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CELL SURFACE OF THE FISH PATHOGENIC BACTERIUM *AEROMONAS SALMONICIDA*

I. RELATIONSHIP BETWEEN AUTOAGGLUTINATION AND THE PRESENCE OF A MAJOR CELL ENVELOPE PROTEIN

DOLF EVENBERG [a,*], RIA VAN BOXTEL [a], BEN LUGTENBERG [a], FRANK SCHURER [b], JOSÉ BLOMMAERT [b] and REMMELT BOOTSMA [b]

[a] Institute for Molecular Biology and Department of Molecular Cell Biology, University of Utrecht, Transitorium 3, Padualaan 8, 3584 CH Utrecht, and [b] Department of Pathology, Faculty of Veterinary Medicine, University of Utrecht, Biltstraat 372, 3572 BP Utrecht (The Netherlands)

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A comparison was made of membrane protein patterns of various *Aeromonas salmonicida* strains, initially isolated from different habitats with respect to fish species affected, pathological entity, and geographic location of the outbreak of the disease. A major protein with a molecular weight of 54000 was found in all autoagglutinating strains, whereas this protein is present in low amounts, or not at all, in non-autoagglutinating strains. Evidence for a causal relationship between the presence of this protein and the phenomenon of autogagglutination came from the observation that a change of the growth medium led simultaneously to an almost complete loss of the additional cell envelope protein and the property of autoagglutination. As it has already been reported that autoagglutination is correlated with the presence of an additional cell surface layer, we hypothesize that the additional cell envelope protein is the (major) subunit of this layer. The application of the gel immuno radio assay, an immunological technique suited to detect antigens in a gel, revealed that the additional cell envelope proteins of all tested strains are immunologically related. The possibility to the use of this protein as a component of a vaccine against *A. salmonicida* infections is discussed.

Introduction

Aeromonas salmonicida subsp. *salmonicida*, the causative agent of septicaemic furunculosis of Salmonidae, was isolated by Emmerich and Weibel in 1894 [1] and still represents a major threat to salmonid fish reared throughout the world. Only in the last decade atypical strains [2] of this bacterium have been reported as the causative agent of another disease, commonly known as ulcer disease in goldfish [3,4], carp erythrodermatitis [5,6] and atypical furunculosis in salmonids [7,8]. These strains are also able to cause mass casualties among several fish species in the wild [9,10]. In contrast to subsp. *salmonicida*, most atypical strains of *A. salmonicida* grow slowly, are fastidious with respect to growth requirements [4,5,8] and autoagglutinate so strongly that the routine serological agglutination technique for whole cells is not applicable to these strains [6,8,9]. The cohesive properties of subsp. *salmonicida* have

* To whom correspondence should be addressed.
Abbreviation: SDS, sodium dodecyl sulphate.

been correlated with the presence of an additional surface layer and with enhanced virulence [11].

The presence of an additional surface layer is not uncommon among both Gram-positive and Gram-negative bacteria (for reviews see Refs. 12 and 13). Freeze-etch studies of such surface layers show often patterned arrays of subunits which generally consist of one protein species [13]. As such a layer of protein can be expected to yield a major band upon analysis of isolated cell envelopes by SDS-polyacrylamide gel electrophoresis, we have compared the cell envelope protein patterns of a large number of *A. salmonicida* strains which vary with respect to autoagglunitation properties, to pathogenicity, to fish species and geographic location. The results show that autoagglutinating strains contain abundant amounts of a cell envelope protein which is absent, or present in only low amounts, in non-autoagglutinating *A. salmonicida* strains. Based on these and other observations and on the above-mentioned published data, we hypothesize that this cell envelope protein is the (major) building block of the additional surface layer and therefore is reponsible for autoagglutination and increased virulence.

Materials and Methods

Strains and growth conditions

The relevant properties and the source of the *A. salmonicida* strains used in this study are listed in Table I. Unless otherwise indicated, the bacteria were grown in tryptose/serum broth which consists of 1% (w/v) tryptose (Difco, Detroit, U.S.A.) supplemented with 10% (v/v) complement-inactivated horse serum, as described previously [5]. The final pH of the medium was 7.3. If necessary, the medium was solidified with 1.2% agar. In some experiments tryptose/glucose/salt broth, which consists of 1% (w/v) tryptose supplemented with 0.1% (w/v) glucose and 0.1% (w/v) synthetic sea salt (an isotonic replacement of horse serum) was used as the growth medium. Stock cultures were maintained on a semi-solid tryptose/serum medium [5]. Unless otherwise indicated, growth was started by diluting a stationary culture 1 : 100, followed by aerobic growth at 27°C for 48 h, as a static culture. The culture was shaken for 5 to 10 s, to resuspend the settled bacteria, and the growth was determined by measuring the turbidity at 660 nm. The autoagglutination properties of the strains were tested using the routine serological slide agglutination technique [14], with whole cells resuspended in a solution of 150 mM NaCl.

Isolation and characterization of cell envelopes

After growth in tryptose/serum broth, cells were harvested, washed and sonically disrupted. Cell envelopes were isolated by differential centrifugation as described earlier [15]. After washing, cell envelopes were resuspended in 2 mM Tris-HCl buffer (pH 7.5) and stored at −20°C. The protein patterns of the cell envelopes were analyzed by SDS-polyacrylamide gel electrophoresis as described before [15]. The periodic acid-Schiff staining procedure was used to detect carbohydrate containing components [16].

Immunological methods

The additional cell envelope protein was purified as described in the accompanying paper [17]. Antisera were raised in rabbits as described before for the PhoE protien of *Escherichia coli* K12 [18]. After inactivation of complement, one volume of antiserum was mixed with one volume of an approx. 100-fold concentrated suspension of cells of the non-autoagglutinating strain 152-69 in a solution of 15 mM NaCl/10 mM $KNaHPO_4$ buffer, pH 7.2. After incubation for 1 h at 25°C, the bacteria were removed by centrifugation. The adsorption procedure was repeated once and the resulting supernatant was used as the antiserum. Since later results showed that no significant differences were observed between adsorbed and unadsorbed antiserum, the adsorption procedure could have been omitted. Antigens in gels were detected by incubation of longitudinal gel slices with an appropriate dilution, approx. 100-fold, of antiserum followed by labelling of the IgG molecules bound to the antigen with ^{125}I-labelled protein A of *Staphylococcus aureus*. For the application of this procedure, designated as the gel immuno radio assay technique [19], we have followed the details described in Refs. 19–21, with the following modifications. (i) The serum diluent was supplemented with 0.5% (w/v) bovine serum albumin and either 0.04% (v/v) Triton X-100 or 0.02% (v/v) Tween-80 and, (ii) the buffer solution

TABLE I

RELEVANT PROPERTIES OF *A. SALMONICIDA* STRAINS [a]

Strain designation [b]	Disease lesion	Fish species [c]	Geographic [d] location	Subspecies or type [e]	Autoagglutination [f]	ACE protein [g]		Source, [h] reference
						Stained	GIRA	
V75/93*(+), V76/73, V76/134, V76/135	Erythrodermatitis	Carp (*Cyprinus carpio*)	YU	atypical	+	+	+	Bootsma [5]
V76/59*(+), V75/160A	Skin ulcer	Minnow (*Phoxinus phoxinus*)	NO	atypical	+	+	+	Håstein [10]
V75/174*(+), V75/175	Ulcer disease	Goldfish (*Carassius auratus*)	YU	atypical	+	+	+	Fijan
V76/65	Ulcer disease	Goldfish (*Carassius auratus*)	NL	atypical	+	+	n.d.	Bootsma
B38/77, M8, B105/78	Skin ulcer	Non-salmonid	SW	atypical	+	+	n.d.	Ljungberg [7]
B2531/77, B2547/77, B2577/77	Atypical furunculosis	Salmonid	SW	atypical	+	+	n.d.	Ljungberg [7]
B3020/77	Atypical furunculosis	Salmonid	SW	atypical	±	+	n.d.	Ljungberg [7]
NCMB 1110*(+)	Furunculosis	Brown trout (*Salmo trutta*)	UK	*achromogenes*	+	+	+	NCMB [25]
NCMB 2020	Furunculosis	Salmon (*Oncorhynchus masou*)	JP	*masoucida*	−	−	−	Kimura [22]
CIP 6713	Furunculosis	Salmonid	n.m.	*salmonicida*	±	+	+	Popoff
TG36/75, TG51/69	Furunculosis	Rainbow trout (*Salmo gairdneri*)	FR	*salmonicida*	±	−	+	Michel [27]
V80/68	Furunculosis	Rainbow trout (*Salmo gairdneri*)	NL	*salmonicida*	±	−	+	Bootsma
153-69, 152-69	Furunculosis	Salmonid	SU	*salmonicida*	−	−	−	Popoff [26]
126-68*(−), 211-67	Furunculosis	Rainbow trout (*Salmo gairdneri*)	FR	*salmonicida*	−	−	−	Popoff [26]

[a] Strains indicated with an asterisk have electronmicroscopically been tested for the presence of an additional cell surface layer; + and − indicate the presence and absence of this layer, respectively (Leunissen-Bijvelt, J. and Schurer, F., unpublished data).

[b] The strain designations used are those used by the donor, unless an accepted type culture collection designation is available.

[c] In a few cases the fish species from which the strain was initially isolated, was not known. Therefore a division in salmonid and non-salmonid fish was made.

[d] The country where the strain was initially isolated is indicated. FR, France; JP, Japan; NL, The Netherlands; NO, Norway; SU, Sowjet Union; SW, Sweden; UK, United Kingdom; YU, Yugoslavia; n.m., not mentioned by donor.

[e] *A. salmonicida* strains are divided into subspecies [28] and unclassed atypical strains.

[f] Autoagglutination as observed in 150 mM NaCl; +, strong (big aggregates and very difficult to resuspend); ±, weak (small aggregates which break easily during resuspension); −, negative (no autoagglutination was observed).

[g] For stained gels: + indicates that the additional cell envelope (ACE) protein can positively be identified and − indicates that no positive identification can be made. For gel immuno radio assay technique (GIRA): +, indicates that a specific reaction with anti-additional cell envelope protein serum was detected; −, no reaction observed. n.d.: not done.

[h] N. Fijan, Veterinary Faculty, University of Zagreb, Yugoslavia; T. Håstein, National Veterinary Institute, Oslo, Norway; T. Kimura, Faculty of Fisheries, Hokkaido University, Hokkaido, Japan; O. Ljungberg, National Veterinary Institute, Stockholm, Sweden; C. Michel, Laboratoire d'Ichtyopathologique, Thiverval-Grignon, France; NCMB, National Collection of Marine Bacteria, Aberdeen, United Kingdom; M. Popoff, Institut Pasteur, Paris, France.

used for washing the gel slices was supplemented with 0.02% (v/v) Triton X-100 or 0.01% (v/v) Tween-80. Addition of low concentrations of detergent to these solutions prevents adsorption of antiserum and protein A [19].

Results

Cell envelope protein patterns and autoagglutination of A. salmonicida strains

The autoagglutination properties of the various strains are listed in Table I, and the protein patterns of cell envelope preparations of most of these strains are shown in Fig. 1. For many of the strains heavy protein bands were observed in electrophoretic positions corresponding with apparent molecular weights (M_r) of about 75000, 54000, 50000, 42000, 22000 and $\leq$ 10000, respectively. A comparison of the protein patterns of autoagglutinating (Fig. 1, slots 1–15 and 17) and non-autoagglutinating strains (Fig. 1, slots 16 and 18–21) indicates the following. The only protein which is present in abundant amounts in autoagglutinating strains and which is absent, or present in only

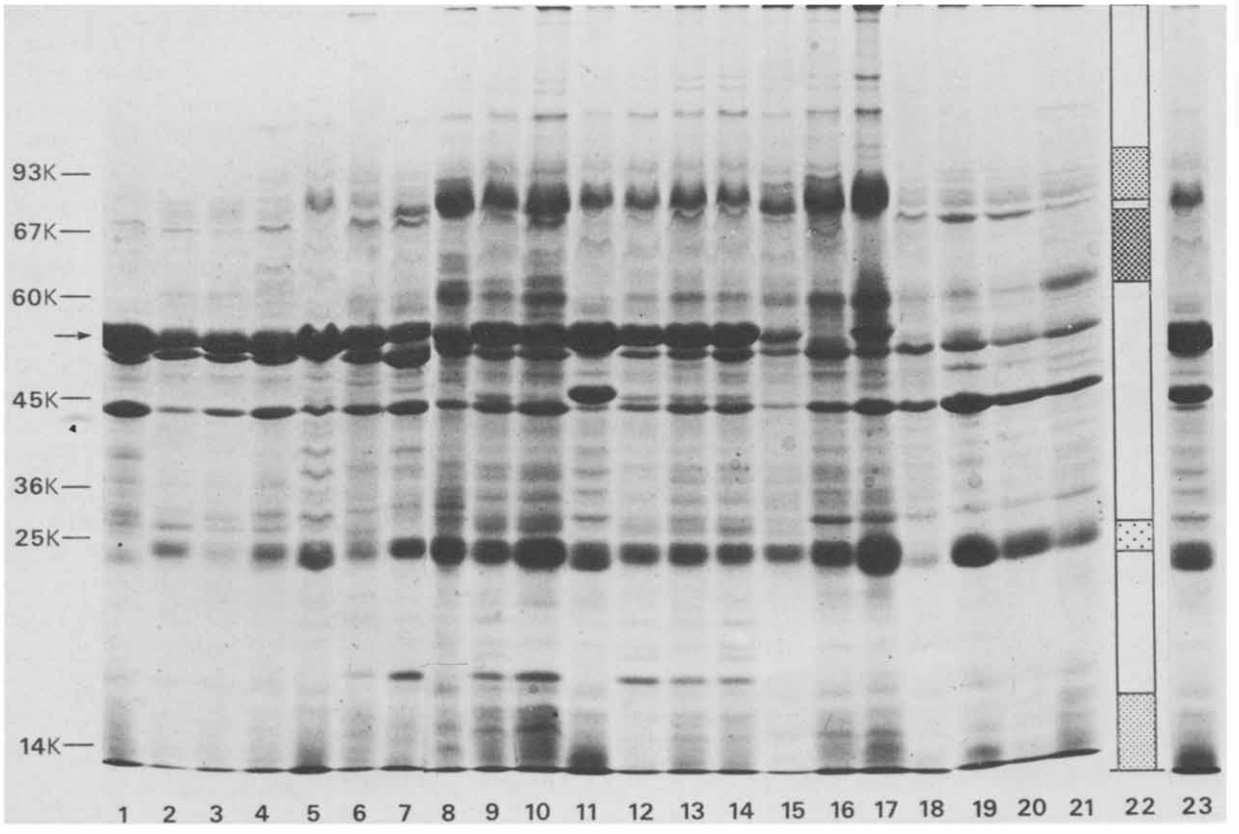

Fig. 1. Cell envelope protein patterns of various strains of *A. salmonicida* analyzed by SDS-polyacrylamide gel electrophoresis. The slots contain cell envelope preparations of the following strains: (1) V75/93, (2) V76/73, (3) V76/134, (4) V76/135, (5) V75/160A, (6) V76/59, (7) V75/174, (8) B38/77, (9) M8, (10) B105/78, (11) NCMB1110, (12) B2531/77, (13) B2547/77, (14) B2577/77, (15) B3020/77, (16) NCMB2020, (17) CIP6713, (18) 211-67, (19) 153-69, (20) 152-69, (21) 126-68. Lane 22 is a schematic representation of the periodic acid-Schiff staining pattern. The difference in density of staining observed is represented similarly by a difference in shading. Lane 23, the same as slot 11, is placed adjacent to lane 22, for purposes of comparison. The position of the molecular weight protein standards are indicated at the left of the gel. The standard protein bands are designated by their molecular weights, multiplied by 10^{-3} and followed by the letter K. The arrow indicates the electrophoretic position of the additional cell envelope protein.

low amounts, in non- or weakly autoagglutinating strains, corresponds with the cell envelope protein of M_r 54000. These results, summarized in Table I, show an absolute correlation between the amount of this protein and the property of the cells to autoagglutinate.

A comparison of the amount of cell envelope protein of cell envelope protein of M_r 54000 between subsp. *salmonicida* and other strains shows that this protein is generally abundantly present in the cell envelopes of subsp. *achromogenes*, and of all analyzed atypical strains. It should be noted that this result is independent of the fish species involved and the geographic location of the outbreak of the disease. Subsp. *masoucida* apparently lacks this protein (Fig. 1, slot 16) and does not autoagglutinate. The atypical *A. salmonicida* strain B3020/77, which has a lesser amount of the cell envelope protein of M_r 54000 (Fig. 1, slot 15) compared to the other atypical isolates, also shows a weaker autoagglutination reaction. Subsp. *salmonicida* was much more heterogenous with respect to the amount of the cell envelope protein of molecular weight 54000, which was present in significant amounts in a few strains (e.g. strain CIP 6713, Fig. 1, slot 17), but which is apparently absent or present in very low amounts in most typical isolates (e.g. strain 153-69, Fig. 1, slot 19). Despite the observed heterogeneity among typical strains, the amount of this protein of M_r 54000 again corresponds well with the tendency of the eells to autoagglutinate (Table I). As this latter property has previously already been correlated, with the presence of an additional surface layer [11], we designated this protein of M_r 54000 as the additional cell envelope protein.

As cell surface proteins can well be glycoproteins [13], we applied the periodic acid-Schiff staining procedure to gels on which cell envelope preparations of *A. salmonicida* strains had been electrophoresed. At least four rather diffuse positive regions (indicated in Fig. 1, slot 22) corresponding with apparent molecular weights of about 72000–95000, 60000–70000, 22000–26000, and $\leq$ 16000 were detected in the strains V75/93, V75/174, B105/78, B2531/77, CIP6731, NCMB1110 and NCMB2020. The periodic acid-Schiff positive material of M_r 72000–95000 was not detected in strains V75/160A, V76/73, V76/134 and 211-67. Strain 152-69 was lacking both the 72000–95000 and the 60000–70000 band. We do not know yet whether the periodic acid Schiff-positive reactions are due to the presence of glycoprotein or lipopolysaccharide. As no periodic acid Schiff-positive reaction was found in or near the electrophoretic position of the cell envelope protein of M_r 54000, the additional cell envelope protein is presumably not a glycoprotein.

Immunological detection of the additional cell envelope protein

Although the data described so far provide strong evidence for the assumption that the additional cell envelope protein detected in cell envelopes of all autoagglutinating strains has the same function in all strains tested, we applied immunological methods to answer the question concerning structural relationship. Moreover, a sensitive immunological technique could answer the question of whether in cases where only a low amount of protein is found in the 54000 position in the gel, traces of the additional cell envelope protein are present. Therefore, using the gel immuno radio assay technique, cell envelope preparations of various strains were incubated with antiserum raised against purified additional cell envelope protein of strain V75/93. The results (Table I and Fig. 2) showed that a 1:100 dilution of the antiserum resulted in a good and practically exclusive reaction at the 54000 (54 K) position in gels containing samples of three strongly autoagglutinating strains (Fig. 2B, slots 1–3). This reaction is specific for the additional cell envelope protein, as the same dilution of the preimmune serum did not react at all (not shown) and as a cell envelope preparation of a non-autoagglutinating strain (Fig. 2B, slot 4) did not react with the antiserum.

A surprising result was observed with cell envelopes of the autoagglutinating isolate V75/174. The antiserum reacted not only with the protein of M_r 54000 but also with that of M_r 50000 (Fig. 2B, slot 8). The stained gel shows that strain V75/174 is exceptional in that it has a heavier band of M_r 50000 (Fig. 2A, slot 8, and fig. 1, slot 7) than other strains. However, the observation that with preparations of other strains no immunological reaction in the gel could be detected at the position of

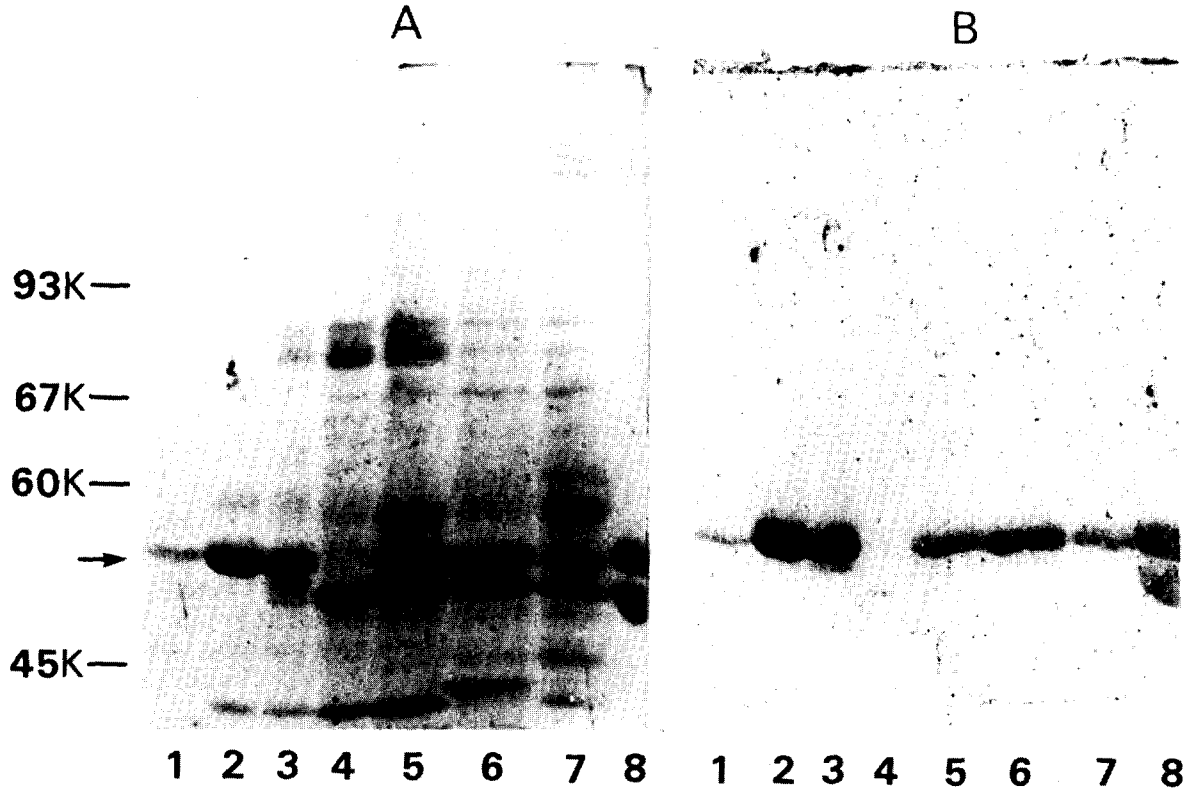

Fig. 2. Immunological detection of the additional cell envelope protein. After electrophoresis, three identical longitudinal slices of the same SDS-polyacrylamide gel were prepared from which one was stained (gel A) whereas the others were treated either with anti-additional cell envelope protein serum (gel B) or with preimmune serum (not shown), followed by incubation with ^{125}I-labelled protein A and autoradiography. The following cell envelope preparations were applied in the slots: (1) V75/93, (2) V76/134, (3) V75/160A, (4) NCMB2020, (5) CIP6713, (6) TG36/75, (7) V80/68, (8) V75/174. Slot 1 contained 3–6 μg protein whereas the other slots contained 15–30 μg protein. Positions of the molecular weight standard proteins are indicated at the left. The position of the additional cell envelope protein is indicated by an arrow. Only the relevant part of the gel is shown. The missing lower part showed no immunological reaction.

M_r 50000, strongly suggests that the increased amount of protein in this band is due to the presence of an extra additional cell envelope-like protein specifically in strain V75/174.

Whereas the additional cell envelope protein is usually difficult or impossible to detect in stained gels containing preparations of subsp. salmonicida cell envelopes (Table I and Fig. 2A slots 5–7), a clear positive reaction at the position of the additional cell envelope protein was observed after using the gel immuno radio assay technique (Fig. 2B, slots 5–7), indicating that small but significant amounts of additional cell envelope protein are present.

Simultaneous loss of additional cell envelope protein and autoagglutinating properties

The results described so far show an excellent correlation between the property of autoagglutination and the presence of the additional cell envelope protein (summarized in Table I). The validity of the correlation between these properties can be tested by using the observation that cultivation of atypical strains in a medium in which serum is replaced by an isotonically equal amount of synthetic sea salt results in loss of autoagglutination property. Comparison of the cell envelope protein pattern of strain V76/73 grown in tryptose/serum broth (Fig. 3, slot 1) with that of the same strain

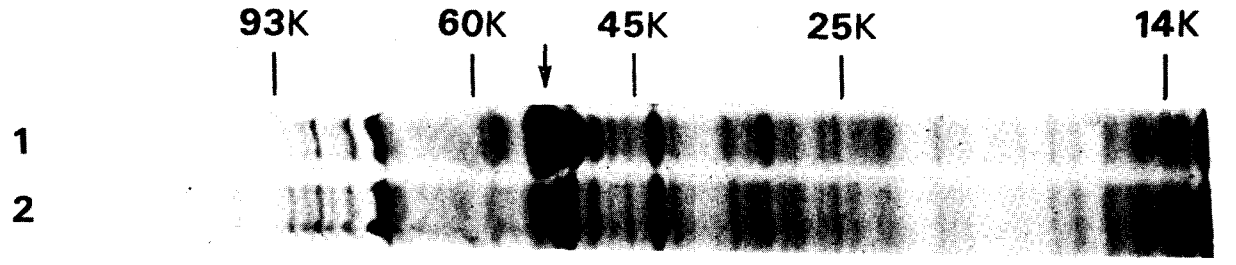

Fig. 3. Influence of the composition of the growth medium on the amount of additional cell envelope protein. Cells of strain V76/73 were grown in tryptose/serum broth and in tryptose/glucose/salt broth as described. After forty transfers cells growing in the latter broth practically did not autoagglutinate anymore. A comparison of cell envelope protein patterns of cells grown in the two media shows that cells grown in tryptose/serum broth (slot 1) produce large quantities of additional cell envelope protein (see arrow) whereas this protein was hardly found after growth in tryptose/glucose/salt broth (slot 2).

grown in tryptose/glucose/salt broth after 40 transfers (Fig. 3, slot 2), clearly shows that the amount of additional cell envelope protein strongly decreased by growth in the latter medium whereas no significant changes in the amounts of the other proteins were observed. Simultaneously, the autoagglutination property of the bacterial cells decreased dramatically. These experiments once more show a correlation between the property of autoagglutination and the presence of the additional cell envelope protein.

Discussion

In contrast to most subsp. *salmonicida* isolates, atypical *A. salmonicida* isolates keep their cohesive properties, even on repeated transfer, provided that the appropriate medium is used e.g. tryptose-serum broth. Autoagglutination of *A. salmonicida* has been correlated with the presence of an additional surface layer (Refs. 11, 24 and Table I). In several bacteria such a layer has been found to consist of protein subunits [12,13]. In order to see whether autoagglutination can be correlated with the presence of a specific cell envelope protein, we initiated a screening of cell envelope protein patterns of 17 autoagglutinating and five non-autoagglutinating strains. The results (Table I and Fig. 1) showed that a protein with an apparent molecular weight of about 54000, was the only candidate as it is present in autoagglutinating strains and absent, or present in only low amounts, in non-autoagglutinating strains. We have designated this protein the additional cell envelope protein. The gel immuno radio assay technique, in which the excellent resolution obtained with SDS-polyacrylamide gel electrophoresis is combined with the specificity of antibodies, enabled us to show that the additional cell envelope protein of strain V75/93 is cross-reactive with a protein of the same electrophoretic mobility present in cell envelope preparations of all tested autoagglutinating strains, but is not present in most preparations of non-autoagglutinating strains (Fig. 2). The goldfish isolate V75/174 even contains a second cross-reactive protein of slightly lower apparent molecular weight (Fig. 2A, slot 8 and Fig. 2B, slot 8). Additional strong support for the notion that the additional cell envelope protein is directly responsible for autoagglutination was provided by experiments which showed that growth of an atypical isolate in a medium in which the serum was replaced by an isotonically equal amount of synthetic sea salt resulted in a strong decreased of cohesive properties. This phenomenon was accompanied by a dramatic decrease of the amount of additional cell envelope protein whereas no significant changes were detected in the amounts of the other cell envelope proteins (Fig. 3). These combined results indicate that the additional cell envelope protein is a major, or even the only, constituent of the additional layer, which is thought to be involved in autoagglutination, in adhesion to the fish epidermal mucus or epidermal cell surface, and in virulence [11,24]. Some of our recent results support and extend this concept. Firstly, using the freeze-etch technique, a regular tetragonal pattern of subunits with a center to center distance of

approx. 10 nm was found on the cell surface of autoagglutinating strains, but not on that of a non-autoagglutinating strain of subsp. *salmonicida* (Leunissen-Bijvelt, J. and Schurer, F. unpublished data). Secondly, preliminary immunofluorescence studies with antisera raised against the purified additional cell envelope protein, indicate that this protein is located at the cell surface (Evenberg, A. and Vos-Maas, M., unpublished data).

The observation that the additional cell envelope protein of strain V75/93 is immunologically related with the same protein of other autoagglutinating isolates from different geographic locations and from a variety of fish species (Table I) is promising from the viewpoint of protection against infection, by vaccination. If this cross-reactivity of additional cell envelope proteins, which so far has been tested only at the level of the purified antigens in gels, can also be observed in intact cells, the additional cell envelope protein would be an excellent candidate to become a constituent of a vaccine. The additional cell envelope protein might even be useful to raise immunity against septicaemic furunculosis, despite the virtual absence of the additional cell envelope protein in several subsp. *salmonicida* (Table I and Figs. 1 and 2). Since it has been described that typical strains easily lose their cohesive properties [9,25–27] as well as their additional layer [11], we interpret the observed differences in the amounts of additional cell envelope proteins between strains of subsp. *salmonicida* in terms of differences in the rate with which the additional layer is lost or in terms of strains which are in different stages of losing this layer. With respect to the mechanism involved in losing the additional layer, an interesting question is whether in a culture of subsp. *salmonicida* with low amounts of additional cell envelope protein either all individual cells contain low amounts of this protein or whether most cells have lost all additional cell envelope protein whereas a few cells still have abundant amounts and therefore a complete additional layer. Studies to answer this question are currently in progress in our laboratory.

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Our prediction that the additional cell envelope protein is surface located was recently supported by Kay et al. (1981) J. Bacteriol. 143, 1077–1084, who reported that a surface localized major protein (M_r 49000) is specifically present in virulent strains.

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