Production and secretion of collagen-binding proteins from Aeromonas veronii

Ascencio, F; Hirst, T R; Wadström, Torkel

Published in:
Journal of Applied Microbiology

DOI:
10.1046/j.1365-2672.2000.01157.x

2000

Journal of Applied Microbiology 2000, **89**, 607–616

Production and secretion of collagen-binding proteins from *Aeromonas veronii*

F. Ascencio[1], T.R. Hirst[2] and T. Wadström[3]

[1]*Department of Marine Pathology, Center for Biological Research, La Paz,Mexico,* [2]*Department of Pathology and Microbiology, University of Bristol, UK, and* [3]*Department of Medical Microbiology, University of Lund, Sweden*

195/2/2000: received 21 February 2000, revised 30 May 2000 and accepted 7 June 2000

F. ASCENCIO, T.R. HIRST AND T. WADSTRÖM. 2000. Collagen-binding protein (CNBP) synthesized by *Aeromonas veronii* is located conserved within the subcellular fraction. The results of this study show that 98% of the total CNBP produced by *Aer. veronii* is present in the extracellular medium, and that the remaining CNBP is distributed either on the cell surface, within the periplasm or anchored on the outer membrane. CNBP is specifically secreted from *Aer. veronii* into the culture medium, because all the *β*-lactamase activity was located in the cells and could be released by polymixin B extraction of periplasmic proteins. CNBP was produced at growth temperatures from 12 °C to 42 °C, but not at 4 °C. The findings indicate that the level of CNBP in the medium increases during the exponential growth phase and reaches a maximum during the early stationary phase. There was less CNBP production in poor nutrient MMB medium than in the rich LB nutrient medium. CNBP secretion, in contrast to aerolysin secretion, was unaffected by the *exeA* mutation of *Aer. hydrophila*. It is concluded that CNBP secretion from *Aer. veronii* must be achieved by a mechanism different from that reported for aerolysin secretion.

INTRODUCTION

Aeromonas veronii causes a variety of human infections, including arthritis, gastroenteritis, meningitis, septicemia and wound infections (Hsueh *et al.* 1998; Janda and Abbott 1998; Steinfeld *et al.* 1998), and is also reported as a pathogen of cold-blooded vertebrates (Trust 1986; Pasquale *et al.* 1994; Sugita *et al.* 1995; Simmacco *et al.* 1998). *Aeromonas veronii* has been reported to produce a variety of biologically-active extracellular products which may be involved in the pathogenesis of *Aeromonas* infections. Among these products are cytotoxins or haemolysins (Stelma *et al.* 1988; Neves *et al.* 1990), enterotoxins (Honda *et al.* 1985; Neves *et al.* 1990), and a number of proteolytic and glycosidic enzymes (Allan and Stevenson 1981; Gobius and Pemberton 1988; Leung and Stevenson 1988). *Aeromonas veronii* produces several adhesion factors, some of them components of pili (Carrello *et al.* 1988; Kirov *et al.* 1995; Kirov and Sanderson 1996) or expressed on the cell surface (Kostrzynska *et al.* 1992). Other *Aeromonas* lectins are also recovered from the extracellular medium (Stewart *et al.* 1986). However, it is still unknown how these non-fimbrial putative adhesins are anchored on the cell surface, how soluble adhesins and lectins are secreted into the surroundings, and what their biological significance is in infectious processes.

In previous work, a number of *Aeromonas* strains, isolated from the environment and from human infections, were screened for collagen-binding activity. The results showed that collagen-binding is a common property among *Aeromonas* species (Ascencio *et al.* 1991). The pathogenicity of *Aer. veronii* in infectious diarrhoea in humans is probably not derived from a single or even a few traits, but from the cumulative or combined contribution of multiple virulence factors (Hoepelman and Tuomanen 1992). From this perspective, adhesion of bacteria is an early process in the development of an infection that involves binding of bacterial adhesins and lectins to gastrointestinal mucosal receptors.

As antigens from *Aer. veronii* with affinity for mucosal constituents and collagenous proteins have recently been found to stimulate the mucosal immune system of rabbit

Correspondence to: Dr F. Ascencio, Marine Pathology Unit, Center for Biological Research, PO Box 128, La Paz, Baja California Sur 23000, Mexico (e-mail: ascencio@cibnor.mx).

(Ascencio *et al.* 1995) and fish (Merino-Contreras *et al.*, personal communication), and are considered as potential vaccine candidates for *Aeromonas* infections in fish farming, the production and secretion of a 98 kDa protein from *Aer. veronii* with affinity for collagen (CNBP) was investigated.

MATERIALS AND METHODS

Chemicals

Water-soluble, rat skin collagen type I was purchased from Serva Feinbiochemica GMBH & Co. (Heidelberg, Germany). Peroxidase-conjugated immunoglobulins and OPD (1,2-phenylenediamine) tablets were from Dakopatts A/S (Glostrup, Denmark). 2-[N-morpholino]ethanesulphonic acid (MES), 1,4-piperazinediethanesulphonic acid (Pipes), and protease from *Staphylococcus aureus* strain V8, were purchased from Sigma. Culture media and individual culture media ingredients were purchased from Difco. Immobilon-PVDF membranes were from Millipore Corp. (Bedford, MA, USA).

Bacteria and culture conditions

Aeromonas veronii strain A186 isolated from a human infection at the Hospital of the University of Lund, Sweden, and which is a high binder for collagen type I and IV (Ascencio *et al.* 1991), was taxonomically identified according to its fatty acid profile by Dr J. McInroy at Auburn University, Alabama, USA. *Aeromonas hydrophila* wild type strain Ah65, and its derived pleiotropic secretory mutants, C5.84 and L1.97, containing a transposon T*n5*-751 insertion in *exeA* (Jiang and Howard 1991), were kindly provided by Prof. S. Peter Howard from the University of Regina, Canada. All strains were grown at 32 °C in Luria-Bertani broth (LB) supplemented with the appropriate antibiotics (Jiang and Howard 1991). Bacterial cells were harvested by centrifugation (5000 *g* at 4 °C for 30 min). Supernatant fluids were filtered through a 0·22 μm nitrocellulose filter and kept at −80 °C until use. Bacterial pellets were washed once with 0·02 mol l^{-1} potassium phosphate buffer (pH 7·2) containing 0·15 mol l^{-1} NaCl (PBS), suspended in PBS to a density of 10^{10} cells ml^{-1} and used immediately for collagen-binding assays (see below).

Growth studies to evaluate the effect of the culture conditions on the production of extracellular proteins

Aeromonas veronii was grown in LB broth at 32 °C in an orbital shaker at 120 rev min^{-1} and maintained until the cell density reached a value of 0·45 optical density (O.D.) units at 540 nm. A 1 litre flask containing 300 ml broth was inoculated with this culture (10 ml) and incubated under similar conditions at temperatures of 4, 12, 22, 32, 37 and 42 °C. Samples were taken at indicated periods of time and centrifuged as described above. Supernatant fluids were stored at −80 °C until use. Other experiments included the growth of cells in a biphasic medium, consisting of a solid phase with 1·5% agarose, 100 μg ml^{-1} collagen type I in 50 mmol l^{-1} Tris–HCl buffer (pH 7·5) containing 5 mmol l^{-1} $CaCl_{2,and}$ a liquid phase containing a minimal medium broth (Mevarech and Werczberger 1985) adjusted to 0·15 mol l^{-1} NaCl. An alternative biphasic medium was made of 100 μg ml^{-1} collagen type I in LB agar for the solid phase and LB broth for the liquid phase. After incubation overnight at 32 °C, the liquid phase was centrifuged and the supernatant fluids were stored at −80 °C until use.

Preparation of subcellular fractions

Aeromonas cells were grown in LB broth at 32 °C and harvested by centrifugation when the culture reached the stationary phase, usually after 18 h. The cell pellet was washed once with PBS and suspended in PBS containing 200 U ml^{-1} polymyxin B sulphate to liberate the periplasmic space proteins (Leece and Hirst 1992). After 4 h incubation in an ice-bath, bacterial cells were centrifuged (8000 *g* at 4 °C for 30 min) and the supernatant fluid dialysed during 24 h, with three changes of PBS. Cell-associated proteins were removed by treating the bacterium with 0·2 mol l^{-1} glycine buffer, pH 3, for 30 min incubation in an ice-bath (Dooley *et al.* 1988), or by simply washing the cells with PBS. Outer membrane proteins (OMPs) were extracted by treating the cell envelope with 0·5% sodium lauroylsarcosine (Filip *et al.* 1973). The cytoplasmic content was released by disrupting the cells with three 30 s sonication bursts (Howard and Buckley 1983).

Production of anti-serum against the *Aer. veronii* CNBP

Anti-serum to the purified 98 kDa CNBP was obtained from adult New Zealand White rabbits injected with 30 μg CNBP emulsified in Freund's complete adjuvant. Booster doses of 20–30 μg protein in Freund's incompleted adjuvant were given 15 and 30 days after the initial immunization. On day 40, the rabbits were bled and the serum was collected and stored at −80 °C. Western blot analysis of whole-cell lysates of *Aer. veronii* strain A186 showed that the anti-sera were specific to the CNBP (data not shown).

Electrophoresis

The separation of proteins by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was done using the discontinuous buffer system of Laemmli (Laemmli 1970). The pooled fractions comprising the peaks that inhibited collagen-binding activity were stacked in 4·5% (w/v) acrylamide and separated in 12·5% (w/v) acrylamide. Electrophoresis was run in a Protean II xi apparatus (Bio-Rad Laboratories, Richmond, CA, USA) at 20 mA (constant current) initially, and at 30 mA when the tracking dye entered the separating gel. Molecular weights were determined from a plot of the logarithm of the M*r* using SDS–PAGE protein molecular weight standards (Bio-Rad).

Western blot analysis

The separated proteins (under denaturating or non-denaturating conditions) were electrophoretically transferred to immobilon-PVDF membranes in a trans-blot cell. Additional binding sites were blocked by incubating the membranes with 3% bovine serum albumin in 10 mmol l^{-1} Tris–HCl-0·15 mol l^{-1} NaCl, pH 7·2, for 1 h at 22 °C. Membranes were washed 3 times with 10 mmol l^{-1} Tris–HCl-0·15 mol l^{-1} NaCl, pH 7·2, containing 0·05% Tween−20 (TNT), at 22 °C. The membranes were probed with horseradish peroxidase (POD)-labelled collagen type I (Hudson and Hay 1989) in 10 mmol l^{-1} Tris-HCl-0·15 mol l^{-1} NaCl (pH 7·2) for 2 h at 22 °C. After washing 3 times with TNT, the reactive bands were visualized with diaminobenzidine as POD substrate.

ELISA to quantify soluble CNBP

ELISA plates (Nunc, Denmark) were coated with 100 μl of a solution of collagen type I (CnI) in 0·1 mol l^{-1} sodium carbonate buffer (pH 8·1) containing 0·15 mol l^{-1} NaCl (10 μg of CnI well^{-1}). Plates were washed 4 times with TNT after 16 h of incubation at 4 °C. Supernatant fluids (100 μl) were added to each collagen-coated well and incubated for 1 h at 37 °C. Wells were washed 4 times with TNT, and 100 μl rabbit polyclonal antibodies raised against purified CNBP (1:1000) were added to each well. After 2 h, wells were washed 4 times with TNT, and 100 μl POD-labelled goat IgG specific for rabbit IgG (diluted 1:5000) were added to each well and incubated at 37 °C for an additional 90 min. Then, the plates were washed 4 times with TNT, a substrate solution of 1 mg ml^{-1} OPD in 50 mmol l^{-1} sodium citrate (pH 5) was added to each well (100 μl well^{-1}), and the plates were incubated for 30 min. The A_{492} value of each well was then determined. The CNBP is expressed in Units, where one U is equivalent to one O.D. unit at 492 nm. These values were corrected for non-specific binding (less than 10%).

Determination of proteolytic activity

Extracellular proteolytic activity in the supernatant fluids was determined using latex beads coated with ^{125}I-labelled collagen type I (Ascencio and Wadström 1994). Briefly, portions (100 μl) of the sample to be assayed were mixed with 100 μl 50 mmol l^{-1} Tris buffer (pH 7) containing 0·15 mol l^{-1} NaCl, 0·5 mmol l^{-1} $CaCl_2$ (TBS) and 10 μl ^{125}I-protein-coated latex-bead suspensions (specific activity of $8{\cdot}5 \times 10^5$ cpm ml^{-1} latex bead suspension diluted to 5×10^5 cpm ml^{-1}) in 96 well (V form) microtitre plates. After 1 h at 37 °C, the incubation mixtures were centrifuged in the plate (2000 *g* at 4 °C for 12 min), and the radioactivity of the supernatant fluids (which contained the ^{125}I-labelled peptides released from the coated latex beads) was measured in a Gamma counter (Clini Gamma, WALLAC, Abo, Finland). Positive controls were tested with purified protease from *Staphylococcus aureus* strain V8. To correct for non-enzymatic hydrolysis of the substrates, the coated latex beads were incubated with the incubation buffer (TBS or uninoculated broth instead of the protease-containing samples). Proteolytic activity is expressed as the percentage of ^{125}I-label released from the ^{125}I-protein-coated latex beads.

RESULTS

Production and cellular distribution of CNBP

The distribution of CNBP between the extracellular medium and the cells, and within the different subcellular compartments, was determined using a CNBP-specific ELISA (Table 1). It was found that 98% of the total CNBP produced by *Aer. veronii* was present in the extracellular medium and the remaining CNBP was distributed either on the cell surface or within the periplasm. It is concluded that under these growth conditions, CNBPs are extracellular proteins.

To examine whether the CNBP was specifically secreted or non-specifically released as a result of cell lysis, besides the release of CNBP, the distribution of a periplasmic marker enzyme, β-lactamase, was also examined. As shown in Table 2, the β-lactamase activity was located inside the cells as it could be released to the same extent by either polymyxin B extraction of periplasmic proteins or by sonication treatment (Table 2). It is therefore concluded that CNBP is specifically secreted from *Aer. veronii* into the culture medium.

The cellular distribution of CNBP in *Aer. veronii* strain A186 was also examined by a collagen-blotting technique

Table 1 Production and distribution of CNBP in subcellular fractions by *Aeromonas veronii* strain A186

Fraction	Total protein (mg)	CNBP (U)	Specific activity
Extracellular (culture supernatant fluid)	14	5130	71
Cell-associated (glycine extract)	0·2	33	145
Periplasmic (polymyxin B treatment)	0·1	27	205
Cytoplasmic (lysed cells by sonication)	0·4	50	125

Bacterial cells grown overnight at 32 °C in a 1 litre flask containing 300 ml LB broth to an O.D.$_{600}$nm of approximately 2·0 were harvested, and subcellular fractions obtained as described in Material and Methods. CNBP was quantified in each fraction and the specific activity is expressed as the amount of CNBP (U) presented in 1 mg of protein.

(Fig. 1). SDS–PAGE analysis of the medium and total cell lysates showed that the medium contained only a few proteins, but when the same fractions were tested by the collagen blot technique, most of the CNBP was present in the extracellular fraction. It is concluded that growth of *Aer. veronii* in LB broth at 32 °C results in the production and secretion of extracellular CNBP.

Effect of growth conditions on the production of CNBP

Growth temperature has been shown to influence the expression of virulence factors from a variety of pathogenic micro-organisms (Kabir and Ali 1983; Gonzalez *et al.* 1988). To examine whether the growth temperature influenced the production of CNBP by *Aer. veronii*, strain A186 was cultured at 32 °C for 18 h, then sub-cultured into fresh broth and maintained at growth temperatures ranging from 4 to 42 °C. CNBP was produced at all growth temperatures from 12 °C to 42 °C, but not at 4 °C (Fig. 2).

At 42 °C, it was observed that the level of CNBP in the medium increased during the early stationary phase, then decreased rapidly over the next 2 h of growth. One explanation for this may be the production of proteinases which might degrade CNBP (Allan and Stevenson 1981; Ascencio and Wadström 1991). Therefore, the level of extracellular proteinases produced during growth at different temperatures was also examined. There appeared to be a relationship between the level of CNBP and the level of extracellular proteases in the medium (Fig. 2).

Table 2 Release of periplasmic proteins from *Aeromonas veronii* A186 treated with Polymyxin B sulphate

	CNBP (U)		Total protein (mg)		β-Lactamase activity (A_{492}nm)	
Time (min)	Polymyxin extraction	Sonication	Polymyxin extraction	Sonication	Polymyxin extraction	Sonication
10	44·2	23·8	0·53	15·7	0·52	0·57
30	53·2	21·1	0·61	18·3	0·53	0·50
120	48·5	28·6	1·06	18·4	0·51	0·55
240	52·1	29·1	1·92	14·1	0·54	0·42
960	65·6	22·8	10·02	12·8	0·51	0·59

Bacterial cells grown overnight at 32 °C in a 1 litre flask containing 300 ml LB broth to an O.D.$_{600}$ nm of approximately 2·0 were harvested and then treated with Polymyxin B sulphate as described in Material and Methods. β-Lactamase activity was determine using the Nitrocefin kit, based on a chromogenic cephalosporin, according to the instructions of the manufacturer (Glaxo Research Limited), and the results expressed in O.D. units at 492 nm.

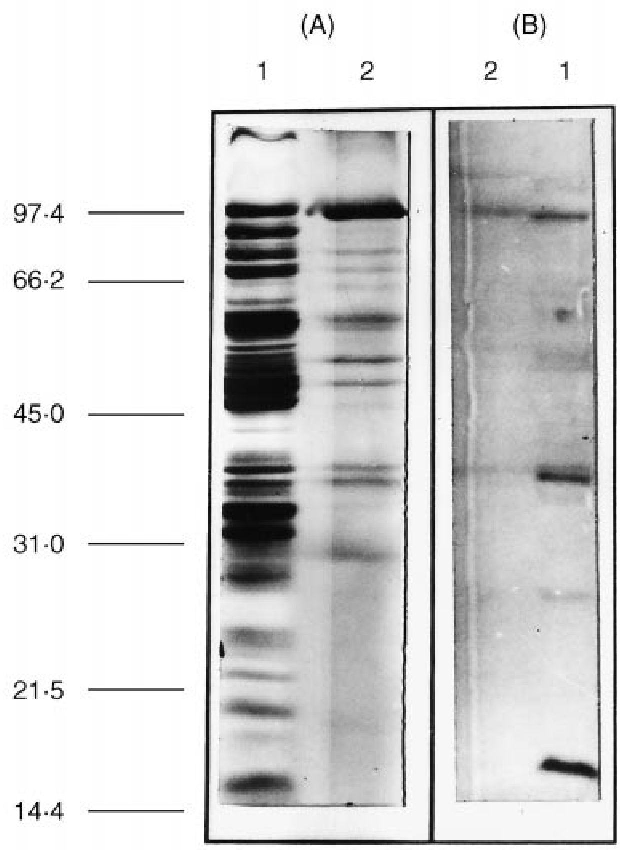

Fig. 1 Detection of CNBP from *Aeromonas veronii* strain A186. (A) SDS–PAGE of sonicate and extracellular proteins. (B) Western blot analysis of expression of *Aer. veronii* CNBP using peroxidase-labelled collagen type I as a probe. Lane 1: sonicate extract; lane 2: extracellular CNBP (culture supernatant fluids). Molecular size markers (in kilodaltons) are indicated on the left side

The reason why CNBP is not present in the medium at 4 °C is not because of its inability to be secreted, since an analysis of the cell growth at this temperature did not show any cell-associated CNBP (Fig. 2), but generally, the level of CNBP in the medium increased during exponential growth and reached a maximum during the early stationary phase (Fig. 2).

To investigate whether the composition of the growth medium affected CNBP production and secretion, *Aer. veronii* was cultured in two culture systems: (i) minimal broth medium supplemented with collagen or deprived of collagen or any other carbone source; and (ii) LB broth supplemented with collagen or without collagen (Table 3). It was found that *Aeromonas* cultures in LB broth gave a greater number of cells. However, CNBP were produced in similar proportions in both minimal and rich media cultures, and the level was not influenced by the presence of collagen in the culture media (Table 3).

Mechanism of CNBP secretion

The mechanism of extracellular protein secretion by *Aer. hydrophila* has been extensively studied by Howard and coworkers (Howard and Buckley 1983, 1985; Jiang and Howard 1991), who recently demonstrated that the secretion of aerolysin and other proteins is dependent on the *exeA* gene (Jiang and Howard 1991). To assess whether CNBP are present in *Aer. hydrophila* culture supernatant fluid, and to compare whether CNBP secretion is dependent on *exeA*, the cellular distribution of CNBP in *Aer. hydrophila* strain AH65 and an isogenic mutant, C5.84, with a transposon insertion in *exeA*, was examined (Jiang and Howard 1991) (Fig. 3). CNBP secretion, in contrast to aerolysin secretion, was unaffected by the *exeA* mutation of *Aer. hydrophila*. It is concluded that CNBP secretion from *Aer. hydrophila* must be achieved by a mechanism different from that reported for aerolysin secretion. Because *Aer. hydrophila* CNBP has a molecular weight homologous to the *Aer. veronii* CNBP, it may be possible that both *Aeromonas* species use similar secretory mechanisms for CNBP secretion.

DISCUSSION

On-going studies in this laboratory focused on the use of *Aer. veronii* antigens as immunogenic and immunoprotective agents against infections produced by *Aeromonas* species in marine fish. As adhesion factors are most important for the establishment of the pathogen in a host, especially during the early stages of an infection process, the possibility of using adhesins and lectins as vaccine candidates is attractive. In fact, extracellular proteins from *Aer. veronii* with affinity for mucin, lactoferrin, IgG and collagen type I have recently been found to stimulate the mucosal immune system of rabbit (Ascencio *et al.* 1995), and are considered as vaccine candidates for *Aeromonas* infections in fish farming as they also stimulate the mucosal immune system of the spotted sand bass, *Paralabrax maculatofasciatus* (Merino-Contreras *et al.*, personal communication).

A major 98 kDa protein band present in both the supernatant fluids and associated with the cell surface of *Aer. veronii* was found to play a role in the interaction of *Aer. veronii* with the collagenous extracellular matrix protein collagen type I. The present results provide some insights into the cell signalling and membrane trafficking of this pathogen. Collagen binding protein (CNBP) production by *Aer. veronii* occurred throughout growth. Although cell-associated CNBP was detected throughout the growth per-

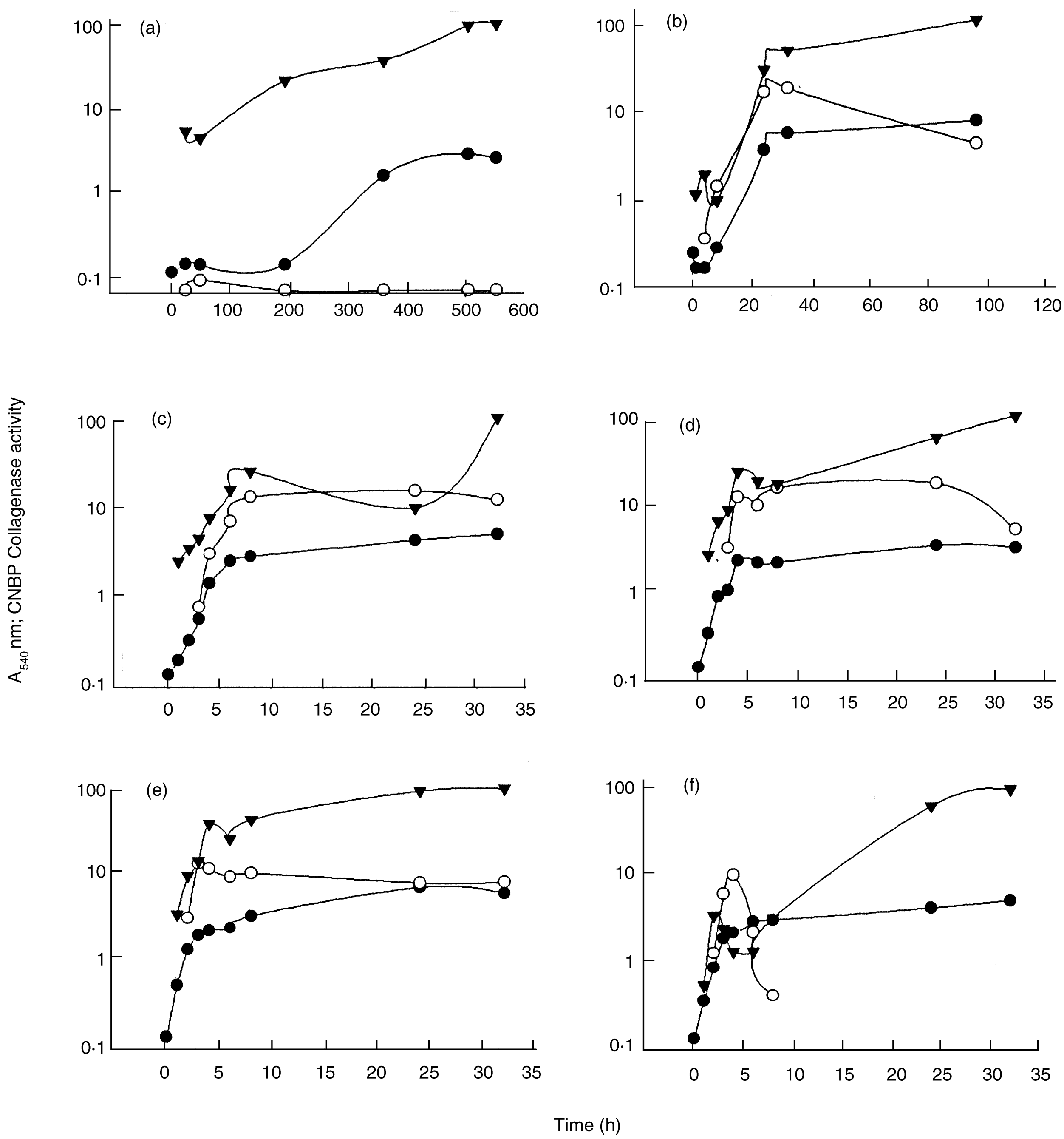

Fig. 2 Effect of the culture temperature on bacterial growth (●), and secretion of CNBP (○) and collagenases (▼), by *Aeromonas veronii* strain A186. Bacterial growth is expressed in O.D. units at 540 nm, CNBP is expressed in Units ml^{-1} and collagenase activity is expressed in percentage of ^{125}I-label released from the ^{125}I-collagen type I-coated latex beads. (a) 4 °C; (b) 12 °C; (c) 22 °C; (d) 32 °C; (e) 37 °C; (f) 42 °C

Table 3 Effect of the culture media on the release of CNBP and proteolytic enzymes into the culture media by *Aeromonas veronii*

Culture media	Total protein (μg ml^{-1}) (CFS/U.B)	Proteolytic activity (U ml^{-1})	CNBP (U ml^{-1})	cfu ml^{-1}
MMB + CN	127/100	69	8	$2\cdot3 \times 10^{10}$
MMB	4/4	6	9	2×10^{10}
MMB w/o C + CN	219/100	51	7	1×10^{10}
MMB w/o C	3·2/4	0	3	8×10^{9}
LB + CN	165/120	69	16	$8\cdot1 \times 10^{10}$
LB	179/24	64	13	8×10^{10}

(MMB + CN) biphasic medium made of a solid phase consisting of 1·5% agar and 100 μg ml^{-1} collagen, and a liquid phase consisting of minimal medium broth; (MMB) minimal medium broth; (MMB w/o C + CN) biphasic medium made of a solid phase consisting of 1·5% agar and 100 μg ml^{-1} collagen, and a liquid phase consisting of minimal medium broth without any carbon source; (MMB w/o C) minimal medium broth without any carbon source; (LB + CN) biphasic medium made of a solid phase consisting of 1·5% agar and 100 μg ml^{-1} collagen, and liquid phase consisting of Luria broth; (LB) Luria broth. (CFS/U.B) Culture supernatant fluid/uninoculated broth media.

iod, CNBP secretion decreased in the stationary phase when protease production reached maximum values.

In a previous study, it was shown that protease inhibitors favour the binding of extracellular matrix proteins (collagens type I and IV, fibronectin and laminin) to *Aer. hydrophila* cells (Ascencio *et al.* 1991). It seems likely that CNBP, collagenase and protease(s) are separate macromolecules operating at different stages in an infectious process.

It might be suggested that *Aer. veronii* expresses both CNBP (extracellular or cell-associated) and proteolytic

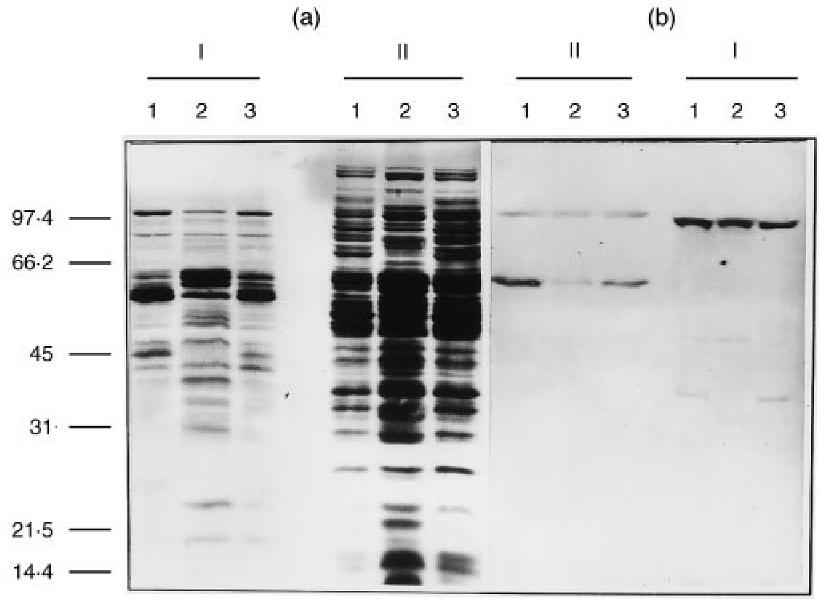

Fig. 3 Mechanism of CNBP secretion. (a) SDS°CPAGE of extracellular and sonicate proteins. (b) Western blot analysis of *Aeromonas hydrophila* CNBPs using peroxidase-labelled collagen type I as a probe. I: extracellular proteins; II: sonicate proteins. Lane 1: *Aer. hydrophila* wild strain Ah65; lane 2: *Aer. hydrophila* pleiotropic secretory mutant C5.84; lane 3: *Aer. hydrophila* pleiotropic secretory mutant L1.97

enzymes early in the infectious process when maximum adherence is needed to successfully colonize a host. However, when the pathogen has overcome this problem, it intensifies the biosynthesis and secretion of proteolytic enzymes to assure its nutritional requirements. Although the secretory activity of CNBP is reduced, the pathogen continues to express the cell-associated pool of CNBP because *Aer. veronii* cells bind to collagen at any stage of growth (data not shown).

The secretion and release of bacterial, cell-associated proteins that bind extracellular matrix components seems to be a common phenomenon among pathogenic micro-organisms. It has been shown that culture supernatant fluids of *Staphylococcus aureus*, *Mycobacterium leprae* and *Streptococcus equisimilis* contain released fibronectin-binding proteins (FNBPs) (Lindberg *et al.* 1992; Lindgren 1992; Thole *et al.* 1992).

Aerolysin secretion by *Aer. hydrophila* has been defined as a general secretory pathway model in this pathogen. Aerolysin has a signal peptide typical of those processed by signal peptidase I; it is rapidly released to the medium (Howard and Buckley 1985, 1986) where the *exeA* gene is required both for the extracellular export and outer membrane assembly, because T*n5*-751 insertion in the *exeA* gene causes pleiotropic defects in aerolysin secretion as well as marked decreases in the quantities of the most abundant outer membrane proteins (Jiang and Howard 1991). The present findings regarding CNBP secretion in isogenic mutants of *Aer. hydrophila* strains C5.84 and L1.97, with T*n5*-751 insertions in its *exeA* gene (Jiang and Howard 1991), strains which are deficient in aerolysin secretion but not in CNBP secretion, suggest CNBP secretion in *Aer. hydrophila* and *Aer. veronii* must be achieved by a mechanism different from the general pathway in aerolysin secretion. Further studies using *exeA* mutants of *Aer. veronii* need to be carried out to probe this hypothesis, particularly because of the potential application of *Aer. veronii* extracellular adhesions for developing effective fish vaccines against *Aeromonas* and *Vibrio* infections.

However, the secretion mechanisms, and the biological significance of why these extracellular matrix (ECM)-binding proteins are liberated by pathogenic micro-organisms into their milieu, remain unclear. It might be asked why these adhesive proteins, which enable the bacterium to bind extracellular matrix components, are secreted into the surrounding environment. A possible explanation is that these ECM-binding proteins may have multivalent functions like the haemagglutinin-protease of *Vibrio cholerae* (Finkelstein *et al.* 1983) or the lectin-toxin (pertussis toxin) of *Bordetella pertussis* (Tuomanen and Weiss 1985). The cell-associated CNBP pool might enable the bacterium to attach onto epithelial surfaces of the mucosal layers, in a manner similar to that in which cytoplasmic lectins from *Pseudomonas aeruginosa* enable the pathogen to adhere to epithelial cells once they are released from lysed bacterial cells (Wentworth *et al.* 1991). The extracellular CNBP pool may help the bacterium in the transport and assimilation of nutrients. An alternative explanation could be that the extracellular CNBP pool confers on the pathogen mimicry abilities to help it evade the immune barrier of the host while the cell-associated CNBP pool gives the pathogen adhesive capabilities. Homologous mechanisms have been proposed for *Candida albicans* (Gustafson *et al.* 1991), as extensively discussed by Hoepelman and Tuomanen (1992).

Western blot analysis of sonicate extracts showed that besides the 98 kDa CNBP, there were another protein bands (60, 40 and 15 kDa) which also gave a positive reaction with the POD-labelled collagen. Immunoblotting analysis, using rabbit polyclonal and monoclonal antibodies raised against the 98 kDa CNBP, demonstrated that the three CNBPs are immunologically homologous (data not shown). However, studies on the molecular basis need to be carried out to determine whether the 60, 40 and 15 kDa proteins are related, and what the gene encoding is for the secretion of CNBPs.

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Medical Research Council (16X04723), the Medical Faculty of the University of Lund and The Swedish Institute. The authors acknowledge S.P. Howard for the donation of *Aer. hydrophila* strain Ah65 and its pleiotropic secretory mutants C5.84 and L1.97 to T.R.H., and the help of Dr Ellis Glazier for editing the English language text.

REFERENCES

Allan, B.J. and Stevenson, R.M.W. (1981) Extracellular virulence factors of *Aeromonas hydrophila* in fish infections. *Canadian Journal of Microbiology* **27**, 1114–1122.

Ascencio, F., Ljungh, Å. and Wadström, T. (1991) Comparative study of extracellular matrix protein binding to *Aeromonas hydrophila* isolated from diseased fish and human infections. *Microbios* **65**, 135–146.

Ascencio, F., Sierra, A., Ruiz-Bustos, E., Romero, M.J., Greene, A. and Wadström, T. (1995) Lectins from *Aeromonas hydrophila* with affinity for mucosal constituents: a possible putative antigen for developing a mucosal vaccine against *A. hydrophila* infections. *Journal of Marine Biotechnology* **3**, 179–181.

Ascencio, F. and Wadström, T. (1991) Effect of proteases on the binding of *Aeromonas hydrophila* strains to connective tissue proteins. *Microbios* **66**, 27–37.

Ascencio, F. and Wadström, T. (1994) Coated-bead assay for microbial cell-associated and extracellular protease activity. *Microbios* **79**, 139–144.

Carrello, A., Silburn, K.A., Budden, J.R. and Chang, B.J. (1988) Adhesion of clinical and environmental *Aeromonas* isolates to Hep-2 cells. *Journal of Medical Microbiology* **26**, 19–27.

Dooley, J.S.G., McCubbin, W.D., Kay, C.M. and Trust, T.J. (1988) Isolation and biochemical characterization of the S-layer protein from a pathogenic *Aeromonas hydrophila* strain. *Journal of Bacteriology* **170**, 2631–2638.

Filip, C., Fletcher, G., Wulff, J. and Earhart, F. (1973) Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *Journal of Bacteriology* **115**, 717–722.

Finkelstein, R.A., Boesman-Finkelstein, M. and Holt, P. (1983) *Vibrio cholerae* hemagglutinin/lectin/protease hydrolyzes fibronectin and ovomucin. *Proceedings of the National Academy of Science USA* **80**, 1092–1095.

Gobius, K.S. and Pemberton, J.M. (1988) Molecular cloning, characterization, and nucleotide sequence of an extracellular amylase gene from *Aeromonas hydrophila*. *Journal of Bacteriology* **170**, 1325–1332.

Gonzalez, E.A., Baloda, S.B., Blanco, J. and Wadström, T. (1988) Growth conditions for the expression of fibronectin and collagen binding to *Salmonella*. *International Journal of Medical Microbiology* **269**, 437–446.

Gustafson, K.S., Vercelotti, G.M., Bendel, C.M. and Hostetter, M.K. (1991) Molecular mimicry in *Candida albicans*: role of an integrin analogue in adhesion of yeast to human endothelium. *Journal of Clinical Investigation* **87**, 1896–1902.

Hoepelman, A.I. and Tuomanen, E.I. (1992) Consequences of microbial attachment: Directing host cell functions with adhesins. *Infection and Immunity* **60**, 1729–1733.

Honda, T., Sato, M., Nishimura, T., Higashitsutsumi, M., Fukai, K. and Miwatani, T. (1985) Demonstration of cholera toxin-related factor of *Aeromonas* species by enzyme-linked immunosorbent assay. *Infection and Immunity* **50**, 322–323.

Howard, S.P. and Buckley, J.T. (1983) Intracellular accumulation of extracellular proteins by pleiotropic export mutants of *Aeromonas hydrophila*. *Journal of Bacteriology* **154**, 413–418.

Howard, S.P. and Buckley, T.J. (1985) Protein export by Gram-negative bacterium: production of aerolysin by *Aeromonas hydrophila*. *Journal of Bacteriology* **161**, 1118–1124.

Howard, S.P. and Buckley, T.J. (1986) Molecular cloning and expression in *Escherichia coli* of the structural gene for the hemolytic toxin aerolysin from *Aeromonas hydrophila*. *Molecular and General Genetics* **204**, 1118–1124.

Hudson, L. and Hay, F.C. (1989) *Practical Immunology*. pp. 46–47. Oxford: Blackwell Scientific Publications.

Hsueh, P.R., Teng, L.J., Lee, L.N. et al. (1998) Indwelling device-related and recurrent infections due to *Aeromonas* species. *Clinical and Infection Diseases* **26**, 651–658.

Janda, J.M. and Abbott, L. (1998) Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentations, and unanswered questions. *Clinical and Infectious Diseases* **27**, 332–344.

Jiang, B. and Howard, S.P. (1991) Mutagenesis and isolation of *Aeromonas hydrophila* genes which are required for extracellular secretion. *Journal of Bacteriology* **173**, 1241–1249.

Kabir, S. and Ali, S. (1983) Characterization of surface properties of *Vibrio cholerae*. *Infection and Immunity* **39**, 1048–1058.

Kirov, S.M., Hayward, L.J. and Nerrie, M.A. (1995) Adhesion of *Aeromonas* sp. to cell lines used as models for intestinal adhesion. *Epidemiology and Infection* **115**, 465–473.

Kirov, S.M. and Sanderson, K. (1996) Characterization of a type IV bundle-forming pilus (SFP) from a gastroenteritis-associated strain of *Aeromonas veronii* biovar *sobria*. *Microbial Pathogenesis* **21**, 23–34.

Kostrzynska, M., Dooley, J.S., Shimojo, T., Sakata, T. and Trust, T.J. (1992) Antigenic diversity of the S-layer proteins from pathogenic strains of *Aeromonas hydrophila* and *Aeromonas veronii* biotype *sobria*. *Journal of Bacteriology* **174**, 40–47.

Laemmli, U.K. (1970) Cleavage of structural protein during assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Leece, R. and Hirst, T.R. (1992) Expression of the B subunit of the *Escherichia coli* heat-labile enterotoxin in a marine vibrio and in a mutant that is pleiotropically defective in the secretion of extracellular proteins. *Journal of General Microbiology* **138**, 719–724.

Leung, K.Y. and Stevenson, R.M.W. (1988) Characteristics and distribution of extracellular proteases from *Aeromonas hydrophila*. *Journal of General Microbiology* **134**, 151–160.

Lindberg, M., Jonsson, K., Muller, H.-P. et al. (1992) Fibronectin-binding proteins in *Staphylococcus aureus.* In *Molecular Biology of the Staphylococci* ed. Novick, R.P. pp. 346–356. New York: VCH Publishers Inc.

Lindgren, P.-E. (1992) Similar genes coding for fibronectin-binding activity in *Staphylococcus dysgalactiae* and *Streptococcus equisimilis*. Fibronectin-binding proteins in Group G streptococci and *Staphylococcus aureus*. PhD Thesis. University of Agricultural Sciences, Department of Microbiology, Uppsala, Sweden.

Mevarech, M. and Werczberger, R. (1985) Genetic transfer in *Halobacterium volcani*. *Journal of Bacteriology* **162**, 461–462.

Neves, M.S., Nunes, M.P., Milhomem, A.M. and Ricciardi, I.D. (1990) Production of enterotoxin and cytotoxin by *Aeromonas veronii*. *Brazilian Journal of Medical and Biological Research* **23**, 437–440.

Pasquale, V., Baloda, S.B., Dumontel, S. and Krovacek, K. (1994) An outbreak of *Aeromonas hydrophila* infection in turtles (*Pseudermis scripta*). *Applied and Environmental Microbiology* **60**, 1678–1680.

Simmacco, M., Mangoni, M.L., Boman, D. and Boman, H.G. (1998) Experimental infections of *Rana esculenta* with *Aeromonas hydrophila*: a molecular mechanism for the control of normal flora. *Scandinavian Journal of Immunology* **48**, 357–363.

Steinfeld, S., Rossi, C., Bourgeois, N., Mansoor, I., Thys, J.P. and Appelboom, T. (1998) Septic arthritis due to *Aeromonas veronii* biotype *sobria*. *Clinical and Infectious Diseases* **27**, 402–403.

Stelma, G.N. Jr, Johnson, C.H. and Spaulding, P.L. (1988) Experimental evidence for enteropathogenicity in *Aeromonas veronii*. *Canadian Journal of Microbiology* **37**, 877–880.

Stewart, G.A., Bundell, C.S. and Burke, V. (1986) Partial characterization of a soluble haemagglutinin from human diarrhoeal isolates of *Aeromonas*. *Journal of Medical Microbiology* **21**, 319–324.

Sugita, H., Tanaka, K., Yoshinami, M. and Deguchi, Y. (1995) Distribution of *Aeromonas* species in the intestinal tracts of river fishes. *Applied and Environmental Microbiology* **61**, 4128–4130.

Thole, J.E., Scholningh, R.R., Mansson, J.A.A. et al. (1992) Molecular and immunological analysis of a fibronectin-binding protein antigen secreted by *Mycobacterium leprae*. *Molecular Microbiology* **6**, 153–163.

Tuomanen, E. and Weiss, A. (1985) Characterization of two adhesins of *Bordetella pertussis* for human ciliated respiratory epithelial cells. *Journal of Clinical and Infectious Diseases* **153**, 118–125.

Trust, T.J. (1986) Pathogenesis of infectious diseases of fish. *Annual Review of Microbiology* **40**, 479–502.

Wentworth, J.S., Austin, F.E., Garber, N., Gilboa-Garber, N., Paterson, C.A. and Doyle, R.J. (1991) Cytoplasmic lectins contribute to the adhesion of *Pseudomonas aeruginosa*. *Biofouling* **4**, 99–104.