Utilization of transferrin and salmon serum as sources of iron by typical and atypical strains of *Aeromonas salmonicida*

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The ability of typical and atypical strains of *Aeromonas salmonicida* to utilize non-haem sources of protein-bound iron was evaluated. (i) In a plate bioassay, the suppression of growth imposed on typical and atypical *A. salmonicida* by addition of the high-affinity iron chelator ethylenediamine-di(o-hydroxyphenylacetic acid) (EDDA) to the growth medium was reversed by the addition of 30% or 90% iron-saturated bovine or human transferrin (Tf) or lactoferrin (Lf) to the growth medium. (ii) The mechanism of obtaining iron from Tf was investigated by the addition of bovine Tf contained within a dialysis bag. The reversal of iron-restricted growth suppression differed between the strains in that the atypical strains were unable to utilize Tf contained within a dialysis bag while the typical strains were able to do so. This suggested a siderophore-mediated uptake of iron from Tf by the typical strains, which are known to produce siderophores while atypical strains do not. (iii) A solid-phase binding assay using horseradish-peroxidase-conjugated or biotinylated Tf or Lf failed to detect Tf/Lf-binding activity using whole typical or atypical cells. (iv) When atypical extracellular products (ECP) plus bovine Tf or salmon serum were enclosed in a dialysis bag, diffusible products were released which could reverse the EDDA-imposed growth suppression of an atypical strain. This reversal was negated by inhibition of the ECP metalloprotease with EDTA. (v) Purified 70 kDa serine protease of a typical strain was able to digest bovine Tf to low molecular mass fragments as observed in SDS-PAGE. These results indicate that typical and atypical strains of *A. salmonicida* differ in their mechanism of utilization of non-haem protein-bound sources of iron. Typical strains utilize Tf via a siderophore-mediated mechanism and are also able to digest Tf with the extracellular serine protease. Atypical strains utilize Tf by a siderophore-independent mechanism probably involving the proteolytic degradation of Tf by the extracellular metalloprotease.

Keywords: *Aeromonas salmonicida*, iron acquisition, transferrin, lactoferrin.

INTRODUCTION

An important factor in bacterial pathogenicity and virulence is the ability of invading pathogens to grow and multiply rapidly *in vivo* by overcoming specific and non-specific host defence mechanisms (Smith, 1990). Although the host contains large amounts of iron, the levels of free

ionic iron (Fe^{3+}) are maintained at very low concentrations due to sequestration by the host iron-binding glycoproteins: transferrin (Tf) in serum and extracellular fluids and lactoferrin (Lf) on mucosal surfaces. These iron-binding glycoproteins have high association constants for Fe^{3+} of the magnitude 10^{36} M (Griffiths, 1987a) and are only partially saturated with iron. This results in the amount of free iron *in vivo* (approximately 10^{-18} M) being insufficient for bacterial growth. Because bacteria have an absolute requirement for iron (Bullen, 1981), successful pathogens must possess iron uptake

Abbreviations: ECP, extracellular products; EDDA, ethylenediamine-di(o-hydroxyphenylacetic acid); HRP, horseradish peroxidase; IROMPs, iron-regulated outer-membrane proteins; Lf, lactoferrin; Tf, transferrin.

mechanism(s) which effectively overcome the host's iron-sequestering mechanisms in order to grow and multiply *in vivo*.

Most pathogenic bacteria growing under conditions of iron restriction *in vitro* possess high-affinity iron uptake mechanisms capable of removing iron for growth from Tf and Lf (Griffiths *et al.*, 1988; Martinez *et al.*, 1990). The most common mechanism, found in many Gram-negative bacteria, involves the production of siderophores (low molecular mass iron chelators) which have high association constants for iron. Uptake of iron into the cell is mediated by specific membrane receptors – iron-regulated outer-membrane proteins (IROMPs) not expressed in iron-replete conditions – which recognize the Fe^{3+} -siderophore complex. Certain pathogens such as *Neisseria meningitidis* (Schryvers & Morris, 1988), *Haemophilus influenzae* (Schryvers, 1988) and *Pasteurella haemolytica* (Ogunnariwo & Schryvers, 1990) are able to obtain iron by direct cell surface binding of Tf or Lf via IROMPs which recognize Fe^{3+} -Tf or -Lf complexes allowing utilization without siderophore production. It appears that the induction of high-affinity iron uptake mechanisms is essential for the growth of bacterial pathogens during the disease process since both siderophores (Griffiths & Humphreys, 1980; Wolf & Crosa, 1986) and IROMPs (Sciortino & Finkelstein, 1983; Chart & Griffiths, 1985; Donachie & Gilmour, 1988) are known to be produced during growth *in vivo*.

Aeromonas salmonicida, the causative agent of furunculosis, is a successful systemic pathogen of both salmonid and non-salmonid fish. Typical strains of *A. salmonicida* (subspecies *salmonicida*) are capable of rapid growth *in vivo* resulting in an acute haemorrhagic septicaemia often associated with extensive liquefaction of host tissues (McCarthy & Roberts, 1980). Atypical strains of *A. salmonicida* (subspecies *achromogenes*) produce a more superficial ulcerative form of furunculosis in both salmonid and non-salmonid fish.

Only typical strains of *A. salmonicida* possess an inducible siderophore-mediated iron uptake mechanism (Hirst *et al.*, 1991), although both typical and atypical strains are able to grow under conditions of iron restriction and express IROMPs (Chart & Trust, 1983; Aoki & Holland, 1985). A second non-siderophore iron uptake mechanism has been described by Chart & Trust (1983) which suggested that direct cell contact with iron-glycoprotein complexes was required.

In this study, the ability of typical and atypical strains of *A. salmonicida* to utilize non-haem sources of protein-bound iron was compared and the possible mechanisms which may enable *A. salmonicida* to obtain iron from Tf and Lf sources and salmon serum were investigated.

METHODS

***A. salmonicida* and growth conditions.** Four typical and two atypical strains of *A. salmonicida* designated in Table 1 and described elsewhere (Hirst *et al.*, 1991) were used. Seed cultures

were grown on Tryptic Soya Agar (TSA; Oxoid) for 24–48 h at 22 °C and suspended in PBS (pH 7.2) to an OD_{540} of 1 (10^9 cells ml^{-1}) for further use. For the identification of a putative Tf/Lf-binding protein, *A. salmonicida* was grown in an iron-restricted medium composed of Tryptone Soya Broth (TSB; Oxoid) containing the high-affinity iron chelator EDDA ($100 \mu\text{g ml}^{-1}$, Sigma). Contaminating iron was removed from EDDA by the method of Rogers (1973). When grown under these iron-restricted conditions all six *A. salmonicida* strains express additional IROMPs (data shown in Hirst & Ellis, 1994). Iron-replete bacteria were grown in TSB lacking EDDA. *A. salmonicida* broth cultures were incubated at 22 °C for 48 h on a rotary shaker. In dialysis bag experiments involving inhibition of the extracellular protease of an atypical strain by EDTA, TSB (Gibco) was used. The atypical strains grew to a higher cell density in Gibco TSB than in Oxoid TSB.

Transferrins (Tf) and lactoferrins (Lf). Purified human and bovine Tfs and Lfs were obtained from Sigma and prepared in the apo-form by the method of Aisen *et al.* (1978). ApoTf and Lf were saturated to 30% (the physiological saturation of Tf; Bezkorovainy, 1987) and 90% by the addition of a calculated amount of ferric citrate and the percentage iron saturation measured by the method of Graham & Bates (1976).

Salmon serum. Four Atlantic salmon, *Salmo salar*, were anaesthetized with MS222 (Sigma) and bled from the caudal vein. Blood was allowed to clot at room temperature and centrifuged. Non-haemolysed serum was collected, pooled and stored at -80 °C until used.

Determination of iron levels in Atlantic salmon serum. Serum iron levels of Atlantic salmon were determined in order to quantify the amounts of uncomplexed ferric iron available to *A. salmonicida* during infection. Serum iron levels were measured using an iron and total iron-binding capacity kit (Sigma) based on the method described by Persijn *et al.* (1971). Free serum iron levels were measured using a bleomycin assay described by Gutteridge *et al.* (1981).

Growth of *A. salmonicida* in salmon serum. *A. salmonicida* was inoculated into heat-inactivated (45 °C for 30 min) Atlantic salmon serum at a concentration of 3×10^8 c.f.u. ml^{-1} and incubated at 22 °C for 48 h. Growth of *A. salmonicida* was monitored by taking 20 μl samples, making serial 10-fold dilutions in PBS and inoculating onto TSA plates. The latter were incubated at 22 °C for 48 h and the number of c.f.u. ml^{-1} (mean of three) calculated to give viable counts and the growth rate of *A. salmonicida* in salmon serum.

Utilization of Tf/Lf-bound iron. A plate bioassay was used to determine which Tf/Lf sources could supply iron to typical and atypical strains of *A. salmonicida*. Approximately 10^5 bacteria were seeded onto TSA containing $200 \mu\text{g EDDA ml}^{-1}$. Thirty microlitres of a 1 mg ml^{-1} solution of 30% or 90% iron-saturated Tf or Lf in 10 mM Tris/HCl, pH 7.4, was added to 3 mm diameter wells punched in the agar. Plates were incubated for 48 h at 22 °C and were then examined for stimulation of growth around the wells. ApoTf, ApoLf and $100 \mu\text{M FeCl}_3$ were included as negative and positive controls respectively.

Siderophore-mediated utilization of Tf-bound iron. Experiments to determine whether Tf-iron acquisition by typical and atypical strains of *A. salmonicida* was mediated by a siderophore-dependent mechanism or required direct cell surface contact were carried out as described by Morton & Williams (1990). *A. salmonicida* was inoculated (to a concentration of 2×10^7 cells ml^{-1}) into TSB (Oxoid) containing $200 \mu\text{g EDDA ml}^{-1}$ with or without 30% iron-saturated bovine

or human Tf (1 mg ml⁻¹). To some broths, a dialysis bag (Medicell; molecular mass exclusion limit approximately 10 kDa) containing 1 ml bovine Tf (5 mg ml⁻¹) or TSB was added. Bacteria were incubated at 22 °C on a rotary shaker and their growth was monitored by OD₅₄₀ measurement. The four typical and two atypical strains listed in Table 1 were used in this experiment.

Tf-binding assays. A solid-phase dot-blot enzyme assay was used to examine the binding of Tf or Lf to *A. salmonicida* as described by Schryvers & Morris (1988). Two microlitres of a 48 h broth culture of iron-replete or iron-restricted whole cells was spotted onto nitrocellulose membranes (0.45 µm; Bio-Rad). Iron-restricted cells express IROMPs (Hirst & Ellis, 1994). Membranes were air-dried at room temperature for 10 min, blocked with 3% (w/v) gelatin in Tris-buffered saline (TBS; 50 mM Tris/HCl, 150 mM NaCl, pH 7.5) and probed either with horseradish-peroxidase-conjugated Tf or Lf (HRP-Tf, HRP-Lf) prepared by a two-step glutaraldehyde coupling procedure described by Avrameas *et al.* (1979), or with biotinylated Tf or Lf (bio-Tf, bio-Lf) prepared as described by Morton & Williams (1990) followed by peroxidase-labelled streptavidin (Sigma). HRP-Tf, HRP-Lf, bio-Tf and bio-Lf immobilized on nitrocellulose in place of whole cells served as positive controls. Dot-blots were developed with a 4-chloro-1-naphthol/H₂O₂ substrate kit (Bio-Rad).

Proteolytic degradation of Tf and salmon serum. Experiments to determine whether iron acquisition from Tf and salmon serum by typical and atypical strains of *A. salmonicida* was mediated by a process involving the extracellular proteases produced by the bacterium were carried out by two methods: the first using dialysis bag experiments, and the second, determining the proteolytic degradation of Tf by SDS-PAGE using a partially purified preparation of an extracellular protease isolated from a typical strain of *A. salmonicida*.

Dialysis bag experiments. The ability of the proteolytic activity of the extracellular products (ECP) of an atypical strain to release iron from Tf or salmon serum enclosed in a dialysis bag was investigated. ECP of the atypical *A. salmonicida* strain MT525 was prepared by harvesting the supernatant from a 48 h culture grown on cellophane overlying Brain Heart Infusion Agar (Oxoid) and proteolytic and inhibition assays using hide powder azure as substrate and 10 mM EDTA as inhibitor were performed as described by Gudmundsdottir *et al.* (1990). EDTA inhibited over 95% of the proteolytic activity.

Tests with the inhibited protease activity involved mixing ECP (1 ml) plus EDTA (10 mM) with 1 ml 30% iron-saturated bovine Tf (5 mg ml⁻¹) or 1 ml salmon serum and placing this in a dialysis bag which was then immersed in a 200 ml flask of TSB (Gibco) containing 10 mM EDTA. Protease-positive tests (ECP + Tf and ECP + salmon serum) and the controls, which included ECP, Tf, salmon serum or TSB, separately, in the bags, omitted the EDTA. The flasks were incubated on a shaker for 16 h at 22 °C and then the bags were removed. EDTA was then added to the protease-positive and control flasks (to 10 mM) and MgCl₂ was added (to 10 mM) to all flasks to saturate the EDTA. EDTA was then added (to 200 µg ml⁻¹) to all flasks. The flasks were then inoculated with the atypical strain MT525 (to a concentration of 2 × 10⁷ cells ml⁻¹) and growth was monitored for 30 h by OD₅₄₀ measurement in a spectrophotometer.

Proteolytic degradation of Tf. A partially purified extracellular protease of the typical *A. salmonicida* strain MT004 (Lee & Ellis, 1989) (approximately 100 µg ml⁻¹) was incubated with an equal volume of bovine Tf (1 mg ml⁻¹) at 22 °C for 2 h and samples were examined by SDS-PAGE.

SDS-PAGE and Western blotting. To detect the proteolytic degradation of Tf by the partially purified 70 kDa protease of the typical strain, samples were incubated with sample buffer (1:2, v/v) comprising 4% (w/v) SDS and 5% (v/v) 2-mercaptoethanol in 0.01 M Tris/HCl, pH 8.0, for 5 min at 100 °C. Aliquots (20 µl) were subjected to PAGE in 4–30% gradient gels (Pharmacia-LKB) at 150 V for 170 min at 10 °C. Electrophoresis buffer (pH 7.4) comprised 0.04 M Tris/HCl, 0.02 M sodium acetate, 0.002 M EDTA and 0.2% SDS. Proteins were transferred to nitrocellulose (0.45 µm; Bio-Rad) by electrophoresis at 30 V for 16 h. Transfer buffer comprised 25 mM Tris/HCl, pH 8.3, 192 mM glycine and 20% (v/v) methanol. After transfer, nitrocellulose membranes were stained for total protein with colloidal gold (Aurodyne, Amersham).

RESULTS

Iron levels in Atlantic salmon

The amount of iron present in Atlantic salmon serum was calculated to be 45.2 ± 14 µg ml⁻¹ (mean ± SE, *n* = 10). Using the bleomycin assay no free serum iron was detected.

Growth of *A. salmonicida* in salmon serum

The ability of two typical and one atypical strain of *A. salmonicida* to grow in heat-inactivated salmon serum was examined (Fig. 1). Both typical and atypical strains were capable of rapid growth in the serum.

Utilization of Tf/Lf-bound iron

The ability of *A. salmonicida* to obtain iron bound to Tf and Lf was examined using a plate bioassay. Both typical

Fig. 1. Growth kinetics of *A. salmonicida* in heat-inactivated Atlantic salmon serum. □, Typical strain MT004; ■, typical strain MT423; △, atypical strain MT194. Results are means of triplicate values.

Table 1. Utilization of non-haem iron proteins by *Aeromonas salmonicida*

Utilization of non-haem iron proteins was determined by measuring the zone of growth (mm) around a 3 mm diameter well containing 30 µl transferrin (Tf) or lactoferrin (Lf) on a plate containing TSA + 200 µg EDDA ml⁻¹ seeded with 10⁶ c.f.u. of one of six *A. salmonicida* strains. Human (H) or bovine (B) Tf or Lf was saturated to 30% or 90% with ferric citrate as indicated.

Strain*	Utilization of							
	HTf		HLf		BTf		BLf	
	30 %	90 %	30 %	90 %	30 %	90 %	30 %	90 %
MT004 (T)	6	22	6	23	7	24	5	23
MT616 (T)	7	21	6	22	8	24	6	21
MT423 (T)	7	21	6	23	8	23	6	20
MT486 (T)	9	23	6	22	8	19	7	21
MT194 (A)	4	23	5	23	5	20	4	20
MT525 (A)	4	21	4	23	6	18	4	17

* (T), typical strain; (A), atypical strain.

and atypical strains were able to utilize iron bound to human and bovine sources of Tf and Lf at 30% iron saturation – the physiological saturation of Tf – and at 90% iron saturation (Table 1). Apo-forms of Tf and Lf did not support the growth of any of the strains although all of the strains were able to utilize 100 µM FeCl₃ (data not shown).

Siderophore-mediated utilization of Tf-bound iron

The ability of the typical and atypical strains listed in Table 1 to utilize Tf-bound iron by a siderophore-mediated mechanism was investigated. Growth kinetics of a representative typical and atypical strain of *A. salmonicida* in the presence of a growth-suppressive concentration of EDDA (200 µg ml⁻¹) are shown in Fig. 2. Results similar to those shown in Fig. 2(a) were obtained with all four typical strains and Fig. 2(b) is representative of both atypical strains. In both typical and atypical strains, suppression of growth imposed by EDDA was reversed to normal by the addition of 100 µM FeCl₃ (data not shown). The inhibition of growth was also reversed upon the addition of a 30% iron-saturated bovine Tf solution (1 mg ml⁻¹) (Fig. 2). However, when the 30% iron-saturated Tf added was contained within a dialysis bag with a 10 kDa molecular mass exclusion limit, the EDDA-imposed growth suppression was reversed only in the typical strain (Fig. 2a), while the growth of the atypical strains remained inhibited (Fig. 2b). The presence of a dialysis bag containing only growth medium had no effect on the growth of *A. salmonicida* in either iron-replete or iron-restricted media (data not shown). The results of the dialysis bag experiments suggest that a low molecular mass soluble factor is responsible for the utilization of Tf-bound iron by typical strains. In contrast, it is possible that atypical strains lacking this soluble factor utilize Tf-bound iron by a mechanism requiring direct contact

between the bacterial cell surface and Tf and/or a proteolytic release of iron from Tf.

Tf-binding assays

The six strains of *A. salmonicida* grown under iron restriction were used to determine whether Tf or Lf could interact directly with the cell surface of *A. salmonicida* via IROMPs. A solid-phase dot-blot enzyme assay was used in which either iron-replete or iron-restricted whole cells were immobilized on nitrocellulose membranes which were then probed with either HRP-Tf, HRP-Lf, bio-Tf or bio-Lf (followed by peroxidase-labelled streptavidin in the latter two cases) to detect either Tf- or Lf-binding activity. Using these binding assays on the four typical and two atypical strains listed in Table 1, it was not possible to detect the binding of Tf or Lf to whole cells. HRP-Tf/Lf and biotinylated Tf/Lf immobilized on nitrocellulose as positive controls gave a positive reaction when stained with 4-chloro-1-naphthol/H₂O₂.

Proteolytic degradation of Tf by atypical *A. salmonicida* ECP

The effects of the ECP of the atypical *A. salmonicida* strain MT525 on 30% saturated bovine Tf were examined in order to determine whether Tf-iron acquisition by atypical strains was mediated by a proteolytic degradation of Tf. ECP of the atypical *A. salmonicida* strain MT525 possessed proteolytic activity due to the presence of a metallo-protease which was almost completely inhibited in the presence of 10 mM EDTA. ECP incubated with bovine Tf enclosed in a dialysis bag reversed the growth inhibition imposed on the atypical *A. salmonicida* by the addition of EDDA (Fig. 3). The ECP of *A. salmonicida* MT525 alone or Tf alone had no effect on growth (data not shown). In a further experiment where EDTA was

Fig. 2. Growth kinetics of *A. salmonicida* in TSB (▲), TSB+EDDA (200 µg ml⁻¹) (△), TSB+EDDA+30% bovine Tf (1 mg ml⁻¹) (■) and TSB+EDDA+30% bovine Tf in dialysis bag (5 mg ml⁻¹) (□). Samples were taken at the times indicated and their OD₅₄₀ measured. (a) Typical strain, MT004; (b) atypical strain, MT194. Data are means of duplicate values.

incorporated to inhibit the ECP proteolytic activity, the growth-stimulating effect of ECP plus Tf inside the dialysis bag was removed (Fig. 4).

Release of iron from salmon serum by atypical *A. salmonicida* ECP

In similar experiments to those using ECP and Tf in the dialysis bag, the conditioning of TSB by ECP and salmon serum within a dialysis bag reversed the suppressed growth rate imposed by EDDA (Fig. 4). However, the inhibition of ECP proteolytic activity by the presence of EDTA removed this effect (Fig. 4). The presence of ECP or salmon serum in the dialysis bag did not reverse the effect of EDDA (Fig. 4).

Fig. 3. Growth kinetics of atypical *A. salmonicida* MT525 in TSB+EDDA (200 µg ml⁻¹) (△), TSB+EDDA+30% bovine Tf (1 mg ml⁻¹) (■), TSB+EDDA+30% bovine Tf in dialysis bag (5 mg ml⁻¹) (□) and TSB+EDDA+30% bovine Tf+ECP in dialysis bag (▲). Samples were taken at the times indicated and their OD₅₄₀ measured. Data are means of duplicate values.

Digestion of Tf by the 70 kDa protease of a typical strain

Similar experiments to determine whether the proteolytic degradation of Tf by ECP of typical strains of *A. salmonicida* was capable of reversing the growth suppression imposed by EDDA were not possible using dialysis bag experiments due to the presence of soluble siderophores capable of reversing growth suppression in the ECP of typical strains. As an alternative approach, a partially purified preparation of the extracellular 70 kDa serine protease isolated from *A. salmonicida* MT004 was incubated with bovine Tf and then analysed by SDS-PAGE. Although the preparation had undergone partial auto-digestion due to prolonged storage, all the proteolytic activity could be attributed to the serine protease because of total inhibition by PMSF. Analysis of the digest by SDS-PAGE indicated total digestion of Tf into a number of low molecular mass components (Fig. 5).

DISCUSSION

In a previous study we confirmed earlier reports that typical and atypical strains of *A. salmonicida* are able to grow and multiply under conditions of iron restriction. Typical strains of *A. salmonicida* possess an inducible siderophore-mediated iron uptake mechanism involving the production of a 2,3-diphenolcatechol siderophore while atypical strains of *A. salmonicida* possess an unidentified siderophore-independent iron uptake mechanism (Hirst *et al.*, 1991). Chart & Trust (1983) reported on one atypical strain of *A. salmonicida* which was able to use Lf as a source of iron but the mechanism did not

Fig. 4. Growth of atypical *A. salmonicida* MT525 in TSB which had previously been conditioned by immersion of dialysis bags containing: TSB; TSB and transferrin (Tf); TSB and salmon serum (SS); TSB and ECP; TSB plus Tf and ECP; TSB plus SS and ECP; TSB plus Tf, ECP and EDTA; TSB plus SS, ECP and EDTA. Following removal of the bags all media were equalized by addition of EDTA (as appropriate; indicated on figure), $MgCl_2$ and EDDA. Results show the growth (OD_{540}) after 30 h incubation. Data are means of duplicate values.

apparently involve the production of a siderophore as the strain was unable to remove $^{59}Fe^{3+}$ bound to Lf across a dialysis membrane, suggesting that Lf utilization involved a direct interaction between Lf and the bacterial cell surface. A number of other non-siderophore-producing bacterial pathogens such as *Neisseria meningitidis* (Schryvers & Morris, 1988), *Haemophilus influenzae* (Schryvers, 1988; Morton & Williams, 1990), *H. pleuropneumoniae* (Niven *et al.*, 1989), *H. somnus* (Ogunnariwo *et al.*, 1990), *Pasteurella haemolytica* (Ogunnariwo & Schryvers, 1990) and *Bordetella pertussis* (Redhead *et al.*, 1987; Redhead & Hill, 1991) are all able to acquire iron bound to Tf via direct cell surface interactions.

In the present study we showed that both typical and atypical strains of *A. salmonicida* were capable of rapid growth in heat-inactivated salmon serum, confirming observations by Ellis & Stapleton (1988). There was no detectable free iron in the serum, as determined with the bleomycin assay of Gutteridge *et al.* (1981). The iron that was present ($45 \mu g ml^{-1}$) was presumably bound by the host iron-binding proteins such as Tf (Congleton & Wagner, 1991) and is comparable to the amounts found in mammalian serum (Persijn *et al.*, 1971).

Using a plate bioassay it was demonstrated that both typical and atypical strains of *A. salmonicida* were able to utilize Tf- and Lf-bound iron from human and bovine sources and to utilize these iron-binding proteins at physiological levels of iron saturation. The ability of *A. salmonicida* to utilize Tf and Lf from mammalian sources

differed from many other bacterial pathogens capable of utilizing Tf via direct cell surface interactions in that it is usual for this mechanism to be specific for the Tf of the host (Schryvers & Gonzalez, 1990).

A solid-phase dot-blot enzyme assay was used to determine whether Tf utilization by typical and atypical strains involved Tf binding. Whole cells immobilized on nitrocellulose membranes were tested for their ability to bind HRP-Tf, HRP-Lf, bio-Tf or bio-Lf from different mammalian sources. These techniques, used to identify the specific cell surface Tf/Lf receptors in *N. meningitidis*, *H. influenzae* and *P. haemolytica*, failed to identify such a receptor in *A. salmonicida*.

Using dialysis bag experiments, other possible mechanisms of Tf utilization by *A. salmonicida* were investigated. The suppression of growth imposed on typical and atypical strains by the addition of EDDA to the growth medium could be reversed by the addition of 30% iron-saturated bovine Tf. When the Tf was contained within a dialysis bag with a molecular mass exclusion limit of 10 kDa, the EDDA-imposed growth suppression of typical strains was still reversed, but growth of atypical strains remained suppressed. This observation confirms the ability of typical strains to utilize Tf-bound iron by a siderophore-mediated mechanism and is similar to the mechanism of Tf-bound iron acquisition described for motile *Aeromonas* species (Massad *et al.*, 1991). Siderophores, being soluble and of low molecular mass (500–1000 Da), can pass through a dialysis mem-

Fig. 5. SDS-PAGE of proteolytic digests of Tf. Proteins were detected by Western blotting and colloidal gold stain. Lane 1, low molecular mass protein standards; lane 2, bovine Tf; lane 3, partially purified 70 kDa protease of typical *A. salmonicida* MT004; lane 4, Tf incubated with the 70 kDa protease showing proteolytic digestion of Tf. The lanes were overloaded (11 µg protein per lane) in order to demonstrate the complete digestion of Tf.

brane to form complexes with iron. The inability of atypical strains to utilize Tf contained within a dialysis bag suggested some other mechanism.

An additional mechanism involved in the utilization of Tf-bound iron by pathogenic bacteria has been described by Griffiths (1987b); it involves the proteolytic degradation of Tf and disruption of the iron-binding site to release Fe^{3+} . Such a mechanism has been described by Döring *et al.* (1988) for *Pseudomonas aeruginosa*, in which only in the presence of the secreted proteolytic enzyme elastase can the *Ps. aeruginosa* siderophore, pyoverdine, facilitate iron release and uptake from Tf. Since both typical and atypical strains of *A. salmonicida* produce extracellular proteases which are regarded as important virulence factors in the pathogenesis of *A. salmonicida* infection (Ellis, 1991), the possibility that proteolytic degradation of Tf may enable typical and atypical strains to utilize Tf-bound iron was investigated.

Experiments examining the ability of both extracellular products of an atypical strain, and of a partially purified extracellular protease of a typical strain, indicated that proteolytic degradation of Tf as a mechanism of Tf-bound iron acquisition by *A. salmonicida* was possible. In atypical strains, ECP containing a metalloprotease was able to interact with Tf contained within a dialysis bag and reverse the growth suppression imposed on atypical *A.*

salmonicida by EDDA; Tf or ECP alone contained within the dialysis bag did not reverse the growth suppression. Inhibition of the ECP proteolytic activity by incorporation of EDTA prevented the ability of the ECP to act upon Tf and release the iron in a form which was able to diffuse through the dialysis bag and stimulate growth of the iron-restricted bacteria. A similar effect was observed with ECP and salmon serum enclosed in the dialysis bag.

The extracellular serine protease produced by typical strains was found to be capable of degrading Tf, resulting in several low molecular mass components observed in SDS-PAGE. It is not known whether the proteolytic degradation described in this study resulted in the disruption of iron-binding sites with the release of Fe^{3+} . It may be the case, as with the elastase produced by *Ps. aeruginosa*, that the serine protease facilitates the release of Fe^{3+} from Tf, enabling uptake by the siderophore produced by typical strains of *A. salmonicida*. However, the experiments with the atypical strain indicate that factors in the ECP which correlated with proteolytic activity can release iron from Tf and salmon serum, making it available for uptake in the absence of siderophores. In this regard, the observation by Neelam *et al.* (1993) that the production of the 70 kDa protease by a typical strain grown *in vitro* under iron restriction was greatly enhanced, may have significance in the utilization of host Tf during infection.

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