Immunochemical analysis and possible biological role of an *Aeromonas hydrophila* surface array protein in septicaemia

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The biochemical, immunological, and biological properties of an S layer purified from an *Aeromonas hydrophila* strain (AH-342) involved in a case of bacteraemia were investigated. The S layer selectively removed from the cell surface was composed of a single acidic (pI 4.65) protein subunit (surface array protein, SAP) with a molecular mass of approximately 52 kDa. Amino acid analysis of this 52 kDa protein indicated a molecule composed of 498 amino acids with 46% hydrophobic residues. No cysteine residues were detected. The first 35 residues of the N-terminus were sequenced by Edman degradation; only 4–24% homology was noted between this sequence and those previously published for SAPs of *Aeromonas salmonicida* (A450) and a strain of *A. hydrophila* (TF7) originally isolated from a moribund fish. Polyclonal antibodies raised against AH-342 SAP were genospecific, reacting only against S layers produced by *A. hydrophila* strains and not those from *Aeromonas veronii*. Acute serum from the bacteraemic patient from whom AH-342 was isolated reacted strongly with the SAP of AH-342 in immunoblot studies. Purified SAP, when intraperitoneally co-inoculated with SAP⁻ strains of *A. hydrophila* into Swiss-Webster mice, could reduce the 50% lethal dose by approximately 30–70 fold. The results suggest that the SAP of *A. hydrophila* strains may play an important role in systemic dissemination after invasion through the gastrointestinal mucosa.

Introduction

We have recently identified a major subgroup of *Aeromonas hydrophila* and *Aeromonas veronii* strains that are preferentially involved in a wide variety of human extraintestinal illnesses, such as septicaemia, peritonitis, and fulminant wound infections (Kokka *et al.*, 1991a). Less frequently, this subgroup has also been implicated in gastrointestinal infections clinically resembling Crohn's disease, enterocolitis, and typhoid fever (Kokka & Janda, 1990). Two useful phenotypic markers which aid in the recognition of this subgroup are the ability to autoagglutinate in broth and the presence of a common somatic antigen of serogroup O:11 (Kokka & Janda, 1990). Seroepidemiologic surveys using these two markers indicate the O:11 group to be one of the two (of over 80 known) most common serogroups associated with human disease, accounting for 20–30% of all *A.*

hydrophila and *A. veronii* (formerly *A. sobria*) infections. Subsequent investigations have also noted that the frequency of isolation of *Aeromonas* O:11 from clinical, as opposed to environmental sources, is at least four-fold higher, suggesting that this serogroup is more likely to cause significant human disease (Paula *et al.*, 1988).

Additional features shared by all O:11 *A. hydrophila* and *A. veronii* strains are the possession of an S layer, and enhanced pathogenicity in mice as determined by 50% lethal dose assays (Paula *et al.*, 1988; Kokka *et al.*, 1991a). The S layer, which is located on the outermost surface of the bacterium, is composed of a single acidic protein molecule (termed the surface array protein, SAP) with a molecular mass of 52–55 kDa (Kokka *et al.*, 1990). Possession of an S layer appears to be unambiguously linked to this single serogroup and to a characteristic molecular architecture of LPS O-polysaccharide side-chains of this somatic group. Pathogenicity studies conducted on a representative number of O:11 strains indicate this subgroup to be more virulent in outbred mice than counterpart non-O:11 strains, further supporting the clinical relevance of this subgroup.

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The amino acid sequence data reported in this paper have been submitted to PIR and have been assigned the accession number A33184.

Table 1. *Bacterial strains used in the present investigation*

Strain	Species	HG†	Source	Comment‡	Reference§
AH-342	<i>hydrophila</i>	1	blood, human	O:11, SAP+	This study
NMRI-7	<i>hydrophila</i>	1	wound, human	O:34, SAP-	<i>a</i>
ATCC 15468	<i>caviae</i>	4	guinea pig	O:11, (atypical), SAP-	<i>b</i>
NMRI-54	<i>hydrophila</i>	1	river	O:11, SAP+	<i>a</i>
AH-121	<i>hydrophila</i>	1	wound, human	O:11, SAP+	<i>a</i>
1113-76	<i>hydrophila</i>	ND	faeces, human	O:11, SAP+	<i>b</i>
A179	<i>veronii</i>	UG	animal	O:11 (atypical), SAP-	<i>b</i>
LL1	<i>hydrophila</i>	1	trout	O:11, SAP+	<i>a</i>
A310	<i>hydrophila</i>	2	well water	O:11 (atypical), SAP-	<i>b</i>
AS-180	<i>veronii</i> *	8	blood, human	O:11, SAP+	<i>a</i>
ATCC 9071	<i>veronii</i> *	8	frog	O:11, SAP+	<i>a</i>
AS-28	<i>veronii</i> *	8	faeces, human	O:11 SAP+	<i>a</i>

* These strains have been reclassified as *A. veronii* biotype *sobria* according to current taxonomic designations (see Kokka *et al.*, 1991*a*).

† HG, hybridization group; UG, unknown group.

‡ SAP, surface array protein.

§ References: *a*, Kokka *et al.* (1991*a*); *b*, Kokka *et al.* (1991*b*).

ND, not determined.

An attractive hypothesis is that the S layer is intimately involved in overt pathogenicity. However, published data from our laboratory to date do not support this concept, based upon the virulence of spontaneous mutants or atypical O:11 strains that are S-layer-negative yet still virulent for mice (Kokka *et al.*, 1991*b*). Furthermore, enzymic or physiological manipulation of the relative amount of SAP produced by typical O:11 strains does not apparently affect virulence, suggesting that the S layer is not directly involved in lethality in mice (Kokka *et al.*, 1991*b*). Nevertheless, the S layer could be involved in earlier steps in pathogenicity, or *in vivo* regulation of S layer synthesis could affect the infectious process. Finally, the virulence potential of S-layer strains could in part be determined by potential antigenic or structural differences in *Aeromonas* SAPs, since we have previously shown two different species to produce structurally similar molecules. To address some of these issues, we have extensively characterized the SAP from a bacteraemic strain of *A. hydrophila* and shown that: (1) this is the immunodominant protein detected during the patient's septic episode; (2) SAPs from genetically related strains (e.g., *A. hydrophila*) show significant immunologic homology; and (3) the SAP appears to enhance the invasive potential of genetically identical strains by overcoming the normal phagocytic barriers.

Methods

Bacterial strains. *Aeromonas* strains used in the present study, with their relevant phenotypic and genotypic characteristics, are listed in

Table 1. All isolates were maintained on extract agar slants [18 g extract broth (Acumedia), 2 g beef extract, 15 g agar per litre].

Protein analysis and purification. Whole-cell (WC) protein extracts of *Aeromonas* were prepared as previously described (Kokka *et al.*, 1990). S-layer proteins were removed from cells by 0.2 M-glycine hydrochloride (pH 4.0) extraction. Briefly, the growth obtained from 60 heart-infusion agar plates was harvested in cold 20 mM-Tris (pH 8) and centrifuged at 7000 *g* for 30 min at 4 °C. The resulting pellet was resuspended in 240 ml 0.2 M-glycine/HCl. This suspension was gently mixed every 10 min for 30 min. Glycine-treated cells were then removed by centrifugation (7000 *g*, 30 min, 4 °C), after which the surface array protein (SAP) material contained in the supernatant fraction was recovered by centrifugation at 30000 *g* for 30 min. The resulting SAP pellets were resuspended in sterile distilled water, the protein concentration of the resuspended SAP was determined and the SAP material was subjected to isoelectric focusing (IEF). Some reconstituted pellets were dialysed against distilled water overnight and lyophilized for subsequent amino acid analysis and sequencing.

Gel electrophoresis and staining. WC (1–30 µg) and glycine-extracted SAPs (0.005–20 µg) were analysed by SDS-PAGE using 10% (w/v) separating gels and 6% (w/v) stacking gels; gels were stained with 0.1% Coomassie brilliant blue R-250 (Bio-Rad Laboratories).

Performic acid oxidation and amino acid composition and sequencing analysis. SAP was prepared for amino acid analysis and sequencing as described by Ho *et al.* (1990). Briefly, 1 ml hydrogen peroxide was added to 9 ml 88% (v/v) formic acid, and the mixture was incubated for 1 h at room temperature before cooling on ice. A 200 µl volume of this chilled preparation was added to a lyophilized sample (30 µg) of S layer protein. After 4 h at 4 °C, the reaction was terminated by addition of 2 ml water to the mixture; the sample was then dried prior to amino acid composition and sequencing analysis.

The amino acid composition was determined after hydrolysing the proteins in 6 M-HCl *in vacuo* at 110 °C for 24, 48, and 72 h, using Beckman 6300 and Durrum D-500 analysers. Methionine and cysteine residues were identified after performic acid oxidation of the proteins, but tryptophan residues are destroyed by this method and were not determined. Amino acid sequencing was performed by Edman

degradation using a Beckman 890M and an Applied Biosystems 470A Sequencer in the presence of polybrene.

Polyclonal SAP antibodies. New Zealand white rabbits were injected intradermally at 10 different sites on the back of the rabbits with 0.1 ml of an equivolume mixture containing SAP (1.0 mg) from AH-342 and Freund's complete adjuvant (Gibco). After four weeks, rabbits were boosted subcutaneously with 1 mg SAP in incomplete Freund's adjuvant at 0.2 ml per site. After three weeks, a trial bleed was performed and the rabbit antisera were initially screened by slide agglutination assays with the homologous isolate to determine if there was a sufficient antibody response. The rabbits were killed by cardiac puncture and the resultant serum was stored at -70°C .

An initial titration of the anti-SAP antibody was performed by immunoblot assay using dilutions of WC suspensions of the homologous isolate and an S-layer-negative spontaneous mutant (AS-180-1) against increasing dilutions of the unabsorbed polyclonal antiserum. Dilutions of the antigen suspensions (10 μl) were placed on nitrocellulose membranes, air-dried, covered with blocking buffer, and kept at room temperature for 1–2 h. The sheets were washed in PBS and dried. Dilutions (10 μl) of the unabsorbed anti-342 SAP antibody in blocking buffer were added to the nitrocellulose–antigen sheets and kept for 1 h at ambient temperature. The blots were subsequently washed with PBS, and dried. A second antibody (anti-IgG-Protein A-gold; E-Y Laboratories) was applied (10 μl) and the blots were incubated for 10–30 min. The blots were then washed with PBS and read macroscopically.

The rabbit anti-342 SAP antisera were absorbed with whole-cell suspensions of homologous and heterologous strains of *Aeromonas* in order to increase the specificity of the antisera for the surface array protein. A total of six successive absorptions were performed using homologous and heterologous *Aeromonas* strains. The absorbed antiserum was centrifuged at 10000 *g* for 15 min and then filtered twice using a 0.45 μm and 0.2 μm Acrodisc filter (Gelman Sciences). Aliquots were stored at -70°C .

Immunoblotting. Electrotransfers on to nitrocellulose of SDS-PAGE gels containing WC and glycine-extracted S layers were performed using a Hoefer TE-50 power lid with the TE 42 transfer unit (Hoefer Scientific) and the Towbin buffer system (Kokka *et al.*, 1991b). Immunoblotting was performed using the respective antisera (rabbit anti-SAP or human patient antisera) and Bio-Rad Immun-Blot Assay Kits as per the manufacturer's directions.

Decreasing amounts of glycine-extracted AH-342 SAP (5.0–0.005 μg) were run on SDS-PAGE gels, electrotransferred to nitrocellulose membranes and immunostained with varying dilutions of the polyclonal rabbit anti-342 SAP antibody. A 1:1000 antibody dilution was considered the optimal use dilution for subsequent immunoblotting experiments. Titrations of patient antisera were performed in a similar manner.

To assess the specificity of the polyclonal anti-SAP antibody, WC protein extracts (0.5–1.0 μg) of the homologous (AH-342) and heterologous (five O:11, SAP⁺, *A. hydrophila*, two O:11, SAP[−] *A. hydrophila*, and three O:11, SAP⁺ *A. veronii*) strains were electrophoresed on 10% SDS-PAGE gels, electrotransferred onto nitrocellulose, and immunostained with the polyclonal anti-342 SAP antibody. Immunoblots of WC protein extracts (1.0 μg) of isolate AH-342 were immunostained with a 1:25 dilution of an acute serum specimen obtained during the bacteraemic phase of the illness.

Biological effects. To assess the potential role of the S layer of AH-342 in pathogenicity, purified SAP from this strain was injected alone (intravenously) or in combination (intraperitoneally) with the homologous (*A. hydrophila* AH-342) or heterologous (*A. hydrophila* NMRI-7; *A. caviae* ATCC 15468) strains into female Swiss-Webster mice as previously outlined. Mortality was evaluated over a seven-day period

Fig. 1. SDS-PAGE (10%, w/v) of Coomassie-blue-stained WC (lane 1, containing 30 μg) and glycine-extracted (lane 2, containing 5 μg) proteins from strain AH 342. Arrow indicates position of the SAP.

and compared to control animals (Paula *et al.*, 1988; Kokka *et al.*, 1991).

Results

Case history and strain identification

The *Aeromonas* strain was isolated from a patient with signs of bacterial sepsis at The Mount Sinai Hospital, NY. The strain was biochemically identified in our laboratory as belonging to the *A. hydrophila* phenospecies, although it possessed several unusual markers for this group, including a negative lysine decarboxylase reaction and failure to produce acid from arabinose or salicin. By both multilocus enzyme electrophoresis and DNA–DNA hybridization (Kokka *et al.*, 1991a), this strain resided in hybridization group (HG) 1 and, therefore, belongs to *A. hydrophila* (*sensu stricto*). The strain was autoagglutination-positive and belonged to serogroup O:11. Transmission electron microscopic studies of thin sections of the bacterium indicated that it possessed an S layer.

Protein analysis and SAP purification of AH-342

Two major proteins of strain AH-342 with molecular masses of approximately 52 and 50 kDa were present in both whole-cell and outer membrane (sarkosyl-treated) fractions, respectively (Fig. 1, lane 1). The 52 kDa SAP

Table 2. *Amino acid composition of tetragonal S layer proteins*

Data for TF7 and A450 were derived from Dooley *et al.* (1988*a*). Hydrophobic residues were calculated from the following amino acids: V, M, I, L, A, F, W, P.

Amino Acid	Molar percentage in:		
	<i>A. hydrophila</i> AH-342	<i>A. hydrophila</i> TF7	<i>A. hydrophila</i> A450
Asx	12.3	15.4	14.9
Thr	7.6	7.1	8.8
Ser	6.0	6.8	3.9
Glx	6.2	8.9	9.5
Pro	2.4	2.4	2.3
Gly	9.7	8.3	4.7
Ala	16.5	12.7	8.4
Val	9.5	7.5	8.4
Met	1.2	1.5	1.0
Ile	3.2	2.8	5.1
Leu	9.2	8.8	10.1
Tyr	2.6	3.2	1.9
Phe	4.0	4.4	7.3
His	0.4	0.6	1.1
Lys	7.0	8.5	6.1
Arg	2.2	2.1	4.3
Cys	0.0	0.0	0.0
Trp	ND	0.7	1.8
Total no. of residues per mol	498	520	473
Apparent molecular mass (Da)	52000	52000	49200
Hydrophobic residues (%)	46	41	45

ND, not determined

was selectively removed from the cell surface by extraction with glycine/HCl buffer (Fig. 1, lane 2). Isoelectric focusing of the SAP indicated a pI of 4.65, which is similar to values previously reported for SAPs from *A. hydrophila* TF7 (4.60) and *A. veronii* biotype *sobria* ATCC 9071 (4.70). A 500 µg quantity of this protein was then prepared for further analysis.

Amino acid analysis

The SAP of strain AH-342 was composed of approximately 498 residues (Table 2) with 61.7% aliphatic, 6.6% aromatic, 1.2% sulphur-containing, 2.4% imino, 18.5% dicarboxylic, and 9.6% basic amino acids. This composition was similar to those of *A. hydrophila* TF7 and *A. salmonicida* A450, the major differences observed being the higher content of alanine and glycine residues in the mesophilic species, while the psychrophilic strain (A450) contained larger amounts of arginine and phenylalanine. No cysteine residues were identified in this analysis, in accordance with previous findings for TF7 and A450 (Dooley *et al.*, 1988*a*); the content of hydrophobic residues (41–46%) was similar for all three SAPs.

A450	1					5						10					15				20
	D	V	V	I	G	P	N	D	N	T	F	V	T	N	S	L	A	S	V	T	
TF7	V	N	L	D	T	G	A	G	V	S	F	K	A	S	G	I	K	V	D	G	
AH342	V	D	L	S	D	A	A	S	V	P	A	S	Y	A	S	Z	I	T	L	P	

Fig. 2. N-terminal sequence of SAP from *A. hydrophila* AH-342. Data from Dooley *et al.* (1988*a*) are shown for comparison; boxes indicate positions of identity. Underlined amino acids have not been positively identified.

Amino-terminal sequence

The N-terminal sequence of the first 35 amino acids of AH-342 SAP was determined by Edman degradation and compared to published sequences for the SAP of TF7 and A450 (Dooley *et al.*, 1988*a*). Over the first 25 amino acids, six positions showed identity between AH-342 and TF7 (24% homology, Fig. 2); in all six instances, neutral amino acids (valine, alanine, leucine, and threonine) were involved. Negligible similarity was

Fig. 3. SDS-PAGE (10%, w/v) of WC proteins from strain AH 342. AH 342 (30 µg) Coomassie blue-stained (lane 1) and AH 342 (5 µg) immunoblotted with anti-342 SAP antibody diluted. 1:1000 (lane 2).

observed between AH-342 and A450 where only a single position (serine at position 15) was identical, with an overall homology of 4%.

Polyclonal antibody

Polyclonal antisera raised against the S layer of *A. hydrophila* 342 reacted with the 52 kDa SAP of this strain when whole cell protein extracts were immunoblotted with these antibodies (Fig. 3, lane 2). Also observed by this staining procedure was a high-molecular-mass LPS band (Fig. 3, lane 2) which was not apparent upon Coomassie blue staining of whole cell protein extracts of AH-342 (Fig. 3, lane 1). This LPS reactivity was due to polysaccharide contamination of the original glycine-extracted preparations of the SAP of AH-342.

To determine the specificity of these antibodies, the whole cell protein extracts from a variety of typical and atypical serogroup O:11 strains of differing genotypes and phenotypes were evaluated by Western blotting. As can be seen in Fig. 4, the SAPs from three S layer-positive *A. hydrophila* strains reacted with SAP antisera (arrow, lanes 1, 2, and 4) with an intensity similar to that of the homologous isolate (lane 3). Only one *A. hydrophila* strain (LL1) had a SAP that was unreactive with these antibodies. Two O:11 *A. hydrophila* strains that were S-layer-negative also failed to demonstrate immuno-reactive bands in the 50–55 kDa region (lanes 6 and 7);

Fig. 4. SDS-PAGE (10%, w/v) of WC proteins (1.0 µg) from serogroup O:11, SAP positive (lanes 1–5 and 8–10) and SAP negative (lanes 6 and 7) *Aeromonas* strains immunoblotted with anti-342 SAP antibody. NMRI-54 (lane 1), 121 (lane 2), 342 (lane 3), 1113-76 (lane 4), LL1 (lane 5), A179 (lane 6), A310 (lane 7), 180 (lane 8), 9071 (lane 9), and 28 (lane 10).

Table 3. *Biological effects of SAP, alone or in combination*

Strain	Dose (c.f.u.)*	Treatment	SAP (μ g)†	Route‡	Mortality	(%)
AH-342	—	—	100	IV	0/5	(0)
NMRI-7	1×10^8	—	—	IP	5/5	(100)
NMRI-7	2×10^7	—	—	IP	0/5	(0)
NMRI-7	2×10^6	—	—	IP	0/5	(0)
NMRI-7	2×10^6	—	50	IP	13/15	(87)
NMRI-7	2×10^6	—	5	IP	2/5	(40)
NMRI-7	2×10^6	—	0.5	IP	0/5	(0)
15468 ^T	2×10^7	—	50	IP	0/5	(0)
AH-342	1×10^8	Anti-SAP	—	IP	20/20	(100)

* The 50% lethal doses (IP) in Swiss-Webster mice for each strain are as follows: *A. hydrophila* AH-342 (1×10^7), *A. hydrophila* NMRI-7 (4×10^7), and *A. caviae* ATCC 15468^T (5×10^8).

† Purified SAP (surface array protein) from *A. hydrophila* AH-342 as described in Methods.

‡ IV, intravenous; IP, intraperitoneal.

all of these strains exhibited high-molecular-mass LPS bands similar to that observed with AH-342 in Fig. 3. For S-layer-positive *A. veronii* biotype *sobria* isolates, no bands were detected whatsoever (lanes 8–10). This finding was repeatedly demonstrated by performing immunoblots of these strains (whole cell) against AH-342 anti-SAP antibodies.

In vivo response to SAP AH-342

Limited serum was available from the patient infected with AH-342 during the course of his bacteraemic episode. When this acute serum was immunoblotted at a 1:25 dilution against the whole cell protein extract of AH-342, immunoreactive epitopes on the 52 kDa SAP were detected (Fig. 5, lane 2). This reactivity was lost when the acute serum was diluted in excess of 1:100. This acute serum also reacted with the 50 kDa OMP-associated protein of AH-342 as well as several other minor species of molecular mass of 45–75 kDa.

Biological effects of SAP

Although a previous study (Kokka *et al.*, 1991*b*) by our laboratory had indicated that the SAP did not appear to be directly responsible for mouse lethality, we investigated the biological effect of purified SAP from AH-342 either alone or when injected in combination with heterologous SAP[−] *Aeromonas* strains (Table 3). When purified SAP was inoculated intravenously into Swiss-Webster mice at concentrations up to 100 μ g per mouse, no morbidity or mortality was observed for 7 d post-inoculation. Because isogenic mutants (SAP[−]) of *A. hydrophila* and *A. veronii* biotype *sobria* are presently unavailable, we evaluated whether purified SAP could enhance the infectivity of selected SAP[−] strains upon

Fig. 5. SDS-PAGE (10%, w/v) of WC proteins from strain AH 342. AH 342 (30 μ g) Coomassie blue-stained (lane 1) and AH 342 (1.0 μ g) immunoblotted with patient acute serum diluted 1:25 (lane 2). Molecular masses of protein standards are shown.

intraperitoneal injection. When 50 μ g SAP was mixed with 2×10^6 c.f.u. of *A. hydrophila* NMRI-7 and co-inoculated into outbred mice, significant mortality was observed (87%) despite the fact that the bacterial concentration was greater than one log₁₀ (approx. 30- to

70-fold) below the LD₅₀ value for the challenge strain. This effect was directly attributable to the SAP as increasing dilutions (10- to 100-fold) of this protein prior to co-infection with NMRI-7 resulted in further decreases in observed mortality (60%–100%); control mice receiving only NMRI-7 also did not succumb to infection. To determine whether this enhancement in the relative infectivity of mesophilic aeromonads was a general phenomenon or strain-specific, we repeated the same assay using *A. caviae* ATCC 15468^T, an isolate that shares somatic epitopes with S-layer-positive *A. hydrophila* strains (atypical O:11). Results of this assay indicate that when ATCC 15468 was injected at a concentration approx. 30-fold below its mean LD₅₀, along with 50 µg SAP, no mortality was observed. Similar results were also found when AH-342 was pre-incubated with a 1:10 to 1:100 dilution of polyclonal anti-SAP (AH-342) prior to mouse inoculation at 10 times the mean LD₅₀.

Discussion

Recent areas of intensified scientific interest concerning the genus *Aeromonas* have centred on the identification and characterization of reputed virulence factors produced by aeromonads that may be operative in disease pathogenesis in warm- and cold-blooded animals (Janda, 1991). Factors currently thought to potentially play such roles in intestinal infections include a cytolytic enterotoxin (beta-haemolysin), a cytotoxic enterotoxin, and a mini-pilin; studies on such factors are hampered by the present lack of an appropriate animal model in which to reproduce faithfully the diarrhoeal syndrome (Janda, 1991).

Another molecule potentially involved in virulence is the SAP, which self-assembles at the cell surface to form an S layer in certain strains belonging to the genospecies *A. salmonicida*, *A. hydrophila*, and *A. veronii*. Although S layers are found in both saprophytic and pathogenic bacteria, an increasing number of species implicated in human disease, including *Campylobacter fetus*, *Mycobacterium bovis*, *Clostridium difficile*, and *Wolinella recta* (Sleytr & Messner, 1988) have been shown to produce such layers. In most instances, the exact function of such layers is unknown; however, in the case of one organism (*A. salmonicida*), possession of an S layer has been unequivocally linked to disease-producing capabilities in salmonids (Trust, 1986; Janda, 1991).

In mesophilic species, S-layer-positive *Aeromonas* strains have been previously linked to invasive disease (notably bacteraemia) and, less frequently, associated with fulminant wound infections and cases of colitis. Studies comparing the frequency of these strains recovered from humans, animals, and environmental

sources indicate that these strains have a particular propensity for causing human illness and may have a selective advantage in causing significant morbidity and mortality in humans (Janda *et al.*, 1987; Kokka & Janda, 1990). On the biochemical level, S-layer-positive strains share additional features, including a homogeneous O-polysaccharide side-chain profile and the ability to autoagglutinate in liquid media (Dooley & Trust, 1988b; Kokka *et al.*, 1990).

Biochemical studies performed on the first SAP purified from a human infection indicate that although *Aeromonas* SAPs share a number of unifying features in common with SAPs (Sleytr & Messner, 1983) from other pathogenic and nonpathogenic species (acidic pI, low content of sulphur-containing amino acids, similar overall amino acid content), their individual N-terminus sequences are remarkably dissimilar (3%–20% homology). Further support for this sequence divergence stems from immunoblot studies conducted on SAPs from different S-layer-containing strains where polyclonal antibodies generated against the SAP of AH-342 were found to be genospecific reacting only against HG1 strains and not HG8. Even within a genospecies, heterogeneity was observed, since one *A. hydrophila* strain, LL1, which was originally isolated from trout, failed to react in Western blot studies against the polyclonal SAP antibodies. This implies wide immunological variation in antigenic epitopes between and even among identical genospecies. Further work is presently in progress to determine the extent of this immunological variation among S-layer-bearing strains.

Previous studies from this laboratory (Kokka *et al.*, 1991b) have suggested that the S layer is not directly responsible for mouse pathogenicity (lethality) based upon the analysis of spontaneous (leaky) mutants and atypical O:11 strains that are SAP⁻. This finding is supported by the present experiments where purified SAP from AH-342, when injected intravenously into individual mice at concentrations up to 100 µg, failed to produce any deleterious effect. The concentration of SAP used far exceeds the amount needed for 50% lethal doses in mice by a variety of bacterial toxins produced by many other Gram-negative and Gram-positive pathogens (Gill, 1982) suggesting that lack of virulence was not due to too small a concentration of SAP. Despite these observations, we cannot at present rule out up-regulation of SAP synthesis *in vivo* by leaky mutants or that the small amount of SAP secreted is sufficient for virulence in mice.

While these cumulative results do not support a major role for SAP in the final events in pathogenesis, other studies do suggest that SAP may contribute to virulence. First, we detected a specific immune response *in vivo* to the SAP from AH-342 during the acute phase of the

patient's bacteraemic illness further extending our observations on the role of these strains in extraintestinal disease. The fact that the acute serum of the patient reacted most strongly against the AH-342 SAP indicates that this protein is highly immunogenic *in vivo*, and suggests an important role for this molecule in the infectious process. Since the completion of the present study, we have demonstrated a similar *in vivo* response to SAPs from other *Aeromonas* strains involved in invasive wound infections, again suggesting an important role in pathogenesis. Secondly, although SAP was not lethal by itself, it enhanced the virulence of SAP⁻ strains when mixed and co-injected with *A. hydrophila* NMRI-7; this effect could be diluted and eventually nullified by adding concentrations of SAP below 0.5 µg. We have not to date determined the actual extent of enhancement of virulence by this molecule with a variety of strains, although from the data on *A. caviae* ATCC 15468^T, the effect appears to be somewhat strain-specific. How SAP enhances virulence *in vivo* is unknown, although one attractive possibility is that SAP is antiphagocytic against peritoneal macrophages. Previous studies conducted on the S layer of *A. salmonicida* have indicated that the paracrystalline surface array may confer resistance to phagocytosis and complement-mediated lysis, and this molecule may additionally function in nature as a barrier to predation by parasitic bacteria such as *Bdellovibrio bacteriovorus* (Koval & Hynes, 1991; Janda, 1991). Furthermore, purified S-layer material has been shown to reassemble on the surface of S-layer-minus strains of *A. salmonicida* to form a single confluent layer (Griffiths & Lynch, 1990). This situation may be similar to the enhancement of virulence observed with *A. hydrophila* NMRI-7 in mice when preincubated with AH-342 SAP. Definitive evidence demonstrating the role of SAP in S-layer-positive strains will require isogenic mutants (SAP⁻) constructed using transposon mutagenesis.

The results of this investigation provide the first demonstration of a humoral response to SAP *in vivo* during the course of systemic infection and provide the first evidence toward a possible role for SAP during the course of disseminated illnesses in humans. With respect to this later observation, the results with *A. caviae* ATCC 15468^T and 50 µg of SAP indicate that 15468 lacks the gene(s) necessary to kill mice under conditions similar to those used for NMRI-7. Since virulence is polygenic in nature (Falkow, 1990) for most micro-organisms, this result is not unexpected. Current studies are in progress to determine how other *Aeromonas* genes and their products of SAP⁺ and SAP⁻ strains interact to cause the observed morbidity and mortality in mice.

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