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## CELL SURFACE OF THE FISH PATHOGENIC BACTERIUM *AEROMONAS SALMONICIDA*

### II. PURIFICATION AND CHARACTERIZATION OF A MAJOR CELL ENVELOPE PROTEIN RELATED TO AUTOAGGLUTINATION, ADHESION AND VIRULENCE

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The purification of the major protein of the membrane fraction of an autoagglutinating strain of *Aeromonas salmonicida* is described. This protein, designated as additional cell envelope protein, is water-insoluble, has a molecular weight of about 54000 and its amino terminal sequence is H<sub>2</sub>N-Asp-Val-Leu-Leu. Neither sulphur-containing amino acids nor sugar residues were detected. Its amino acid composition, which shows that the additional cell envelope protein is hydrophobic in nature, is remarkably similar to those of various proteins known to be present in additional surface layers of other bacteria, to the adhesive K88 fimbriae of enteropathogenic *Escherichia coli* and to a pore protein of the outer membrane of *E. coli* K12.

#### Introduction

Isolates of the Gram-negative bacterium *Aeromonas salmonicida* subsp. *salmonicida* are the etiological agent of the classical septicaemic furunculosis of salmonid fish [1], whereas so-called atypical variants of *A. salmonicida* [2] cause an ulcerative type of furunculosis in a variety of fish including salmonids [2–8]. These pathogenic bacteria contain an additional cell envelope layer located outside their outer membrane [4,9,10], which has been implicated in adhesion [9], in enhanced virulence [9,10] and in autoagglutination [9–11]. Assuming that this additional layer con-

sists of protein subunits, as has been described for several other bacteria [12–17], we have recently analyzed the cell envelope protein patterns of a large series of *A. salmonicida* strains. The results showed that a protein with an apparent molecular weight of 54000 is the most abundant protein in strongly autoagglutinating strains whereas it is absent, or present in only low amounts, in non-autoagglutinating isolates [11]. As the presence of the additional cell envelope layer in *A. salmonicida* has been correlated with autoagglutination, a phenomenon which, in turn, has been correlated with the presence of the protein with a molecular weight of 54000, we have designated this protein as the additional cell envelope protein [11].

As a reasonable working hypothesis seems to be that the additional cell envelope protein is a major, or the only, constituent of the additional layer, which is involved in autoagglutination, adhesion

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Abbreviation: SDS, sodium dodecyl sulphate.

and increased virulence, we decided to purify and characterize this protein. The results are described in this paper.

## Materials and Methods

### *Strains and growth conditions*

The strongly autoagglutinating atypical *A. salmonicida* strain V75/93, the causative agent of carp erythrodermatitis [3], was grown at 27°C in 1% (w/v) tryptose broth supplemented with 10% (v/v) complement-inactivated horse serum [3]. The medium was inoculated 1:100 and the cells were grown for 48 h under vigorous aeration, harvested at 4°C by centrifugation at  $5800 \times g$  for 20 min, washed with 110 mM NaCl and either used directly or stored at -20°C.

### *Isolation of membrane fractions*

Frozen bacteria were thawed, resuspended in 50 mM Tris-HCl buffer (pH 8.5) containing 2 mM EDTA, and disrupted by passing through a French press. Cell envelopes were isolated by differential centrifugation as described previously [18], except that ultracentrifugation was replaced by centrifugation for 1 h at  $43000 \times g$  in a refrigerated centrifuge.

### *Purification of the additional cell envelope protein*

A detailed description of the final procedure is described in Results.

The solutions used for differential extraction of cell envelopes had the following composition. Buffer I contains 2 mM Tris-HCl, pH 7.8/2% (v/v) Triton X-100/0.25 mM dithiothreitol/10 mM MgCl<sub>2</sub> and buffer II contains 5 mM Tris-HCl, pH 7.5/0.5% (w/v) SDS/1.25 mM EDTA/0.2 mM dithiothreitol. Insoluble envelope material was recovered by centrifugation for 45 min at  $240000 \times g$  at 23°C and subsequently resuspended in buffer III which contains 2 mM Tris-HCl, pH 7.2/0.25 mM dithiothreitol. The protein was eluted from the gels with buffer IV which contains 25 mM Tris-HCl, pH 6.8/0.2% (w/v) SDS/5% (v/v) glycerol/0.1% (v/v) 2-mercaptoethanol. The dialysis buffer used consists of 5 mM NH<sub>4</sub>HCO<sub>3</sub>, pH 7.7/0.025% (w/v) SDS.

Dansylation was carried out as described [19] at 45°C, after solubilization of the cell envelope frac-

tion at a protein concentration of 8–12 mg/ml in 100 mM NaHCO<sub>3</sub>, pH 8.8/2% (w/v) SDS by incubation for 10 min at 60 to 65°C. Excess reagent was separated from the dansylated protein fraction either by acetone precipitation [20] or by gel chromatography on a Sephadex G-25 (fine) column using 10 mM NaHCO<sub>3</sub>, pH 8.8/0.1% (w/v) SDS as the eluent.

### *Analytical methods*

The preparation of samples and the subsequent analytical SDS-polyacrylamide gel electrophoresis was performed as described [18]. Sugar-containing cell envelope constituents were detected by application of the periodic acid-Schiff staining procedure on gels [21]. For preparative gel electrophoresis the following modifications were introduced. (i) The protein sample was dissolved in sample buffer [18] by incubation for 10 min at 60–65°C. (ii) The gel thickness was 5 mm. (iii) Electrophoresis was carried out at a current of 10 mA/cm<sup>2</sup>. Protein was determined either by a modification [22] of the procedure described by Lowry et al. [23] or by scanning of stained gels [18]. Amino acid analyses were carried out as described [24]. The amounts of serine and threonine were extrapolated to zero time using values of 90 and 95%, respectively, for a hydrolysis time of 24 h. The NH<sub>2</sub>-terminal amino acid was determined by the dansyl procedure [25] and the sequence was established using the Edman degradation procedure [26], after solubilization of the protein by citraconylation [27]. Phenylthiohydantoin derivatives were identified according to the method of Frank and Strubert [28].

## Results and Discussion

### *Preliminary results*

Several methods for the isolation of additional bacterial cell surface layers and for the purification of their protein subunit have been described [13,17]. Our attempts to apply these and similar methods for the purification of the additional cell envelope protein of *A. salmonicida* were unsuccessful. Neither mechanical 'shaving' of intact fresh cells by treatment in a Sorvall Omnimixer blender [29], nor treatment of the cells with 10 mM EDTA [14], nor differential extraction of cell envelopes with 1.5 M guanidine hydrochloride [13], 3 or 5.4 M

urea [14,15] or 0.5% or 2% SDS [17] resulted in a specific solubilization of the additional cell envelope protein or of another cell envelope protein. The additional cell envelope protein is not heat-modifiable as the same electrophoretic mobility was observed after preincubation in sample buffer for 2 h at 37°C and after boiling for 5 min. Thus a purification procedure based on differences in electrophoretic mobility, depending on the temperature used for solubilization, as was used for the OmpA protein of *E. coli* [24], could not be used for the additional cell envelope protein. Differential extraction of cell envelopes yielded a considerable enrichment of the additional cell envelope protein to the extent that only one major protein with an apparent molecular weight of 42000 was the most significant protein contaminant. The latter protein was even more resistant to extraction with solutions containing detergents than the additional cell envelope protein, and it was the only protein which remained associated with peptidoglycan during extraction in 2% SDS at 40°C. The final step in the purification procedure consisted of separation of the two proteins by preparative polyacrylamide gel electrophoresis and the subsequent elution of the additional cell envelope protein from the gel.

#### *Purification of the additional cell envelope protein*

The results of the final procedure adopted for the purification of the additional cell envelope protein are illustrated in Fig. 1. The procedure consisted of the following steps. (i) Cell envelopes (1 g wet weight, 90 mg total protein, 40% additional cell envelope protein; see Fig. 1, slot 1 for protein pattern) were extracted twice with 5 ml buffer I for 30 min at 40–45°C followed by ultracentrifugation. The resulting pellet (0.3 g wet weight, 65 mg total protein, 50% additional cell envelope protein; see Fig. 1, slot 3) was washed once with 5 ml buffer III, then extracted for 10 min at 22°C with 5 ml of buffer II and washed again with buffer III (Fig. 1, slots 4 and 5). (ii) Ten to twenty percent of the resulting pellet (50 mg total protein, 60% additional cell envelope protein) was separately dansylated and then added to the remaining non-dansylated material (Fig. 1, slot 6). (iii) The preparation was solubilized in sample buffer for SDS-polyacrylamide gel electrophoresis

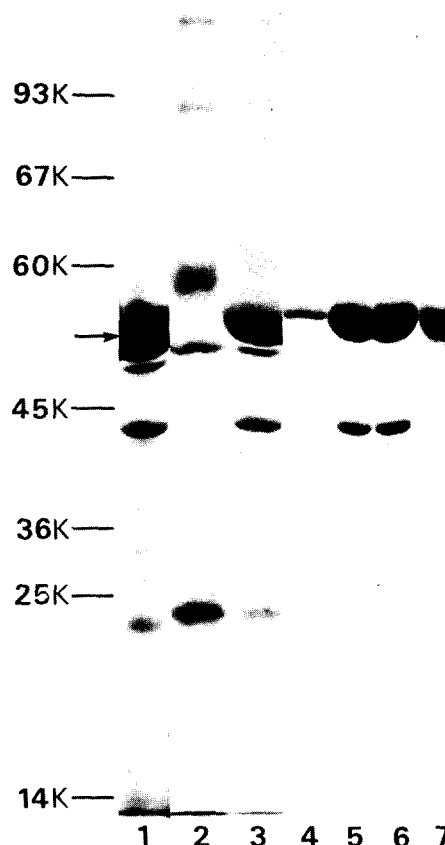


Fig. 1. Purification of the additional cell envelope protein from *A. salmonicida* strain V75/93, monitored by SDS-polyacrylamide gel electrophoresis. The position of the additional cell envelope protein is indicated by the arrow. The slots contain the following fractions. (1) cell envelope; (2) buffer I extract; (3) buffer I insoluble fraction; (4) buffer II extract; (5) buffer II insoluble fraction; (6) mixture of dansylated (10%) and non-dansylated (90%) buffer II insoluble fraction; (7) purified additional cell envelope protein.

by incubation for 10 min at 60°C and applied to 5 mm thick gels at a protein concentration of 1 to 2 mg/cm<sup>2</sup>. The subsequent electrophoretic separation was monitored with a long wave (366 nm) ultraviolet lamp. After 5 to 6 h, when the bromophenol blue front marker already had left the gel, the separation was satisfactory, the electrophoresis was stopped and the additional cell envelope protein band was cut out. (iv) The gel slice was transferred to a syringe containing 5–10 ml of buffer IV. The gel was macerated by passing the mixture several times through a needle (diameter

TABLE I

COMPARISON OF THE AMINO ACID COMPOSITION (MOL %) OF THE ADDITIONAL CELL ENVELOPE (ACE) PROTEIN WITH THOSE OF THE PROTEIN SUBUNITS OF REGULAR SURFACE LAYERS OF THE GRAM-NEGATIVE BACTERIA *ACINETOBACTER* AND *SPIRILLUM* AND THE GRAM-POSITIVE BACTERIUM *CLOSTRIDIUM*, THAT OF A PORE PROTEIN OF THE OUTER MEMBRANE OF *E. COLI* K12 AND WITH THAT OF THE K88 FIMBRIAE OF ENTEROPATHOGENIC *E. COLI*

Amino acid	Composition of proteins: protein, source, apparent molecular weight <sup>a</sup> and reference					
	ACE protein <i>A. salmonicida</i> <sup>b</sup>	$\alpha$ -layer protein <i>Acinetobacter</i> sp.	HP-layer protein <sup>c</sup> <i>Spirillum serpens</i> sp.	S-layer protein <i>Clostridium thermohydrosulfuricum</i>	PhoE protein <i>E. coli</i> K12	K88 ad fimbriae <sup>e</sup> <i>E. coli</i> H56
	54 000	65 000 [14]	150 000 [13]	140 000 [15]	40 000 [31]	25 000 [32]
Lys	7.25	5.84	3.15	5.74	7.16	4.18
His	0.98	0.34	0.31	0.50	0.40	0.84
Arg	4.85	1.17	1.26	2.13	4.14	2.51
Half Cys	0	0	0	0	<0.10	0
Asx <sup>e</sup>	12.6	17.41	16.0	14.16	17.76	12.13
Thr	9.45	8.45	13.7	10.01	5.61	10.04
Ser	5.85	3.68	5.4	6.97	4.66	6.69
Glx	7.94	9.06	4.1	6.19	9.59	7.53
Pro	2.49	0	1.47	4.47	1.31	2.93
Gly	9.64	21.10	13.4	7.90	11.50	14.23
Ala	11.8	12.67	14.4	8.71	8.53	10.88
Val	7.51	9.31	7.15	10.71	3.63	7.95
Met	0	0	0.21	0.95	1.94	0.83
Ile	4.64	2.98	4.63	4.79	3.99	4.60
Leu	9.23	5.13	7.68	6.41	6.61	7.53
Tyr	1.02	0	1.15	0	6.66	2.93
Phe	4.73	2.86	1.89	3.63	6.51	4.18
Trp	n.d. <sup>d</sup>	n.d.	3.89	5.25	n.d.	n.d.

<sup>a</sup> Molecular weights as estimated by SDS polyacrylamide gel electrophoresis.

<sup>b</sup> The results presented in the table are the average of duplicate analyses. Hydrolysis of the protein was conducted for 24 and 72 h, respectively. Serine and threonine contents are corrected for losses as described in Methods. Neither sulphur-containing amino acids nor amino sugars were detected.

<sup>c</sup> Since no amide content was determined the values for aspartate and asparagine (Asx) and glutamate and glutamine (Glx) respectively, are taken together.

<sup>d</sup> Not determined.

<sup>e</sup> Calculated from the data shown in Refs. 13 and 32.

1.3 mm). The resulting mixture was transferred to a screwcapped vial using another 5–10 ml of buffer IV. The vial was rotated slowly and elution was carried out at 22°C for 16 h. The gel particles were collected by centrifugation for 10 min at  $880 \times g$  and extracted twice more at 22°C during 3 h. It should be noted that presence of glycerol in the elution buffer was essential for obtaining satisfactory yields (50–60%) whereas the yield was 4-times lower in the absence of glycerol. The pooled eluates were extensively dialyzed against buffer V for 48 h with several changes of buffer. The contents of the dialysis bags were filtered using a Millipore filter (pore size  $5 \mu\text{m}$ ) in order to remove small gel particles and the resulting filtrate was subsequently lyophilized. (v) the dry material was dissolved in a solution of 10 mM  $\text{NH}_4\text{HCO}_3$ , pH 7.7, to a final SDS concentration of 2%. The protein was freed from SDS by acetone precipitation [20] and from the coprecipitated salts by three successive washings with distilled water. The resulting preparation consisted of practically pure additional cell envelope protein (Fig. 1, slot 7), of which the overall yield was mainly dependent on the efficiency with which the protein was extracted from the gel slices.

#### *Chemical characterization of the additional cell envelope protein*

The final preparation of the water-insoluble protein hardly contained protein impurities as analysis of the preparation by densitometrical scanning showed that, depending of whether the preparative gel had been overloaded or not, the purity of the additional cell envelope protein varied between 92% and  $\geq 98\%$ . The N-terminal amino acid sequence of the additional cell envelope protein was  $\text{H}_2\text{N-Asp-Val-Leu-Leu}$  indicating that the preparation was homogenous. Since amino sugars were not detected by amino acid analysis, and no periodic acid-Schiff positive material was detected on gels overloaded with purified additional cell envelope protein, this protein is probably devoid of sugar residues. The amino acid composition of the purified protein is given in Table I. The least abundant amino acids are tyrosine and histidine. From the calculated minimum molecular weight of 10750 and the estimated apparent molecular weight on gels of 52000–55000, it must be con-

cluded that the real molecular weight must be close to 54000. The results of Table I also show that sulphur-containing amino acids are not present in the additional cell envelope protein. The protein is likely to be hydrophobic in nature, because of its insolubility in water.

Comparison of the amino acid composition of the additional cell envelope protein with those of regular surface layer proteins from other bacteria (Table I) shows interesting similarities despite considerable differences in molecular weights. As a pore function has been suggested for the surface layer protein of *Spirillum* species [30], the amino acid composition of the PhoE protein [31], one of the pore proteins of *E. coli* K12, has also been listed in Table I. Since it has been reported that *A. salmonicida* strains that possess an additional cell wall layer show adhesive properties towards various types of eukaryotic cells [9], the amino acid composition of ad adhesive K88 fimbriae of enteropathogenic *E. coli* [32] has also been included in Table I. Comparison of these data with those of the additional cell envelope protein again shows striking similarities, especially with the K88 fimbriae. Immunological studies could help answer the question of whether these similarities are based on structural relationships.

The purified protein and antibodies raised against it should enable us to test several parts of our hypothesis that the additional cell envelope protein is (one of) the building block(s) of the additional surface layer and that it therefore is involved in adhesion to fish cells. Moreover, the observation that antibodies also react with a protein of approximately the same molecular weight from *A. salmonicida* isolates derived from other habitats [11] raises the possibility that the additional cell envelope protein may be used as (part of) a vaccine against *A. salmonicida* infections, provided that the antigenic relationship also exists at the level of the cell surface.

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