Biochimica et Biophysica Acta 815 (1985) 233–244
Elsevier

BBA 72582

Biochemical and immunological characterization of the cell surface of the fish pathogenic bacterium *Aeromonas salmonicida*

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(Received November 2nd, 1984)
(Revised manuscript received February 7th, 1985)

Key words: Cell surface; Lipopolysaccharide; Bacterial antigen; Furunculosis; Fish disease; (*A. salmonicida*)

(1) The identification of lipopolysaccharide as periodic acid-Schiff positive material, present in the membrane fraction of the fish pathogenic Gram-negative bacterium *Aeromonas salmonicida*, analyzed by SDS-polyacrylamide gel electrophoresis, is shown. Such analysis has revealed several periodic acid-Schiff positive bands and many membrane proteins among which a pathogenicity-related M_r 54 000 protein as a constituent of an additional surface layer outside the outer membrane (Evenberg et al., (1982) Biochim. Biophys. Acta 684, 241–248). The latter protein, designated as additional cell envelope protein or ACE protein, has been purified and characterized in our laboratory (Evenberg and Lugtenberg, (1982) Biochim. Biophys. Acta 684, 249–254). (2) Most strains produce both high and low molecular weight lipopolysaccharide species, presumably corresponding with the presence and (virtual) absence, respectively, of an O-antigenic chain. The property to produce high molecular weight lipopolysaccharide can be lost upon subculturing in laboratory growth media and such is greatly enhanced by the prior loss of the ability to produce ACE protein. (3) Lipopolysaccharide and ACE protein were identified as the major antigens. A new polysaccharide-like antigen, designated as PS-antigen, was detected. Moreover, immunological indications for the presence of a lipoprotein in *A. salmonicida* are described. (4) The surface localization of the antigens was determined by testing whether preadsorption of antisera by intact cells decreased the binding of IgG to these antigens, or decreased the ability of the sera to agglutinate cells. According to these criteria lipopolysaccharide, ACE protein and PS-antigen are the major surface-located antigens. (5) Material cross-reactive with lipopolysaccharide, ACE protein and PS-antigen has been found in a large number of strains. (6) Several lines of evidence indicate the presence of interactions between ACE protein and lipopolysaccharide. (7) Based on these results a molecular model of the cell envelope of virulent *A. salmonicida* is presented.

Introduction

The Gram-negative bacterium *Aeromonas salmonicida* is the causative agent of major fish diseases affecting a large variety of fish species. Typical strains are involved in systemic furunculosis, whereas atypical strains are associated with ulcerative furunculosis in cyprinids [1].

In addition to the three cell envelope layers

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Abbreviation: ACE protein, additional cell envelope protein.

present in all Gram-negative bacteria, namely cytoplasmic membrane, peptidoglycan layer and outer membrane (for a review, see Ref. 2), cells of virulent *A. salmonicida* possess an additional regularly patterned cell surface layer outside their outer membrane [3–5]. This so-called A-layer consists mainly or entirely of repeating units of one protein species, designated as the additional cell envelope protein or ACE protein [6]. The presence of this layer has been correlated with virulence [5,7], autoagglutination [6] and adhesion to fish cells [5]. *A. salmonicida* cells have the tendency to loose the property of producing ACE protein upon repeated subculturing under laboratory conditions, the atypical strains being more stable than those of the subspecies *salmonicida* [6,8].

In our studies on the cell surface of *A. salmonicida*, as part of a project meant to develop a vaccine against furunculosis, we have described the characterization of the cell envelope proteins of various *A. salmonicida* strains using SDS-polyacrylamide gels in which the protein bands were either stained or detected immunologically [6]. In addition, using a periodic acid-Schiff staining procedure on gels, several sugar-containing bands were observed and we speculated that they might represent glycoprotein and/or lipopolysaccharide [6]. As it can be expected that such components are immunogenic and are located at the cell surface, they might be protective antigens. Therefore we have further characterized these periodic acid-Schiff positive constituents and in the present paper we show that they represent lipopolysaccharide. In addition, our results provide evidence for an interaction between ACE protein and lipopolysaccharide. Moreover, using a combination of biochemical and immunological methods, surface-exposed antigens are characterized. We could show for a large number of *A. salmonicida* strains that these antigens showed immunogenic cross-reactivity. A molecular model explaining these data is presented.

Materials and Methods

Bacterial strains and growth conditions

The relevant properties of *Aeromonas salmonicida* strains used in this study are listed in Table I. Strains lacking ACE protein were isolated after repeated subculturing at 27–30°C [6] by plating suitable dilutions and screening for grey-white colonies after growth for 48 h at 22°C on tryptic soy agar plates (Difco, Detroit, MI, U.S.A.), containing 0.1 mg/ml Coomassie Brilliant Blue R250 (Bio-Rad, Richmond, CA, U.S.A.). Colonies from ACE protein-positive strains stain dark blue (Udey, L.R., personal communication) with a faint halo around the colony on prolonged standing (4–5 days). Clones which lack both the ACE protein and the periodic acid-Schiff positive band in the electrophoretic position corresponding with that of proteins with M_r 60000-70000 were obtained spontaneously and with a high frequency after serial subculturing of ACE protein negative mutants.

Unless mentioned otherwise, cells were grown at 22°C under aeration as described previously

TABLE I

RELEVANT PROPERTIES OF *AEROMONAS SALMONICIDA* STRAINS

+ and − indicates presence or absence, respectively, of the indicated constituents. LPS, lipopolysaccharide.

Strain	ACE-protein	High M_r LPS [a]	Source, reference
V75/93	+	+	[6]
V75/93-W2	−	−	This study
V234/81	+	+	Wiedemann [b]
V75/174	+	+	[6]
V75/174-W1	−	+	This study
V75/174-W2	−	−	This study
V76/135	+	+	[6]
NCMB1110	+	+	[6]
1110-W2	−	−	This study
NCMB2020	−	+	[6]
TG51/79	+	+	[6]
TG51-W1	−	+	This study
TG51-W2	−	−	This study
TG36/75	+	+	[6]
TG36-W1	−	+	This study
TG36-W2	−	−	This study
126-68	−	+	[6]
152-69	−	−	[6]
ATCC14174	−	+	[c]
CIP6713	+	+	[6]

[a] Absence of high molecular weight lipopolysaccharide (virtually) lacking O-antigenic chains.
[b] Wiedemann, G., Bavarian Animal Health Service, F.R.G.
[c] American Type Culture Collection, Rockville, MD, U.S.A.

[6,9]. Turbidity of the cultures was measured at 660 nm. The viability of the cells was determined as the number of colony-forming units. In order to obtain reproducible counts from autoagglutinating strains, the cells were diluted in a low ionic strength solution consisting of 240 mM glycine/30 mM NaCl/3 mM Na_2HPO_4 (pH 6.8)/0.1% Tween 20, and vortexed for 30 s at maximal speed. Microscopic examination showed that in addition to single cells aggregates of up to approximately 50 cells were obtained.

Isolation and characterization of membrane fractions

After ultrasonic disruption of cells, cell envelopes were isolated by differential centrifugation as described previously [10], resuspended in phosphate-buffered saline (7 mM Na_2HPO_4/3 mM KH_2PO_4/140 mM NaCl (pH 7.2)) and stored at −20°C. SDS-polyacrylamide gel electrophoresis was carried out according to Ref. 10. Protein bands were stained with Fast Green FCF [10] and sugar-containing material was detected using the periodic acid-Schiff staining [11]. Protein bands are not stained by the latter procedure provided that fresh solutions are used. To establish the positions of the periodic acid-Schiff positive material and protein bands relative to each other, the proteins were stained after the Schiff procedure had been applied to the gel. Often good photographic recording of the periodic acid-Schiff positive band turned out to be difficult. Staining of this material is improved considerably upon proteolytic digestion of cell envelopes.

Proteolytic digestion was performed by incubating cell envelope suspensions (1.4 mg protein) with 0.2 mg of a protease, like trypsin, protease I or proteinase K, for 1 h at 37°C in a total volume of 120 μl. The reaction was terminated by the addition of 20 μl 0.1 M diisopropylphosphorofluridate in anhydrous isopropanol. After incubation for 5 min the solution was acidified with 100 μl ice-cold 0.1 M acetic acid/acetate buffer (pH 3.5). The suspensions were centrifuged for 30 min at 4°C in a table-top Eppendorf centrifuge at maximal speed. The pellets were washed twice with and finally resuspended in 100 μl ice-cold phosphate-buffered saline.

In later stages of the work the more sensitive silver-staining procedure of Tsai and Frasch [12], as modified by Hitchcock et al. [13], was used to stain lipopolysaccharide bands.

For the isolation of lipopolysaccharide cell envelopes were isolated from cells grown for 2 to 3 days on solid medium [9]. Lipopolysaccharide was extracted from cell envelopes [14] using the phenol-water procedure of Westphal and Jann [15]. Further purification was achieved by treatment with RNAase followed by repeated ultracentrifugational sedimentation at $105\,000 \times g$ at 4°C [15] for 3 h. The purified lipopolysaccharide was resuspended by sonication in a small volume of distilled water and stored at −20°C. Lipopolysaccharide fractions were further analyzed by SDS-polyacrylamide gel electrophoresis. Protein determinations were carried out as described before [16].

Purified lipoprotein [17] was a kind gift from N. Overbeeke and R. van Boxtel.

Antisera

Three procedures were used to raise antibodies against cells of *A. salmonicida*. In the first one, rabbit antisera were obtained according to the so-called OK-antiserum schedule [18], using formaldehyde(0.37%, w/v)-killed cells of *A. salmonicida* strains V75/93 and 126-68. Rabbits were injected intravenously twice a week, during 3 weeks. Starting with a dose of 0.1 ml of a suspension of approx. $3 \cdot 10^8$ cells/ml, the injected volume was increased 2-fold at every following event. The rabbits were killed by heart puncture on day 24. A variation of this procedure was used to raise antisera against viable cells of strains V75/93 and 126-68 after priming with formaldehyde-killed bacteria. Immunization was carried out as described in the first procedure except that the interval between two successive injections was increased to 5 days. These rabbits were killed 6 days after the fourth injection. In the third procedure cells of strains 152-69 and V76/135 were used, which had been killed by heating for 10 min at 80°C and washed twice prior to a formaldehyde treatment. Rabbits were immunized using the OK-antiserum immunization regimen.

Antisera against ACE protein or cell envelope preparations from strain V75/93 were raised as described previously [6].

Antisera against *A. salmonicida* reference strains

ATCC14174, NCMB1110(As29), NCMB2020, and atypical strains (As 8) and *Hemophilus piscium* strain ATCC15711, identified as an atypical *A. salmonicida* strain [19], were a generous gift of Dr. W.D. Paterson (Connaught Laboratories, Willowdale, Ontario, Canada).

For the absorption of antibodies to whole cells, bacteria were grown as described, washed with phosphate-buffered saline and resuspended to a cell density of $(1-5)\cdot 10^{10}$ cells/ml. To saturate aspecific adhesion sites which was especially required in the case of ACE protein producing cells, bacteria were preincubated at room temperature for 2 h with 0.5 ml normal rabbit serum/ml cell suspension. The cells were pelleted and the supernatant fluid was discarded. These preincubated cells were used to absorb antisera as described [18]. The absorbed antiserum was then filtered through a Millipore nitrocellulose filter (pore size 0.45 μm) and the filter was washed with an equal volume of sterile phosphate-buffered saline/0.1% NaN_3, resulting in 2-fold diluted absorbed serum.

Immunological assays

Bacterial agglutination was measured in U-bottom polyvinyl microtiter plates (Linbro, Flow Laboratories Ltd., Irvine, U.K.) as described [19]. Volumes of 50 μl of a suspension of freshly harvested bacteria in phosphate-buffered saline ($2\cdot 10^9$ cells/ml) were mixed with 50-μl serial dilutions of antiserum in phosphate-buffered saline. The titer was determined after incubation for 16 h at 4°C as the ^{2} log value of the highest serum dilution which showed a positive agglutination. *A. salmonicida* strains which are ACE protein-positive could not be used in this test since their cohesive properties caused autoagglutination [6].

Gel immuno radio assay was performed as described before [20], with some modifications to reduce the time from 5 to 3 days. The overnight incubation with the antiserum was reduced to 2 h and the periods of washing required to remove the excess of antiserum or of ^{125}I-protein A from the gel slices were reduced to 10 min. The gel slices were washed under constant shaking in polystyrene urine-sample beakers containing 20–50 ml phosphate-buffered saline/0.05% (v/v) Tween 80. The exposure time was reduced from 70 h to 16 h by application of an Illford tungstate intensifying screen for fluorography. Unless otherwise stated the antiserum dilution was 1 : 100. In order to exclude any positive reaction due to unknown cross-activity, preimmune rabbit serum was diluted 1 : 10.

Iodination of protein A

Staphylococcus aureus protein A (Pharmacia, Uppsala, Sweden) was labelled with 1 mCi ^{125}I (The Radiochemical Centre, Amersham, U.K.) as described before [20]. The labelling efficiency was approximately 40–50%, resulting in labelled protein A with a specific activity of 2.2–2.5 mCi/mg protein.

Results

Identification of periodic acid-Schiff stained positive material as lipopolysaccharide

We previously reported that, after electrophoresis of SDS-solubilized cell envelopes of *A. salmonicida*, periodic acid-Schiff positive material could be detected as purple areas in the gel. The heaviest periodic acid-Schiff positive area, designated as H1, corresponded with the region for proteins of M_r 60000 to 70000, two areas of moderate intensity were found at regions for proteins of M_r values < 16000 (L) and to M_r values between 72000 and 95000 (H2), whereas a weak band at M_r 22000 to 26000 was observed occasionally [6]. The latter band was only visible on heavily overloaded gels and for technical reasons we have neglected it in subsequent experiments. Capsular polysaccharides, lipopolysaccharide and glycoproteins are the components which can be responsible for the periodic acid-Schiff positive materials. As capsular polysaccharide can hardly or not at all enter the gel, the possibility that it is responsible for the stained bands is unlikely. Moreover, capsular polysaccharides can be resistant towards periodic acid oxidation, as is reported by How [21], Poolman and Zanen [22] and P. Van der Ley (personal communication) and therefore will not be stained by Schiff's reagent. Glycoproteins are also candidates, although their presence in bacteria is rare. To investigate this possibility, cell envelope preparations were subjected to various proteolytic treatments, using trypsin, protease I and proteinase K. Analysis of

the polypeptide patterns showed that proteinase K treatment was the most effective one as it almost completely degraded the cell envelope proteins (compare Figs. 1a and 1b). The M_r 42000 protein appears to be the most resistant major protein towards tryptic treatment (not shown). Comparison of periodic acid-Schiff stained gels of samples representing proteinase K-treated and control cell envelopes showed that proteolytic treatment neither altered the mobility nor reduced the intensity of the periodic acid-Schiff stained areas (compare Figs. 1c and 1d). In fact, the intensity of the periodic acid-Schiff stain increased upon proteolytic degradation of cell envelopes as is best illustrated by the easier detection of band H2 (Fig. 1d), the presence of which is strain-dependent [6]. These results exclude a major contribution of glycoprotein to the periodic acid-Schiff positive regions and they therefore strongly suggest that the stained material is lipopolysaccharide.

Definite proof for this was obtained by purifying lipopolysaccharide from cell envelopes by aqueous phenol extraction and analyzing these preparations on gels. The results shows that purified lipopolysaccharide preparations contain the same periodic acid-Schiff positive areas as found in proteinase K-digested cell envelopes (compare Figs. 1e and 1d). In view of the very high similarity between those Schiff positive areas of purified lipopolysaccharide and those of proteinase K-digested cell envelopes, a contribution of other polysaccharides can be excluded. Apparently, lipopolysaccharides of *A. salmonicida* consists of high and low molecular weight fractions, all of which seem to be heterogeneous. Using the more sensitive silver-staining procedure to detect lipopolysaccharide [12,13], purified lipopolysaccharide and proteinase K-digested cell envelope material also gave identical patterns. With this procedure a multiple band pattern is observed more easily (Fig. 1f and 1g).

Significant differences in the electrophoretic positions of both high and low molecular weight lipopolysaccharide bands are found among various strains (compare, for example, Figs. 1f and 1g). The electrophoretic mobilities of H1 and H2 LPS bands of type strain NCMB2020 and five typical *A. salmonicida* strains tested, 126-68, TG51/79, TG36/75, ATCC14174, CIP6713, were consistently lower than those of the five atypical strains tested (strains V79/93, V234/81, V75/174, V76/135 and type strain NCMB1110) strains tested. Moreover, after periodic acid-Schiff treatment the lipopolysaccharide of strain V75/93 stains pink purple in contrast to the dark purple colour for lipopolysaccharide of the other strains listed in Table I.

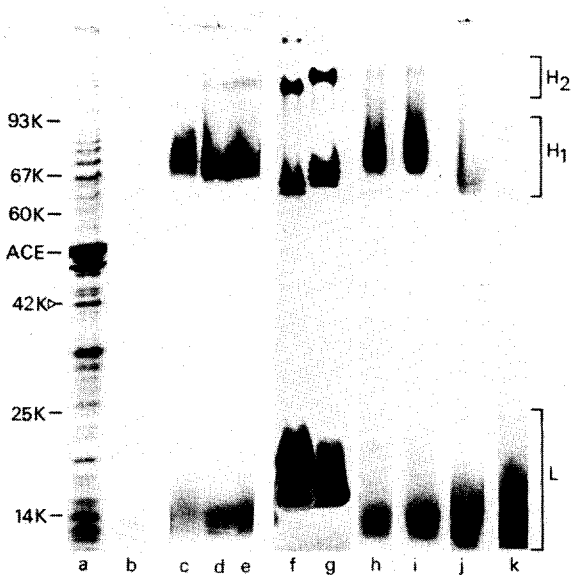

Fig. 1. SDS-polyacrylamide gel electrophoresis patterns of variously treated cell envelope preparations of several *A. salmonicida* strains visualized by different staining methods. Lanes a and b contain Fast Green-stained cell envelope preparations of the virulent strain V75/93 before (a) and after (b) proteinase K treatment. The apparent molecular weights of a series of standard proteins are indicated at the left. Lanes c and d represent the periodic acid-Schiff-stained patterns of the same preparations as used in lanes a and b, respectively, whereas lane e represents periodic acid-Schiff-stained lipopolysaccharide purified from the same strain. The positions of lipopolysaccharide regions H1, H2 and L are indicated at the right. Lanes f and g represent periodic acid-silver-stained patterns of purified lipopolysaccharide from atypical strain V75/93 (lane f) and the typical strain TG51/79 (lane g). Lanes h–k represent the periodic acid-Schiff patterns of cell envelopes of typical, virulent strain TG51/79 (lane h) and its avirulent, ACE protein negative mutant TG51-W1 (lane i) and of the atypical, reference type strain NCMB1110 (lane j) and its avirulent mutant strain 1110-W2, which lacks both ACE protein and high molecular weight lipopolysaccharide (lane k).

Loss of O-antigen in ACE protein negative mutants

ACE protein negative mutants can be obtained

after growing cells at a relatively high temperature [8]. Initially these mutants have the same lipopolysaccharide pattern as the ACE protein positive strains (compare, for example, Figs. 1h and 1i), but the ability of these mutants to produce the H1 and, if present, the H2 form of lipopolysaccharide, presumably both corresponding with lipopolysaccharide with long O-antigen side chains [25,26], is easily lost upon subculturing (compare, for example, Figs. 1j and 1k). Apparently, the presence of high molecular weight lipopolysaccharide is a selective disadvantage in the absence of ACE protein. The opposite situation, namely the lack of high molecular weight lipopolysaccharide in the presence of ACE protein, has never been observed.

Immunogenicity of A. salmonicida cell envelope constituents

The immunogenicity of *A. salmonicida* cell envelope constituents was investigated to determine which cell envelope antigens could be protective. Viable, formaldehyde-treated or formalinized heat-killed cells were used as the immunogens. O-antigen-containing, high molecular weight, lipopolysaccharide and ACE protein are expected to be immunogenic. Therefore cells of strains missing one or both of these constituents were also used for immunization as such antisera may be useful for the detection of other, minor, antigens. Furthermore, purified ACE protein, or detergent (SDS)-extracted cell envelopes were used as antigens in order to facilitate the identification of the immunogens. The gel immuno radio assay was used to reveal the molecular identity of the immunogens. Cell envelope constituents of strain V75/93 solubilized by boiling in sample buffer, were separated by electrophoresis and SDS-free gel sections were allowed to react with antiserum raised against formaldehyde-treated cells of the homologous strains. Fig. 2 shows that antigens (lane b) are present in positions corresponding with periodic acid-Schiff positive bands (lane a). In addition to the known lipopolysaccharide bands H1, H2 and L, whose identity was confirmed by using purified lipopolysaccharide preparations as antigens (lanes c and d), another weak antigenic activity with low electrophoretic mobility, designated as PS in Fig. 2 (lane e), was also present. This antigen which can be present as a smear (Fig.

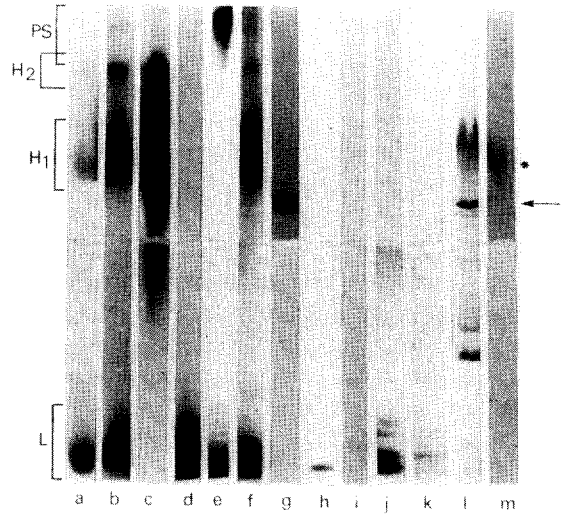

Fig. 2. Immunogenicity of *A. salmonicida* cell envelope constituents. Whole or fractionated cell envelopes were solubilized in sample buffer either by boiling (a–k) or by incubation at 37°C (l and m) and the individual constituents were electrophoretically separated in 3-mm thick SDS-polyacrylamide gels. Antigens were detected using the gel immuno radio assay technique with a variety of antisera. For experimental details see Materials and Methods. Lane (a) contains a periodic acid-Schiff-stained preparation of cell envelopes of strain V75/93. Lanes b–f show antigens detected with a serum raised against formalinized cells of strain V75/93. The slots were loaded with cell envelopes of strain V75/93 (b), lipopolysaccharide purified from the wild-type, typical, strain TG51/79 (c), lipopolysaccharide purified from strain TG51-W2, an ACE protein-deficient mutant of the latter strain which in addition lacks high molecular weight lipopolysaccharide (d), cell envelopes of the ACE protein-deficient, high molecular weight lipopolysaccharide-deficient strain 152-69 (e) and cell envelopes of the atypical reference strain NCMB1110 (f). Lane g is the same as lane b except that the antiserum was preabsorbed with intact cells of the ACE protein-deficient strain 126-68 and diluted 1:16, resulting in a specific reaction with the ACE protein. Lanes h, i and j show antigens detected with antibodies raised against heat-killed formalinized cells of the ACE protein-deficient, high molecular weight lipopolysaccharide-deficient strain 152-69 after preadsorption with homologous cells. The slots were loaded with cell envelopes of strain V75/93 (h), the same cell envelopes after digestion with proteinase K (i) and purified *E. coli* lipoprotein (j). It should be noted that the purified bound form of lipoprotein, in contrast to the free form which is presumably observed in slot h, gives more than one band (slot j). Lane k showes the latter preparation after staining with Fast Green. Lanes l and m contain cell envelopes of strain NMCB1110 solubilized at 37°C and stained with Fast Green (slot l) and after reaction with antiserum raised against pure 54K protein (slot m). The positions of the lipopolysaccharide bands L, H1 and H2 of the PS-antigen, of the ACE protein (arrow) and of the presumed ACE protein-lipopolysaccharide complexes (asterisk) are indicated.

2f) has the following properties. It is present in variable amounts in various strains, the most abundant amounts being detected in cell envelope preparations of strain 152-69, which lacks high molecular weight lipopolysaccharide (Fig. 2e). The antigen is proteinase K-resistant and is virtually lacking in preparations of purified lipopolysaccharide (Figs. 2c and 2d) and in cell envelopes of a phage AsP1-resistant mutant of strain 126-68 which still produces high molecular weight lipopolysaccharide bands (not shown). These properties strongly suggest a non-lipopolysaccharide polysaccharide-like nature for this antigen. We therefore will tentatively refer to this antigen as PS-antigen. As expected, another antigen was detected in the position of ACE protein after preadsorption of the anti-lipopolysaccharide immunoglobulins from the antiserum, anti-V75/93, with whole cells of strain 126-68 (Fig. 2g). Its identity as ACE protein was confirmed by the observations that the absorbed antiserum also reacted with purified ACE protein and that no reaction was observed with electrophoretically separated cell envelope constituents of the ACE protein deficient strain 126-68 (results not shown).

When an antiserum raised against heat-killed formalinized cells of strain 152-69, lacking both ACE protein and high molecular weight lipopolysaccharide, was used, the observed gel immuno radio assay pattern was quite different from that obtained with antiserum against formaldehyde-treated cells of strain V75/93. In addition to a moderate activity against a background smear, the strongest activity is formed against material at the front of the gel (not shown). Its immunogenicity and its electrophoretic position are consistent with low molecular weight lipopolysaccharide as well as with lipoprotein [20,23,24]. To distinguish between these two antigens the antiserum was preabsorbed with intact cells of the homologous strain, as the major antigenic determinants of lipopolysaccharide, in contrast to those of lipoprotein are exposed to the cell surface [2,24]. As no reaction was observed with (homologous) purified low molecular weight lipopolysaccharide (not shown) and as the antigenic activity was destroyed by treatment with proteinase K (compare Fig. 2h with 2i) the antigen must be a protein or contain a protein moiety. The observation that the pre-adsorbed antiserum reacts with purified *Escherichia coli* B/r lipoprotein (Fig. 2j) in positions exactly corresponding with the Fast Green stained protein pattern (Fig. 2k) strongly suggests that the antigen is a lipoprotein.

The way in which the cellular antigens had been treated prior to immunization had some influence on the specificity of the raised antibodies. The major differences were the following.

(i) A very strong reaction against the ACE protein is obtained with rabbit antisera raised against SDS-EDTA washed cell envelope preparations, which have a low lipopolysaccharide content. When formalinized whole cells were used to immunize rabbits, the raised antisera showed a very weak reaction with the ACE protein, and when viable cells or formalinized heat-killed cells were used no antibody response against the ACE protein was detected.

(ii) The strongest reaction towards *E. coli* lipoprotein was observed in antisera raised against formalinized heat-killed cells. It was also present in antisera raised against live cells, but virtually absent in antiserum raised against formaldehyde-fixed whole cells. A summary of the relevant results is shown in Table II.

As part of the antigenic determinants could well be irreversibly inactivated by boiling in SDS, the standard method for solubilization of cell envelopes prior to gel electrophoresis was replaced in a number of cases by incubation for 30 min at 37°C. The latter method can be expected to be less destructive to antigenic determinants, but it can have the disadvantage that solubilization is incomplete, resulting in antigenic complexes too large to enter the gel or in antigenic complexes whose molecular identity is hard to establish. The results showed that the only striking influence of the lower solubilization temperature is that a new irregular Fast Green positive smearing band appears at the approximate position of M_r 56 000–70 000, whereas the ACE protein band became very weak (Fig. 2l). These alterations were found only for the ACE protein positive strains (e.g. V75/93, NCMB1110 and TG51/79), but not for the seven ACE protein negative strains tested (Table I). This result strongly suggests that the smearing band contains ACE protein which was confirmed by the use of an ACE protein specific

TABLE II

EFFECT OF PRETREATMENT OF THE IMMUNOGEN AND PREADSORPTION OF THE ANTISERUM ON THE REACTIONS WITH ELECTROPHORETICALLY SEPARATED ANTIGENS

Cells were treated in various ways and used as immunogens as described previously. Rabbit sera [19] donated by Dr. W.D. Paterson (Connaught Research Institute, Willowdale, Ontario, Canada) showed very similar gel immunoradioassay patterns. Antisera with high bacterial agglutination titres showed a very strong and predominant activity against high molecular weight lipopolysaccharide (LPS), whereas the antisera with low titres showed a strong reactivity against low molecular weight lipopolysaccharide. Antibodies directed against ACE protein were detected in anti-As29 (NCMB1110). Anti-*Hemophilus piscium*, ATCC15711, showed a gel immunoradioassay pattern with *A. salmonicida* antigens, which is very similar to those obtained with anti-*A. salmonicida* sera. The antigens were detected using the gel immunoradioassay on boiled electrophoretically separated antigens. PS-antigen is a high molecular weight antigen, presumably acid polysaccharidic in nature, abundantly present in strain 152-69, which lacks high molecular weight lipopolysaccharide. In the other strains (Table I) no such defined area was observed, but rather a continuous (background) smear (Fig. 2f), from the top to the bottom of the gel, which could be reduced effectively by preadsorbing the antisera with cells of strain 152-69 (Fig. 3b).

Antiserum			Antigen				
Cells	Pretreatment	Preadsorption	High M_r LPS	Low M_r LPS	PS-antigen	ACE protein	Lipoprotein
I. V75/93	Formalinized	None	+ + + + [a]	+	+ +	±	−
		V75/93 cells	−	−	−	−	n.d. [b]
		126-68 cells	−	−	−	+	n.d.
	None	None	+ + +	+	+	−	+
II. 126-68	Formalinized	None	+ + +	+	+	−	n.d.
		V75/93 cells	−	±	−	−	−
	None	None	+ + +	+	+	−	+
		V75/93 cells	−	±	−	−	+
III. 152-69	Heat-killed and formalinized	None	−	+ + +	+ +	−	+ +
		V75/93 cells	−	±	−	−	+

[a] Usually antisera were diluted 1 : 100, the absorbed antisera 1 : 50, except for anti 152-69 sera which were diluted 1 : 32 and 1 : 16. As a rule the antisera were diluted 10-fold less than the measured bacterial agglutination titre. Since the gel immunoradioassay is a semi-quantitative procedure, antiserum dilution, exposure time and the quality of the radiolabelled ^{125}I-protein A influence the final result. The intensity of darkening of the film on the respective antigen positions are scored as follows: −, no reaction; ±, no visible reaction detected under the standard conditions. However, a positive reaction was detected using a 4-fold less antiserum dilution, a 2- to 3-fold longer exposure time or alternatively the presence of antibodies was detected by bacterial agglutination. +weak reaction: + +, moderate reaction; + + + and + + + +, respectively, strong and very strong reactions.

[b] n.d., not determined.

antiserum (Fig. 2m). As incomplete solubilization often leaves protein-lipopolysaccharide complexes intact [2,27], it is tempting to assume that the smear is caused by ACE protein/lipopolysaccharide complexes. Consistent with this assumption is the observation that it reacts with anti-high molecular weight lipopolysaccharide serum (not shown). However, as completely solubilized lipopolysaccharide also runs in a similar electrophoretic position, this observation does not provide absolute proof.

It is concluded that the major immunogenic constituents of *A. salmonicida* cells in rabbits are high and low molecular weight lipopolysaccharides, ACE protein, lipoprotein and an antigen, probably polysaccharide in nature, designated as PS-antigen.

Surface-exposed antigens

One of the criteria that has to be fulfilled by a protective immunogen is that it must be localized at the cell surface of intact cells. The question whether the detected immunogens (Fig. 2 and Table II) are surface-localized and accessible for antibodies was approached by measuring whether incubation of antisera with intact ACE protein-containing cells removed certain populations of antibodies as judged from the gel immunoradioas-

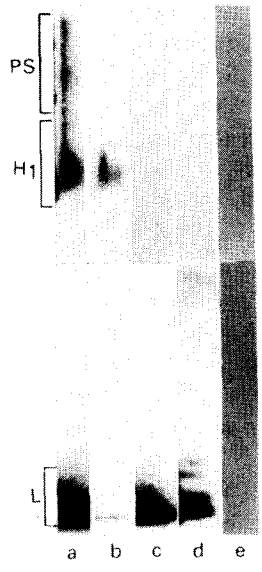

Fig. 3. Surface localization of antigens. Two examples are shown to test the accessibility of antibodies to intact *A. salmonicida* cells. Immunogens are detected with an antiserum. If preincubation of this antiserum with intact cells specifically prevents the detection of a certain antigen, this antigen must be exposed at the surface of these cells. Cell envelopes of strain V75/93 (lanes a–c and e) and *E. coli*, purified bound form, lipoprotein (lane d) were subjected to SDS gel electrophoresis and subsequently tested in the gel immuno radio assay using antiserum against live cells of the ACE protein-negative strain 126-68 (lane a), diluted 1 : 100, the same antiserum as used in lane a but preadsorbed with cells of the ACE protein and high molecular weight lipopolysaccharide negative strain 152-69 (lane b) or the same antiserum as used in lane a but now preadsorbed with cells of the virulent, wild-type strain V75/93 and diluted 1 : 16 (lanes c and d). Antiserum raised against formalinized cells of the wild type strain V75/93 and preadsorbed homologously was used in lane e, and served as a control.

says (GIRA) and from agglutination tests (not shown). An example of the former analysis is shown in Fig. 3.

Pre-absorption of anti-126-68 serum with cells of the ACE protein deficient and high molecular weight lipopolysaccharide deficient strain 152-69 removes most or all antibodies reacting with the PS-antigen and with low molecular weight lipopolysaccharide, whereas antibodies against high molecular weight lipopolysaccharide are still present (compare Figs. 3a and 3b). The latter observation shows that aspecific binding of antibodies by these cells hardly occurs. In contrast, cells of ACE protein-containing strains showed aspecific absorption, absorbing even anti-*E. coli* K12 pore protein and other unrelated antibodies. This aspecific absorption by such A-layered cells will be the subject of another paper. Here it suffices to say that the aspecific binding can be strongly decreased by incubation of the cells in preimmune serum prior to incubation with hyperimmune sera. Using cells of the four different A-layer-containing strains V75/93, V234/84, NCMB1110 and TG51/79, which gave the same results, it is observed that antibodies directed against ACE protein, high molecular weight lipopolysaccharide and PS-antigen and to a less extent, those against low molecular weight lipopolysaccharide (compare Figs. 3a with 3c and 3e) and not those against lipoprotein were adsorbed by these cells (Fig. 3d). These results show that at least certain sites of the former two antigen molecules are surface-exposed in intact cells of pathogenic *A. salmonicida* strains. The remaining antibodies were still capable to agglutinate cells of strain 152-69, which lacks ACE protein and high molecular weight lipopolysaccharide. Further analysis showed that these mutant cells are able to remove anti-low molecular weight lipopolysaccharide antibodies. These results indicate that low molecular weight lipopolysaccharide, which is accessible at the cell surface of the mutant strain, but less or not all at that of the parent strain, which is shielded by the A-layer. In conclusion, ACE protein and part of the O-antigenic chain of high molecular weight lipopolysaccharide and PS-antigen are easy accessible for antibodies in intact A-layered cells. Low molecular weight lipopolysaccharide is accessible in intact ACE protein negative cells whereas lipoprotein is not accessible at all.

Cross-reactivity of lipopolysaccharide and PS-antigen at the level of isolated molecules

One of the criteria that has to be fulfilled by a practically effective protective immunogen is that antigenic determinants must be shared by cells of most strains against which protection is required. By showing that antibodies against ACE protein of strain V75/93 react in gels of many other strains, we concluded that these strains shared antigenic determinants, at least at the level of isolated molecules [6]. Using antisera against

strains V75/93 and 126/68, both made specific for high molecular weight lipopolysaccharide by absorption with cells of strain 152-69, it was observed by gel immunoradioassay that cross-reacting material against this lipopolysaccharide was found in 24 out of the 26 strains listed in Ref. 6. Exceptions were the two strains 152-69 and 153-69, which are lacking high molecular weight lipopolysaccharide. In similar experiments, using antisera raised against formaldehyde-treated cells, cross-reacting material with the PS-antigen was detected by comparing the gel immunoradioassay patterns before and after preabsorbtion with intact cells of strain 152-69, which lacks high molecular weight lipopolysaccharide. Material cross-reactive with PS-antigen was found in all wild-type isolates listed in Table I.

Discussion

The periodic acid-Schiff positive materials present in cell envelopes of *A. salmonicida* strains [6] was identified as lipopolysaccharide and appeared to be present as a high molecular weight and a low molecular weight fraction, both of which are heterogenous. The fractions are likely to correspond with O-antigen-containing lipopolysaccharide and with core-lipid A lipopolysaccharide (see Ref. 28 for lipopolysaccharide structures). Compared with lipopolysaccharides from other Gram-negative bacteria, which appears as a large number of bands, so-called ladder structures; throughout the gel as a result of a varying numbers of O-antigen repeating units [25,26], the high molecular weight lipopolysaccharide of *A. salmonicida* appeared relatively homogenous (Fig. 1 and Ref. 29). However, using very sensitive immunological techniques we could show that these ladder structures can also be observed for *A. salmonicida* lipopolysaccharide (Fig. 2c). Based on the electrophoretic mobility, lipopolysaccharides from typical and atypical strains could be distinguished (compare, for example, Figs. 1f and 1g), which is interesting as the two groups of strains are responsible for different diseases.

To survey possibilities for future vaccine development rabbits were immunized with cells or cell envelope fractions pretreated in various ways. The availability of mutants missing ACE protein or both ACE protein and high molecular weight lipopolysaccharide increased the possibility to detect minor immunogens. The results (Fig. 2 and Table II) showed that high molecular weight lipopolysaccharide, low molecular weight lipopolysaccharide and ACE protein are good immunogens. Using preadsorbed antisera a new immunogen, preliminary designated as PS-antigen was also detected. Moreover, material cross-reactive with *E. coli* lipoprotein and having a similar electro-

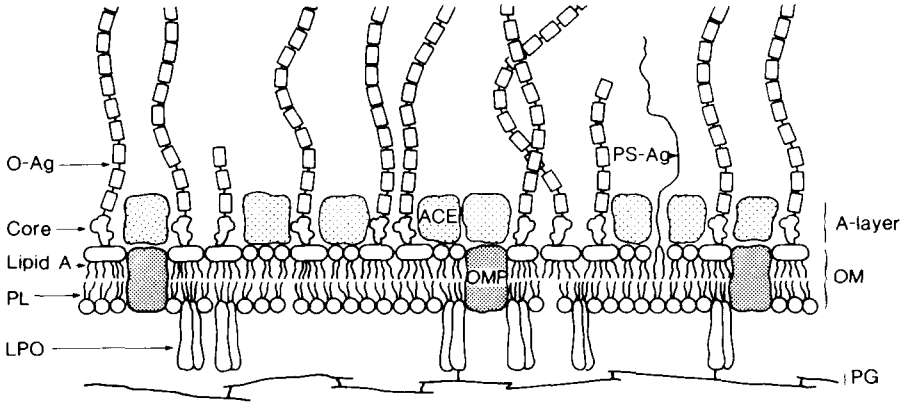

Fig. 4. Model of the cell surface of *A. salmonicida*. Outside the peptidoglycan layer (PG), the outer membrane (OM) and A-layer are drawn. The outer membrane consists of outer membrane proteins (OMP), from which lipoprotein (LPO) is specifically indicated, phospholipid (PL). Lipopolysaccharide (LPS, consisting of three regions, lipid A, core and O-antigen). The PS-antigen is assumed to be inserted in the outer membrane in a similar way [31] as assumed for capsular polysaccharide. The A-layer consists of regularly arranged subunits of the ACE protein.

phoretic mobility was detected in *A. salmonicida* cell envelopes. Using binding to intact wild-type cells as a criterium (Fig. 3), it was shown that high molecular weight lipopolysaccharide, ACE protein and PS-antigen are well accessible, that low molecular weight lipopolysaccharide is moderately accessible and that the lipoprotein-like antigen is not accessible. These results are in excellent agreement with current models on outer membranes in which this latter protein is not facing the outside of the cell [2]. The observations that high molecular weight lipopolysaccharide, ACE protein and PS antigen are immunogenic and in intact cells accessible to antibodies make these three components candidates for vaccines. The observation that antigenic determinants of these components are common in most or all of the tested strains (Ref. 6 and this paper) even increases the chances to develope a successful vaccine based on one or more of these constituents.

Based on the results described in this paper, we can design a model of the cell surface of *A. salmonicida* (Fig. 4). The model is based on the following considerations.

(i) It is assumed that the architecture of the outer membrane of *A. salmonicida* is similar to that of other Gram-negative bacteria [2] and that the A-layer is localized external to the outer membrane layer [30].

(ii) Experiments on the accessibility of antigens to antibodies have shown that high molecular weight lipopolysaccharide, ACE protein and PS-antigen are exposed at the cell surface of wild-type cells. The observation that low molecular weight lipopolysaccharide is accessible in cells lacking both ACE protein and high molecular weight lipopolysaccharide, is incorporated in the model.

(iii) Several lines of evidence indicate that ACE protein is complexed with high molecular weight lipopolysaccharide. The observation that ACE protein negative mutants are unstable in that they loose the capacity to produce high molecular weight lipopolysaccharide with high frequency is likely to reflect a relation between the biogenesis and/or structural stabilization of high molecular weight lipopolysaccharide and ACE protein. Moreover, our result that ACE protein and high molecular weight lipopolysaccharide comigrate in SDS-polyacrylamide gels of incompletely solubilized cell envelopes is also consistent with a non-covalent interaction between these two constituents.

(iv) Our results have indicated the presence of the free form of lipoprotein in *A. salmonicida* (Fig. 1h). It is not shown whether this bacterium also contains the bound form like *E. coli* and many other Gram-negative bacteria [23]. This information together with the observation that the lipoprotein is not accessible to antibodies in intact cells of either wild-type or mutant strains, suggests a location of the lipoprotein similar to that in *E. coli* (for a discussion, see Ref. 2).

Acknowledgements

We thank Dr. L.R. Udey and Peter van der Ley for communicating their findings on, respectively, the election of ACE protein negative mutants and capsular polysaccharide resistance towards periodic acid-silver staining, prior to publication, Dr. W.D. Paterson for the gift of anti-*A. salmonicida* rabbit antisera, Ria van Boxtel and Nico Overbeeke for purified *E. coli* B lipoprotein and Oscar Kuipers and Peter Dols for technical assistance. The interest of Dr. R. Bootsma in this work is acknowledged. These investigation were supplemented (in part) by the Netherlands Foundation for Technical Research (STW), future Technical Science Branch/Division of the Netherlands Organization for the Advancement of Pure Research (ZWO).

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