The susceptibility of S-layer-positive and S-layer-negative *Aeromonas* strains to complement-mediated lysis

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Forty strains of *Aeromonas hydrophila* and *Aeromonas veronii* recovered from invasive and non-invasive infections were tested for their susceptibility to complement-mediated lysis by 65% pooled human serum (PHS). Based upon the results of this assay, two major populations could be defined. The first group ($n = 20$) consisted of serogroup O:11 strains, all of which possessed a paracrystalline surface layer (S layer); all of these strains were refractory to the bactericidal activity of 65% PHS with the exception of *A. hydrophila* strain AH-121, which was composed of mixed subpopulations of serum-susceptible and serum-resistant clones. A second collection of isolates ($n = 20$), all of which were S-layer-negative, contained a subgroup of strains ($n = 7$) that were highly susceptible to complement-mediated lysis, showing a greater than 100-fold reduction of viable progeny within 30 min of exposure to 65% PHS. Serum-resistant strains from both groups could not be lysed by exposure of bacterial cells to polyclonal somatic or whole cell antisera or to $30 \mu\text{g ml}^{-1}$ of polymyxin B nonapeptide prior to challenge with 65% PHS. Analysis of selected serum-resistant and serum-susceptible strains from both groups showed that all isolates activated the complement pathway and most bound C3b to the cell surface, indicating that the inability of complement to lyse serum-resistant strains was related to a defect in the terminal portions of the complement pathway. The major differences noticed between serum-resistant and serum-susceptible strains were a lack of a definable lipopolysaccharide side chain profile and higher 50% lethal dose values in strains that were susceptible to complement-mediated lysis.

Keywords: *Aeromonas*, S layers, serum resistance, pathogenicity

INTRODUCTION

One of the most intriguing appendages on the outer surface of many Gram-negative and Gram-positive bacteria is the S layer, or paracrystalline surface layer (Messner & Sleytr, 1992). While the majority of recently described S layers have been associated with saprophytic bacteria, an increasing number of human and animal pathogens have also been reported to contain such structures. Pathogenic bacteria having such S layers include *Clostridium botulinum*, *Aeromonas salmonicida*, *Campylobacter fetus* subsp. *fetus*,

Mycobacterium bovis and *Campylobacter* (*Wolinella*) *recta* (Messner & Sleytr, 1992; Kobayashi *et al.*, 1993).

Very little information is presently available on the role(s) S layers may play in microbial pathogenesis. Several functions for these layers have been proposed or alluded to and include action as a barrier or molecular sieve, and involvement in adhesive (attachment) activities or in resistance to normal immune functions such as complement-mediated lysis or phagocytosis by professional or non-professional phagocytes (Messner & Sleytr, 1992). Munn *et al.* (1982) assessed the bactericidal activity of normal and immune fish sera against *A. salmonicida* and found that the S layer in conjunction with lipopolysaccharide (LPS) played an integral part in the overt resistance of the bacterium to complement-mediated lysis. In similar studies performed with *Camp. fetus*,

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Abbreviations: PHS, pooled human serum; PMBN, polymyxin B nonapeptide; S-layer, paracrystalline surface layer; SAP, surface array protein.

Table 1. Characteristics of *Aeromonas* strains used in this study

Defining character	Genomospecies	Source	Strain(s)
Serogroup O:11; S layer-positive*	<i>A. hydrophila</i>	Blood	AH-342
		Wound	AH-121, AH-357
		Faeces	AH-205, AH-321, AH-332
		Animals	LL1, AH-252
		Water	NMRI-54
	<i>A. veronii</i> biotype sobria	Blood	AS-39, AS-76, AS-86, AS-114, AS-180
		Wound	AS-143, AS-151, AS-218
		Faeces	AS-28
		Animals	ATCC 9071, AS-174
Serogroup non-O:11; S layer-negative*	<i>A. hydrophila</i>	Blood	AH-88, AH-106, AH-145, AH-184, AH-186, AH-211, AH-351, AH-360, AH-361
		Wound	NMRI-7, AH-178
		Faeces	AH-208, AH-319
		Urine	AH-78
	<i>A. veronii</i> biotype sobria	Blood	AS-127, AS-315
		Faeces	AS-320
		Unknown	AS-53
	<i>A. hydrophila</i> complex	Animals	NCMB 1134, ATCC 14715

* As determined by transmission electron microscopy (thin section).

isogenic mutants lacking the 100 kDa surface array protein (SAP) which forms the S layer were found to be serum-susceptible, unlike their parental counterparts (Blaser *et al.*, 1987).

Recently, several other species within the genus *Aeromonas* have been found to possess S layers (Paula *et al.*, 1988; Kokka *et al.*, 1991a). Strains of these species (*A. hydrophila*, *A. veronii* biotype *sobria*) possessing S layers cause diseases in humans and animals (particularly fish) and are often associated with invasive systemic infections (septicaemia, peritonitis) in these vertebrates. Since resistance to complement-mediated lysis is thought to play an important role in disseminated disease caused by Gram-negative bacteria (Figueroa & Densen, 1991), and in light of the remarkable similarities in surface characteristics and disease spectrum of SAP-positive *Camp. fetus*, *A. hydrophila* and *A. veronii*, we have investigated whether or not the S layer of the latter two species is responsible for serum resistance in a fashion analogous to that previously determined for S layer-positive *Camp. fetus* and *A. salmonicida*.

METHODS

Organisms. Forty mesophilic strains of *A. hydrophila* or *A. veronii* were investigated (Table 1). These strains were identified biochemically to genomospecies using a recently described scheme of phenotypic tests (Abbott *et al.*, 1992). Strains were further subdivided into two groups based upon their reactivity against O:11 antisera in slide agglutination assays (Kokka & Janda, 1990). All O:11 strains were autoagglutination-positive (AA⁺) and possessed an S layer as determined by thin-section

transmission electron microscopy (Kokka *et al.*, 1991a). The remaining 20 strains were S-layer-negative and displayed diverse phenotypes and were designated non-O:11 strains; some of these non-O:11 strains were subsequently serogrouped through the courtesy of T. Shimada (National Institutes of Health, Tokyo, Japan).

Serum susceptibility studies. The susceptibility of all 40 *Aeromonas* strains to complement-mediated lysis by 65% pooled human serum (PHS) was determined by microassay as previously described (Paula *et al.*, 1988). Briefly, exponential-phase bacteria (6×10^6 – 3×10^7 c.f.u.) suspended in phosphate-buffered saline (PBS, pH 7.2) were mixed with 65% (v/v) PHS in microfuge tubes and incubated for 120 min at 35 °C. Susceptibility or resistance to PHS was determined at 120 min by the criteria of Taylor (1983) after determining the number of surviving bacteria (c.f.u. ml⁻¹) when compared to initial inoculum ($t = 0$).

Some isolates were additionally challenged against PHS after presensitization (25 °C, 30 min) with a 1:32 dilution of polyclonal antisera (rabbit, complement-inactivated) raised against whole cell (WC) or somatic (O) antigen preparations of the homologous strain. The effect of polymyxin B nonapeptide (PMBN, Boehringer Mannheim) on the serum susceptibility status of some strains was determined by the simultaneous incubation of the challenge inoculum with 65% PHS and 30 µg PMBN (Viljanen *et al.*, 1986).

For several strains (AH-78, AS-178, AH-121), surviving bacteria from a single exposure (cycle) to 65% PHS were centrifuged, rechallenged with fresh 65% PHS, and reincubated for an additional 120 min. Surviving colonies (clones) from single (120 min) or double (240 min) exposures to 65% PHS were picked and regrown in brain heart infusion broth (BHIB). These cultures were then rechallenged as above with one or two

C3b binding. *Aeromonas* strains were grown in BHIB for 18–20 h at 35 °C and after overnight incubation were diluted into fresh BHIB and reincubated for an additional 2–4 h at 35 °C to obtain exponential-phase bacteria. Such cultures were then harvested, centrifuged, and resuspended in PBS (pH 7.4) and spectrophotometrically (OD_{610}) adjusted to approximately 5×10^6 to 1×10^7 c.f.u. ml⁻¹; cells were then pelleted in a Beckman Microfuge E and the supernatant was discarded. Exponential-phase cells were then resuspended in 300 µl of 30 % fresh serum (in PBS) and incubated for 30 min at 37 °C (Horwitz & Silverstein, 1980; Agüero *et al.*, 1984). Following incubation, 1 ml cold 10 mM EDTA in PBS was added. Cells were then centrifuged and washed once in PBS before incubation for 15 min in 100 µl of a 1:10 dilution of FITC-conjugated goat anti-human C3 IgG (Jackson). Following incubation with conjugate, cells were washed once and resuspended in 100 µl PBS. C3 coating of bacteria was determined by placing a 25 µl portion of this suspension on a slide with a cover slip and then examining cells for fluorescence using an epifluorescence microscope (Horwitz & Silverstein, 1980; Agüero *et al.*, 1984).

Mouse pathogenicity. The relative pathogenicity of selected aeromonads was determined by LD-50 studies performed in outbred Swiss-Webster mice (Janda & Kokka, 1991). Sets of five mice were challenged intraperitoneally with concentrations of the respective strain ranging between 10^5 and 10^9 c.f.u. Mice were observed daily for 1 week. All mice succumbing to *Aeromonas* infection died within 72 h of inoculation; LD-50 values for each strain were then calculated according to the method of Reed & Muench (1938).

Serum susceptibility

95% (19/20) were resistant to complement-mediated lysis, the only exception being one *A. hydrophila* strain (AH-121) whose numbers decreased fivefold during the incubation period. For non-O:11 strains, 45% (9/20) were found to be susceptible to complement-mediated lysis, with most of these serum-susceptible strains (88%, 8/9) showing a 100-fold (two logs) or greater decrease in viable bacteria over the course of the incubation with PHS.

Exposure to polyclonal antisera

To see whether immune sera raised in rabbits against either whole-cell or somatic antigen preparations could activate the lytic response, two strains, *A. veronii* biotype *sobria* ATCC 9071 and *A. hydrophila* NMRI-54, were challenged in microassays against rabbit immune sera to either antigenic preparation. As can be seen in Table 2,

Table 2. Effect of various treatments on the susceptibility of O:11 *Aeromonas* to lysis

Challenge strain	Pretreatment*	Serum source	Susceptibility (Δ c.f.u.)†
<i>A. veronii</i> ATCC 9071	None	PHS	+0.7
	None	RIS(O)	+1.0
	None	RIS(WC)	+0.1
	RIS(O)	PHS	+0.6
	RIS(WC)	PHS	+0.5
<i>A. hydrophila</i> NMRI-54	None	PHS	+0.3
	None	RIS(O)	+0.3
	None	RIS(WC)	+0.8
	RIS(O)	PHS	+0.6
	RIS(WC)	PHS	+0.2

* Preincubation of indicated strain with a 1:32 dilution of the indicated antiserum (complement-inactivated) prior to PHS challenge. RIS, rabbit immune serum against somatic (O) or whole-cell (WC) antigens of the homologous strain.

† Change in viable progeny ($\log_{10}$ c.f.u.) at 120 min of incubation when compared to $t = 0$.

neither strain was inactivated in the presence of whole-cell or somatic antiserum; the same held true when each strain was preincubated with a 1:32 dilution of antiserum prior to challenge with normal 65% PHS.

Effect of PMBN

PMBN is a nine-amino-acid cationic peptide that is a derivative of polymyxin B (Viljanen *et al.*, 1986). It has previously been shown to enhance the bactericidal activity

Fig. 2. Kinetics of growth of *A. hydrophila* AH-78 in the presence of normal (▲) and complement-inactivated (△) 65% PHS. For comparison, growth of the Ra mutant *S. typhimurium* TV 119 in normal (●) and complement-inactivated (○) serum (56 °C, 30 min) is also shown.

of complement in several Gram-negative species including *Salmonella* and *Escherichia* (Viljanen *et al.*, 1986). We therefore challenged several serum-resistant, O:11, S-layer-positive *Aeromonas* strains with PHS in the presence of PMBN (Table 3). Of the four O:11 strains challenged, only one (strain AS-28) showed a quantitative decrease in c.f.u. when incubated in the presence of 30 µg PMBN and 65% PHS; this decrease amounted to a 63-fold reduction in c.f.u. When strain AS-28 was incubated in the presence of PMBN and 65% PHS (complement-inactivated), lytic activity was destroyed, indicating that lysis was the result of complement and not the PMBN itself.

Table 3. Effect of PMBN on the susceptibility of *Aeromonas* to pooled human serum

Organism	Serogroup	PHS*	PHS(CI)†	PMBN‡	Δ C.f.u.§
<i>A. hydrophila</i> NMRI-54	O:11	+	—	—	+1.3
		+	—	+	+0.9
<i>A. hydrophila</i> LL1	O:11	+	—	—	+0.6
		+	—	+	+0.9
<i>A. veronii</i> AS-76	O:11	+	—	—	+0.5
		+	—	+	+0.1
<i>A. veronii</i> AS-28	O:11	+	—	—	+1.1
		+	—	+	-0.7
		—	+	+	+0.4
<i>A. veronii</i> AS-228	O:23	+	—	—	+1.3
		+	—	+	-0.2
		—	+	+	+0.9

* Exposure to 65% pooled human serum.

† Exposure to 65% pooled human serum (complement-inactivated).

‡ Addition of polymyxin B nonapeptide (30 µg ml⁻¹).

§ Change in viable progeny ($\log_{10}$ c.f.u.) at 120 min.

Table 4. Effect of multiple cycles of PHS on the susceptibility of *Aeromonas* to complement-mediated lysis

Organism	Serogroup	Challenge inoculum	Percentage survival	
			Cycle 1	Cycle 2
<i>A. hydrophila</i> AH-78	O:22	1.2×10^7	1	0
<i>A. veronii</i> AS-178	O:36	1.7×10^7	22	100
<i>A. hydrophila</i> AH-121	O:11	2.1×10^6	48	31
Clone 1*		8.4×10^6	2	NT
Clone 3*		3.7×10^6	1	NT
Clone 4*		5.3×10^6	6	NT
Clone 6*		9.8×10^7	100	NT

NT, Not tested.

* Clone 1 was derived from the parental strain AH-121 and had never been exposed to serum; clones 3 and 4 were recovered as viable progeny after exposure of AH-121 to PHS for 120 min; clone 6 was recovered after 240 min exposure (two cycles) to PHS.

Clonal susceptibility

We next investigated the one strain of serogroup O:11 that had previously been determined to be susceptible to complement-mediated lysis. Strain AH-121 was exposed to multiple cycles of fresh 65% PHS and the percentage viable progeny (compared to $t = 0$) were measured and compared to a serum-susceptible strain (AH-78) and a serum-resistant isolate (*A. veronii* AS-178). As can be seen in Table 4, no viable progeny could be detected for strain AH-78 after two cycles of 65% PHS. In contrast, for strain AS-178 a significant decrease (78%) was noted after one cycle; however, the remaining viable bacteria when subjected to another round of exposure to PHS rapidly multiplied to yield c.f.u. slightly exceeding the initial inoculum. For strain AH-121, successive cycles of ex-

posure to 65% PHS resulted in continued decreases in c.f.u., although the results of a second cycle of exposure to PHS were not as dramatic as those obtained from the first challenge. Clones of strain AH-121 recovered from successive cycles of PHS exposure showed major differences in susceptibility to complement-mediated lysis. Clone 121-6 recovered from a second cycle exposure of parental strain AH-121 to PHS (at 240 min) was completely refractory to PHS upon rechallenge with PHS. This was dramatically different from results obtained with clones 121-3 and 121-4, which were recovered from single-cycle exposure to PHS.

Correlation of virulence properties with serum susceptibility

Finally we studied six strains (three serum-resistant, three serum-susceptible) in regard to their activation of complement, LPS chemotype, and pathogenicity in mice (Table 5). All six strains were able to activate complement (94–100%) based upon complement depletion assays regardless of their susceptibility to complement-mediated lysis; five of these six strains also bound C3b to their cell surface. The only exception was *A. hydrophila* LL1, an isolate originally recovered from a moribund fish, that has previously been demonstrated by immunoblotting to contain an S layer antigenically distinct from those of most other S-layer-positive *A. hydrophila* strains (Kokka *et al.*, 1992). With regard to LPS chemotypes and mouse pathogenicity, differences were found that appear to be directly related to serum susceptibility status. All three serum-resistant strains were found to be moderately pathogenic for mice and to have a defined LPS pattern of either the A or B chemotype. In contrast, all three serum-susceptible strains were of low pathogenicity and did not exhibit a detectable O polysaccharide side chain profile upon silver staining (type C pattern).

DISCUSSION

The bacteriolytic activity of human serum is one of several natural host defence mechanisms that are active against Gram-negative bacterial pathogens. This activity

Table 5. Correlation of serum susceptibility with various structural and pathogenic properties

Strain	Serum susceptibility	Complement activation (%)	C3b binding	LPS type*	LD-50†
LL1 (O:11)	+0.9	100	—	A	7.2
AS-28 (O:11)	+1.1	97	+	A	7.2
NMRI-7 (O:34)	+1.0	100	+	B	7.6
AH-78 (O:22)	—5.0	100	+	C	8.3
ATCC 14715 (O:22)	—4.5	94	+	C	8.5
AS-53 (R)	—5.0	100	+	C	8.4

* LPS types: type A, homogeneous side chain profile; type B, heterogeneous side chain profile; type C, side-chain deficient.

† $\log_{10}$ 50% lethal dose (Swiss Webster mice).

is largely dependent upon complement-mediated lysis or killing of intact bacteria, and individuals that are deficient in one or more complement components, most notably C3 and C5, are at increased risk of developing serious life-threatening infections caused by *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Haemophilus influenzae* (Lachmann, 1990; Figueroa & Densen, 1991). In individuals with alcoholic or non-alcoholic cirrhosis, diminished bactericidal and opsonic activity has been noted (Akalin *et al.*, 1985). Underlying liver disease, particularly Laennec's cirrhosis, is a well-recognized predisposing condition leading to invasive disease caused by the genus *Aeromonas* (Janda & Duffey, 1988).

Earlier studies have established that both *A. hydrophila* and *A. veronii* biotype *sobria* (formerly referred to as *A. sobria*) are inherently more resistant to complement-mediated lysis by fresh serum than is *A. caviae* (Janda *et al.*, 1984; Brenden & Janda, 1986; Massad *et al.*, 1991). This difference in susceptibility appears to correlate *in vivo* with the greater propensity of the former two species to cause serious invasive infections including septicemia (Janda *et al.*, 1994). Recently we identified a group of highly invasive strains of *A. hydrophila* and *A. veronii* that are characterized by a number of unique phenotypic and structural properties including possession of an S layer (Kokka *et al.*, 1991a). Previous studies by Munn *et al.* (1982) on S-layer-positive *A. salmonicida* indicated that resistance to the bactericidal activity of fresh serum was partly attributable to this layer. We therefore set out to compare the susceptibility properties of a group of these O:11 strains to those of a control group which were S-layer-negative and belonged to serogroups other than O:11.

The results in Fig. 1 indicate that there is a significant difference in these two groups in regard to their ability to resist the normal lytic action of complement. Overall, O:11 S-layer-positive strains were significantly more resistant to PHS than non-O:11 strains. Within the non-O:11 group, however, two distinct populations could be discerned. The larger of these two groups was also refractory to complement-mediated lysis despite the fact that the strains were S-layer-negative; selected strains analysed by SDS-PAGE and stained for O polysaccharide side chains by silver staining were shown to have an intact LPS side chain profile. In contrast, the slightly smaller group was extremely susceptible to complement-mediated lysis, showing kinetics of inactivation similar to rough mutants of the Ra or Rc chemotypes of *E. coli* or *S. typhimurium* (Hitchcock *et al.*, 1986). These strains had a somatic antigen definable by serogrouping. Upon LPS analysis, no side chain activity was detectable. This result may indicate that the total amount of polysaccharide side chains present is either diminished or of a truncated form, which would explain both results. Regardless of which alternative is correct, these strains were less pathogenic in mice and acted like attenuated or non-virulent isolates (Janda & Kokka, 1991).

For O:11 strains, all isolates tested (with one exception) were serum-resistant under the defined conditions. The

serum-susceptible strain was found, upon agar cloning, to be comprised of both serum-susceptible and serum-resistant progeny, with the former predominating in unselected populations. When continuously challenged with successive cycles of PHS, the serum-resistant clones predominated suggesting that a similar situation would occur *in vivo*. This contrasts sharply with non-O:11 serum-susceptible isolates, where complete elimination of infectious progeny was apparent within two cycles of PHS incubation. For O:11 strains, susceptibility to complement-mediated lysis could not be enhanced with somatic or whole-cell antibodies nor (with one exception) in the presence of PMBN, a peptide known to enhance the lytic activity of complement for many Gram-negative bacteria at a concentration of $30 \mu\text{g ml}^{-1}$ (Viljanen *et al.*, 1986). The inability of these strains to be lysed by complement was not due to failure to activate complement or bind C3b to the cell surface. Rather, it appears that the failure to be lysed by fresh pooled serum is due to a defect in the activation of the terminal components of the complement pathway.

The results suggest that S-layer-containing aeromonads are inherently resistant to complement-mediated lysis. This effect is probably not directly due to the presence of the S layer for the following reasons: (1) many non-O:11 strains that are S-layer-negative are serum-resistant; (2) serum-susceptibility of strains generally correlated with the absence of detectable LPS side chains as determined by silver staining of SDS-polyacrylamide gels of proteinase-K-treated whole-cell extracts; and (3) a previously analysed spontaneous mutant of an O:11 strain that was autoagglutination- and S-layer-negative was still resistant to the lytic activity of fresh serum, presumably by maintenance of an intact homogeneous O polysaccharide side chain profile (Kokka *et al.*, 1991b). The fact that strain AS-28 was lysed by PHS in the presence of PMBN and that strain LL1 failed to bind C3b to the cell surface implies some heterogeneity in regard to physiological and biological functions. Support for this concept stems from recent molecular investigations which have demonstrated extensive amino acid sequence variation in the N-termini of a large number of SAPs (Kostrzynska *et al.*, 1992). Previous studies with the S layer of *A. salmonicida* (formerly referred to as the A layer) indicate that the paracrystalline surface layer of this psychrophilic species appears to have many more functions associated with virulence than do the SAPs of *A. hydrophila* and *A. veronii* biotype *sobria*. Some of these proposed functions for the S layer of *A. salmonicida* include a role in iron transport, binding of IgG and IgM from serum, providing entry into phagocytic cells, a protective barrier against the harmful effects of proteases and complement, and the binding of high molecular mass basement membrane molecules such as laminin, fibronectin and collagen (Kay & Trust, 1991). Although the S layer of *A. hydrophila* and *A. veronii* does not at present appear to have such a major role in pathogenesis as demonstrated for *A. salmonicida* (e.g. anti-lytic properties) it may well be that the S layer possesses anti-phagocytic activity which may aid in its systemic dissemination once invasion through the gas-

trointestinal mucosa has occurred. A recent study from our laboratory, demonstrating enhanced virulence of S-layer-negative strains in mice upon intraperitoneal injection when mixed with purified S layer, suggests that this is a distinct possibility (Kokka *et al.*, 1992). Further studies on the opsonic and phagocytic activity of professional and non-professional phagocytes with O:11 strains await the construction of isogenic mutants lacking a paracrystalline surface array.

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Received 14 February 1994; revised 12 April 1994; accepted 26 May 1994.