C. Strains AH-53, AH-54, AH-159, AH-8, AH-51 and AH-151 showed a marked decrease in viability (under 1% survival) after 2 h. No decrease was observed with wild-type strains AH-3, Ba5 or AH-101 or strains AH-34, AH-301, AH-302, AH-303, AH-304 and AH-305 (serum-resistant mutants derived from strains AH-8, AH-53, AH-51, AH-54, AH-151, and AH-159, respectively). These isogenic serum-resistant mutants were still devoid of the O-antigen polysaccharide chains on their LPS, and their LPS-core showed a lower migration rate on SDS-PAGE gels than their isogenic rough serum-sensitive strains (Fig. 1). Further, the serum-resistant mutants showed alterations in the chemical composition of their LPS core oligosaccharides versus their isogenic serum-sensitive strains. Table 3 shows the chemical composition of LPS from wild-type strains (O:34),

Table 2. Survival of *A. hydrophila* strains in NHS and in complement-depleted NHS

Complement depletion was achieved either by heating NHS for 30 min at 56 °C or by treating the NHS with 20 mM-EDTA for 1 h at 37 °C.

Strain	Percentage of inoculum surviving on:					
	NHS			Complement-depleted NHS		
	1 h	2 h	3 h	1 h	2 h	3 h
AH-3	96	99	101	102	103	109
AH-8	1.8	0.2	<0.1	104	103	112
AH-53	0.8	<0.1	<0.1	103	105	108
AH-34	94	100	103	106	113	120
AH-301	95	101	105	102	107	114
Ba5	98	102	105	104	107	116
AH-51	1.9	0.3	<0.1	100	106	108
AH-54	0.7	<0.1	<0.1	102	104	107
AH-302	96	100	103	103	109	118
AH-303	97	99	102	104	109	117
AH-101	97	100	108	102	110	116
AH-151	1.7	0.1	<0.1	100	102	104
AH-159	0.9	<0.1	<0.1	99	101	105
AH-304	98	101	106	103	111	118
AH-305	97	102	106	105	112	120

Fig. 1. SDS-PAGE of LPS purified from different *A. hydrophila* O:34 strains, detected according to Tsai & Frasch (1982). Lanes: 1, LPS from strain AH-3; 2, LPS from strain AH-8; 3, LPS from strain AH-34; 4, LPS from strain AH-53; 5, LPS from strain AH-301; 6, complete LPS from strain AH-3; 7, LMM-LPS from strain AH-3 (pooled fractions); and 8, HMM-LPS from strain AH-3 (pooled fractions).

phage-resistant mutants (serum-sensitive) (Merino *et al.*, 1990*a, b*) and serum-resistant mutants. The main change observed in the LPS composition of the serum-resistant mutants versus the serum-sensitive phage-resistant mutants is the substitution of glucose for galactose in the LPS core region.

To determine whether differences in survival in NHS among strains were due to nutritional rather than bactericidal factors, we measured survival in serum which was heated or treated with 20 mM-EDTA to destroy complement. These results (Table 2) showed no significant differences between the wild-type strains and the rough isogenic mutants (AH-8, AH-53, AH-51, AH-54, AH-151, and AH-159).

Fig. 2. The bactericidal effects of NHS on the serum-sensitive strain *A. hydrophila* AH-53. Control serum (●) was unaltered prior to the addition of cells; otherwise serum was pretreated as follows: heated at 56 °C for 30 min (○), 20 mM-EDTA (□), 20 mM-EGTA plus 2 mM-MgCl₂ (△), 2 mg ml⁻¹ inulin (▲), or heated at 50 °C for 20 min (■).

Mechanism of complement activation by *A. hydrophila* strains.

The rough serum-sensitive strain AH-53 was rapidly killed in the presence of NHS (Fig. 2). However, serum treated with EDTA, or heated at 56 °C for 30 min (which inhibits both the CCP and ACP), was virtually nonbactericidal. Also, serum treated with Mg²⁺-EGTA (which selectively inhibits CCP), was nonbactericidal, whereas serum pretreated either with inulin or by heating at 50 °C for 20 min (which depletes ACP), was strongly bactericidal (Fig. 2). Similar results were obtained with another serum-sensitive strain AH-8. These results suggested that serum killing of sensitive *A. hydrophila* O:34 strains is mediated by the CCP in the absence of specific antibody.

Table 3. Chemical composition of purified LPS from *A. hydrophila* strains

Strain	Content [$\mu\text{mol (mg LPS)}^{-1}$] of:							
	KDO*	L-Heptose†	D-heptose†	Pentose†	Glucose†	Galactose†	Rhamnose†	Hexosamines†
AH-3	0.018	0.31	0	0	1.57	0	0	0.41
AH-8	0.043	1.12	0	0	1.48	0	0	0
AH-53	0.051	1.25	0	0	1.32	0	0	0
AH-34	0.041	0.98	0	0	0	1.96	0	0
AH-301	0.048	1.02	0	0	0	1.80	0	0
Ba5	0.019	0.31	0	0	1.55	0	0	0.43
AH-51	0.042	1.10	0	0	1.49	0	0	0
AH-54	0.050	1.26	0	0	1.29	0	0	0
AH-302	0.043	1.01	0	0	0	1.94	0	0
AH-303	0.046	1.06	0	0	0	1.84	0	0

* Assayed by the colorimetric method of Karkhanis *et al.* (1978).

† Assayed by GLC.

Table 4. Inhibition of bactericidal activity of serum against serum-sensitive strains of *A. hydrophila* by homologous and heterologous LPS, and pooled fractions of LPS

The LPS concentration used was 100 µg ml⁻¹. Results are means of three independent experiments.

Strain	Percentage survival of bacteria after 180 min incubation in:						
	NHS plus LPS from the following strains					NHS plus LPS fractions	
	Control (NHS)	Serum-sensitive		Serum-resistant		HMM-LPS	LMM-LPS
		AH-8	AH-53	AH-3	AH-34		
AH-8	<0.1	101	103	99	101	0.6	98
AH-53	<0.1	100	98	98	102	0.7	99
AH-51	<0.1	104	102	97	104	0.8	102
AH-54	<0.1	100	103	98	100	0.6	96
AH-151	<0.1	102	100	99	103	0.6	98
AH-159	<0.1	103	103	96	101	0.9	101

Table 5. Concentration of complement components C1q and C3 in fresh NHS untreated or treated with whole cells, or LPS fractions, from different *A. hydrophila* O:34 strains

Results are means from experiments done at least twice in triplicate. Standard deviations were all <0.07.

Component	Untreated	Concentration of C1q or C3 in arbitrary units (A ₄₀₅ ELISA measurements) in NHS treated with:							
		Whole cells			Complete LPS			LPS fractions	
		AH-3	AH-8	AH-34	AH-3	AH-8	AH-34	HMM*	LMM*
C1q	1.32	0.35	0.27	0.29	0.41	0.32	0.35	1.29	0.33
C3	1.98	0.44	0.39	0.41	0.46	0.40	0.42	1.87	0.41

* Isolated from strain AH-3.

Inhibition of serum bactericidal activity by *A. hydrophila* O:34 LPS

Various concentrations of purified whole LPS (a mixture of O-antigen-containing and O-antigen-deficient LPS molecules) from *A. hydrophila* AH-3 inhibited the bactericidal activity of NHS in a dose-dependent manner when tested against the sensitive strain AH-53. The survival of AH-53 cells in NHS after 3 h of incubation at 37 °C was 0, 26, 79, 98, 101 and 102% at LPS concentrations of 0, 0.025, 0.05, 0.1, 0.2 and 0.4 mg ml⁻¹, respectively. Purified whole LPS at 0.1 mg ml⁻¹ from serum-resistant (AH-3 and AH-34) or serum-sensitive (AH-8 and AH-53) *A. hydrophila* strains also inhibited the bactericidal activity of serum when tested against the sensitive strains AH-8, AH-53, AH-51, AH-54, AH-151 and AH-159 (Table 4).

Inhibition of bactericidal activity by LPS fractions

LPS from *A. hydrophila* AH-3 or Ba5 was fractionated using Sephacryl S-300, and the presence of LPS in

column fractions was monitored by determination of KDO and total hexose, and by SDS-PAGE. Pooled fractions containing HMM-LPS and pooled LMM-LPS are shown in Fig. 1. Serum-sensitive strains of *A. hydrophila* showed a high percentage survival (>96%) in NHS treated with pooled LPS fractions having a low hexose:KDP ratio (LMM-LPS), whereas if NHS was pretreated with pooled LPS fractions having a high hexose:KDO ratio (HMM-LPS) the percentage survival of serum-sensitive strains was always less than 1% (Table 4), although some residual effect on the bactericidal activity remained.

Anticomplementary activity of *A. hydrophila* O:34 LPS

The complement-absorbing activity of LPS from *A. hydrophila* AH-3 or Ba5 was measured to determine whether inhibition of serum bactericidal activity was due to depletion of serum complement. The complement-absorbing activity of LPS from *A. hydrophila* AH-3 and the serum-sensitive strains AH-53 and AH-54 was dose-dependent (Fig. 3). Furthermore, concentrations of

Fig. 3. Inhibition of complement-mediated haemolysis of sensitized sheep erythrocytes. Erythrocytes were incubated for 30 min in NHS as control, or with NHS containing whole LPS from *A. hydrophila* AH-3 (Δ) or AH-53 ($\circ$), LMM-LPS from strain AH-3 ($\blacksquare$), or HMM-LPS from the same strain ($\square$) or HMM-LPS from strain Ba5 ($\bullet$).

Table 6. Interaction of complement components C3b and C5b-9 with *A. hydrophila* O:34 whole cells, measured as A_{405} in an ELISA assay

Results are means from experiments done at least twice in triplicate.

Strain	Relative concentration A_{405} ELISA values)	
	C3b	C5b-9
AH-3	0.32 $\pm$ 0.06	0.08 $\pm$ 0.03
AH-8	1.52 $\pm$ 0.13	1.32 $\pm$ 0.15
AH-53	1.63 $\pm$ 0.14	1.36 $\pm$ 0.13
AH-34	0.09 $\pm$ 0.04	0.09 $\pm$ 0.02
AH-301	0.10 $\pm$ 0.04	0.07 $\pm$ 0.03
Ba5	0.30 $\pm$ 0.07	0.07 $\pm$ 0.03
AH-51	1.54 $\pm$ 0.15	1.42 $\pm$ 0.14
AH-54	1.60 $\pm$ 0.12	1.45 $\pm$ 0.15
AH-302	0.08 $\pm$ 0.03	0.09 $\pm$ 0.02
AH-303	0.09 $\pm$ 0.04	0.07 $\pm$ 0.01

complement components C1q and C3 were depleted when NHS was treated with whole cells or complete LPS from *A. hydrophila* serum-sensitive or serum-resistant strains, as well as LMM-LPS (but not HMM-LPS) from strain AH-3 (Table 5).

Binding of C3b and C5b-9 to *A. hydrophila* O:34 whole cells

As shown in Table 6, serum-resistant strains of *A. hydrophila* O:34 did not bind C3b or C5b-9, whereas the serum-sensitive strains interacted strongly with C3b or C5b-9, producing A_{405} values in ELISA which were all >1.31 .

Discussion

The bactericidal effects of immune or nonimmune sera are mediated by activated components of the CCP and ACP (Pangburn, 1983; Rowley, 1968; Taylor, 1983, 1988). Activation of either pathway can lead to membrane damage, usually resulting in cell death. Our study of complement activation by *A. hydrophila* O:34 strains indicates that only the CCP was involved in serum killing of sensitive strains. Selective inhibition of the CCP by treatment with Mg^{2+} -EGTA abolished serum bactericidal activity, and serum treated with inulin, or heated at 50 °C for 20 min, was still bactericidal. Thus the ACP is probably not involved in serum-killing of sensitive strains of *A. hydrophila* O:34. Other Gram-negative bacteria such as *Neisseria gonorrhoeae* (Schafer *et al.*, 1984), *Pseudomonas aeruginosa* (Schiller *et al.*, 1984), and *Haemophilus ducreyi* (Odumeru *et al.*, 1985), have also been shown to activate only the CCP; however, other bacteria, such as *Haemophilus influenzae* (Quinn *et al.*, 1977), *Salmonella* spp. (Tomás *et al.*, 1988), *Escherichia coli* (Tomás *et al.*, 1988), and *Klebsiella pneumoniae* (Ciurana & Tomás, 1987), activate both pathways.

The susceptibility of many Gram-negative bacteria to the bactericidal activity of immune and non-immune sera has often been attributed to their LPS composition. LPS from serum-resistant and serum-sensitive strains of *A. hydrophila* O:34 inhibited the serum bactericidal activity against sensitive strains, and activated complement, as measured either by the inhibition of haemolysis with sensitized sheep erythrocytes, or by direct measurement of components C1q and C3 in treated serum.

Only the low-molecular-mass fractions of *A. hydrophila* O:34 LPS inactivated serum bactericidal activity, prevented haemolysis of sensitized sheep erythrocytes, and depleted the levels of C1q and C3 complement components from NHS after 30 min incubation at 37 °C; this indicates a relationship between blocking of serum bactericidal activity and complement-absorbing activity of these LPS fractions. On the other hand, *A. hydrophila* O:34 LPS fractions containing HMM-LPS, i.e. only O-antigen-containing LPS molecules, neither inactivated the serum bactericidal reaction, nor inhibited the haemolysis of sensitized sheep erythrocytes, nor depleted C1q or C3 complement components in treated NHS.

Serum-sensitive strains of Gram-negative bacteria may activate the CCP, leading to the formation of C5b-9 complexes which cause membrane damage and cell death (Taylor, 1988). For this reason, the serum-sensitive strains are rapidly killed by NHS. However the wild-type strains (serum-resistant) also activated the CCP, but showed only minor binding of C3b and no formation of C5b-9 complexes. This may be due to binding of C3b by O-antigen (HMM-LPS) sufficiently far away from the

membrane to exclude the formation of C5b-9 complexes, so that the cells are still resistant to NHS. Although purified LPS abolishes the bacterial activity of sera when fresh bacteria are subsequently added, it does not necessarily follow that LPS inhibits the bacterium: complement interaction *in vivo*.

Finally, the isogenic serum-resistant mutants (rough strains) derived from serum-sensitive strains activated the CCP, but no C3b binding was observed under our experimental conditions. In these mutants there is a substitution of glucose for galactose in the LPS core oligosaccharides, a change that also influences the electrophoretic mobility of the LPS. At present we can not explain how changes in the LPS core oligosaccharides can prevent C3b binding, but without binding of C3b to whole cells there is no formation of C5b-9 complexes.

This study has more closely defined the role of LPS, and specially the core oligosaccharides from the LPS core, in the resistance of *A. hydrophila* O:34 strains to serum-killing of these strains.

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