Acquisition of Iron by Aeromonas salmonicida

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The ability of six typical and three atypical strains of Aeromonas salmonicida to sequester Fe\(^{3+}\) from the high-affinity iron chelators ethylenediaminedi-hydroxyphenylacetic acid, lactoferrin, and transferrin was determined. Typical strains were readily able to sequester Fe\(^{3+}\) and used two different mechanisms. One mechanism was inducible and appeared to involve production of a low-molecular-weight soluble siderophore(s). Iron uptake by this mechanism was strongly inhibited by ferricyanide. One virulent strain displayed a second mechanism which was constitutive and required cell contact with Fe\(^{3+}\)-lactoferrin or -transferrin. This strain did not produce a soluble siderophore(s) but could utilize the siderophore(s) produced by the other strain. Fe\(^{3+}\) uptake by this stripping mechanism was strongly inhibited by dinitrophenol. Atypical strains displayed a markedly reduced ability to sequester iron from high-affinity chelators, although one of them was able to utilize the siderophores produced by the typical strain. In all strains examined, Fe\(^{3+}\) limitation resulted in the increased synthesis of several high-molecular-weight outer membrane proteins.

Aeromonas salmonicida is an important pathogen, causing the systemic disease furunculosis in fish (11). In acute forms of the disease, the organism grows rapidly in the major body organs, producing a terminal septicemia which is often complicated by severe tissue necrosis. Although best recognized as producing disease in the salmonidae, the organism is also able to infect a wide range of other fish species (11). In recent years, a variety of atypical strains of A. salmonicida have been isolated, especially from carp species. These strains differ from typical A. salmonicida strains in several biochemical characteristics (30) and by their poor growth on conventional laboratory media.

The primary virulence factor of this fish pathogen is a surface protein array with tetragonal symmetry (A-layer) (14, 17). This protein array appears to have an important role in protecting the organism from the host defenses (17). However, in addition to withholding host defense mechanisms, successful systemic pathogens such as A. salmonicida must obtain essential growth factors. Bacteria have an absolute requirement for iron (18, 19, 29). In host animals, iron is tightly bound by glycoproteins such as transferrin and lactoferrin which have a very high affinity for ferric iron (5, 32). Consequently, the free iron levels are well below the limits required for bacterial growth (3, 5). So, to survive and grow in the host, pathogens such as Escherichia coli, Neisseria meningitidis, and Vibrio anguillarum have evolved highly specific mechanisms which allow them to obtain Fe\(^{3+}\) from host iron-binding proteins (2, 5, 6).

Because of its ability to grow rapidly in fish and produce disease, A. salmonicida clearly should have highly efficient mechanisms for iron acquisition. We therefore investigated the ability of A. salmonicida to obtain iron from various chelating agents with high association constants for Fe\(^{3+}\).

MATERIALS AND METHODS

Bacteria. Strains of A. salmonicida used in this study are listed in Table 1. V. anguillarum strains 775 and 775-E39 were provided by M. H. Schiewe (Northwest Fisheries Center, National Marine Fisheries Service, Seattle, Wash.). Strain 775 was virulent in salmonid fishes and carried the 47-megadalton plasmid pJM1, coding for a high-affinity iron-sequestering mechanism (6). Strain 775-E39 was an ethidium bromide-cured plasmidless derivative of 775(pJM11), pJM11 being a transposition derivative of plasmid pJM1 (7). This strain was avirulent and unable to grow under conditions of iron limitation (6). Salmonella typhimurium strain LT2 was obtained from K. D. Sanderson (Department of Biology, University of Calgary, Alberta, Canada).

Media and culture conditions. Strains were maintained at -80°C in tryptic soy broth (GIBCO Diagnostics, Madison, Wis.) containing 15% (vol/vol) glycerol as the cryoprotectant. Bacteria were cultured on tryptone soy agar (GIBCO) or in tryptone soy broth (100 ml in 500-ml conical flasks) with agitation (orbital shaker at 150 rpm; New Brunswick Scientific Co., New Brunswick, N.J.). A. salmonicida and V. anguillarum were cultured at 20 and 25°C, respectively,
whereas *S. typhimurium* was cultured at 37°C. For anaerobic culture, strains were incubated in a carbon dioxide-hydrogen atmosphere (GasPak; BBL Microbiology Systems, Cockeysville, Md.).

Iron chelation. Iron (Fe³⁺)-limited culture conditions were provided by the addition of 0.28 mM ethylenediaminedihydroxyphenylacetic acid (EDDA), lactoferrin (1 mg/ml), or transferrin (5 mg/ml). Fe³⁺ limitation was determined by culturing *V. anguillarum* strain 775-E39 in concentration gradients of chelator in tryptone soy broth; minimum inhibitory concentrations were used for further study. In addition, a similar protocol was employed to determine the amount of FeCl₃ required to saturate chelator Fe³⁺-binding capacity (0.54 mM). Before experimentation, strains were subcultured three times across media containing chelator.

Iron chelators used in this study were obtained from Sigma Chemical Co. (St. Louis, Mo.). Contaminating Fe³⁺ was removed from EDDA by the method of Rogers (25) and from glycoproteins by the method of Aisen et al. (1).

**Uptake of ⁵⁹Fe³⁺.** ⁵⁹FeCl₃ (833 μCi/mol) was obtained from Amersham Corp., Oakville, Ontario, Canada. Kinetics of ⁵⁹Fe³⁺ uptake were monitored by providing mid-exponential-phase cultures with ⁵⁹FeCl₃ bound to EDDA or to lactoferrin. **Cellular ⁵⁹Fe³⁺** was determined by applying 20-μl volumes of culture medium to nitrocellulose filters (0.45-μm pore size; Millipore Corp., Bedford, Mass.) and washing with 5 ml of sterile tryptone soy broth-EDDA medium. The cell-associated ⁵⁹Fe³⁺ on these filters was quantitated in a gamma counter (model 8000; Beckman Instruments, Inc., Fullerton, Calif.). Values obtained by applying ⁵⁹Fe³⁺ solutions alone to Millipore filters were subtracted from test data.

In some experiments, the effects of metabolic inhibitors were tested on cells grown aerobically or anaerobically in tryptone soy broth-EDDA to the exponential phase. After 30 min, ⁵⁹Fe³⁺ bound to EDDA was added, and after a further 30 min, cells were collected and cellular radioactivity was determined. The inhibitors tested were iodoacetamide (Sigma Chemical Co., St. Louis, Mo.), p-chloromercuribenzoic acid (pCMBA, Sigma), 2,4-dinitrophenol (DNP, Sigma), K₂Fe(CN)₆, NaNO₃, NaCN, NaF, NaAsO₃, and N,N′-dichlorohexylcarbodimide (DDC) (Aldrich Chemical Co., Milwaukee, Wis.).

**Binding of ⁵⁹Fe³⁺ to lactoferrin.** Fe³⁺ was removed from lactoferrin by the method of Aisen et al. (1) before reacting it with ⁵⁹FeCl₃ (2 μCi/mg of lactoferrin) at 4°C (2 h). ⁵⁹Fe³⁺-lactoferrin complexes were dialyzed against distilled water (4°C) until extraneous activity approached background radioactivity. The inherent activity of ⁵⁹Fe³⁺-lactoferrin was found to be 8 × 10⁶ cpm/mg.

**Uptake of ⁵⁹Fe³⁺ across a dialysis membrane.** Ability of strains to obtain across a dialysis membrane iron bound to lactoferrin was examined by placing cultures (10⁶ bacteria in 1 ml) opposite a dialysis membrane (14,000-molecular-weight exclusion limit) separating them from lactoferrin (1 mg/ml, in tryptone soy broth) saturated with ⁵⁹Fe³⁺. Samples (200 μl) were taken after 3.0 h of incubation.

**Siderophore detection.** Hydroxamate synthesis was examined by the method of Csáky (8) with and without the modification of Gibson and Magrath (10) and Holzberg and Arts (12); culture supernatants, concentrated (×10) by freeze-drying, were analyzed with hydroxylamine hydrochloride (Sigma) as a standard (10).

Possible catechol production was assessed in ethyl acetate extracts of culture supernatants (21, 22) by the colorimetric method of Arnow (4) with dihydroxybenzene (Sigma) as a standard.

**Cross-feeding.** Cells of *A. salmonicida* or *S. typhimurium* were grown in tryptone soy broth-EDDA for 30 h and removed by centrifugation (4,000 × g, 30 min). A 1-ml amount each of DNase (Sigma) and RNase (Sigma) was added per ml, and the supernatant fluid was sterilized by filtration through a 0.25-μm filter (Millipore). A 1-ml volume was added to 5 ml of tryptone soy broth-EDDA, which was then inoculated with atypical strain A406. Growth was assessed by determination of culture absorbance at 590 nm.

**Isolation of outer membranes.** Cells were harvested from agar plates or broth cultures, washed in 20 mM Tris-hydrochloride (Sigma) (pH 7.2), and resuspended in this buffer containing 10 mM EDTA (Sigma) and 0.1 μg each of DNase and RNase per ml. After disruption of cells by three passages through a precooled French pressure cell (16,000 lb/in²), unbroken cells were removed by centrifugation at 4,000 × g for 30 min. The total cell membrane (envelope) fraction was then collected by centrifugation at 40,000 × g at 4°C for 30 min. Cytoplasmic membranes were selectively solubilized with sodium lauryl sarcosinate (W. R. Grace and Co., Organic Chemicals Division, Nashua, N.H.) by the method of Filip et al. (9), and the remaining outer membrane fraction was sedimented by centrifugation at 40,000 × g for 1 h. The outer membrane was washed in 20 mM Tris-hydrochloride (pH 7.2) and stored at −20°C until required.

**Outer membrane samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were separated according to the method of Laemmli (16). Proteins solubilized in sample buffer (62.5 mM Tris-hydrochloride [pH 6.8/5%), [vol/vol] 2-mercaptoethanol, 3% [wt/vol] sodium dodecyl sulfate) were stacked in 4.5% acrylamide (10 mA) and separat-

### Table 1. Strains of *Aeromonas salmonicida*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source*</th>
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<tbody>
<tr>
<td>A438</td>
<td>T. P. T. Evelyn (strain 76-30)</td>
</tr>
<tr>
<td>A449</td>
<td>C. Michel (strain TG36/75)</td>
</tr>
<tr>
<td>A449-3</td>
<td>Attenuated derivative of A449 (17)</td>
</tr>
<tr>
<td>A450</td>
<td>C. Michel (strain TG72/78)</td>
</tr>
<tr>
<td>A450-3R</td>
<td>Attenuated derivative of A450 (17)</td>
</tr>
<tr>
<td>A470</td>
<td>D. Rockey (strain AsSC14)</td>
</tr>
<tr>
<td>A400</td>
<td>This laboratory (30)</td>
</tr>
<tr>
<td>A406</td>
<td>This laboratory (30)</td>
</tr>
<tr>
<td>A419</td>
<td>E. B. Shotts, Jr. (30)</td>
</tr>
</tbody>
</table>

* T. P. T. Evelyn, Pacific Biological Station, Nanaimo, B.C., Canada; C. Michel, Laboratoire d’Ictyopathologie, Thiverval-Grignon, France; D. Rockey, University of Wyoming, Laramie, Wyo.; E. B. Shotts, Jr., University of Georgia, Athens, Ga.
RESULTS

Growth under Fe³⁺ limitation. The ability of typical and atypical strains of *A. salmonicida* to grow under conditions of Fe³⁺ limitation was tested on media containing the high-affinity Fe³⁺ chelators EDDA, lactoferrin, and transferrin. The chelators produced Fe³⁺ limitation in the medium as demonstrated by the inability of *V. anguillarum* 775 E-39 to grow in the absence of added Fe³⁺. All typical strains of *A. salmonicida* tested grew well in the presence of each of the Fe³⁺ chelators. In contrast, the growth of the normally slower growing atypical strains tested was perceptibly reduced under conditions of Fe³⁺ limitation, as imposed by EDDA. In the case of atypical strain 406, growth was totally inhibited by EDDA. The addition of FeCl₃ to the medium alleviated growth inhibition in all cases.

Uptake of ⁵⁹Fe³⁺. The ability of *A. salmonicida* cells to take up ⁵⁹Fe³⁺ was demonstrated by using mid-exponential-phase cells of strain A450 growing in tryptone soy broth-EDDA (Fig. 1). When these cells were provided with excess ⁵⁹Fe³⁺ (1 μM) bound to EDDA, the cells immediately began to take up the radiolabeled Fe³⁺, and this uptake continued linearly for 10 min, at which time cells became saturated with iron. In a parallel experiment, total cell membranes (envelopes) and outer membrane fractions were isolated from cells collected at the various time intervals, and the membrane-associated ⁵⁹Fe³⁺ was determined. A linear increase in membrane-associated ⁵⁹Fe³⁺ was seen during the first 10 min of cell exposure to ⁵⁹Fe³⁺ (data not shown). By 10 min, 32 ng of ⁵⁹Fe³⁺ per mg of protein was envelope associated, of which only 14% was associated with the outer membrane. Exponential-phase cells growing in broth without EDDA and membrane fractions isolated from these cells failed to accumulate ⁵⁹Fe³⁺ over the same time period.

Inducibility of Fe³⁺ uptake systems. Cells were grown to exponential phase under Fe³⁺ limitation in tryptone soy broth-EDDA or Fe³⁺-sufficient broth, harvested by centrifugation, and suspended in broth containing ⁵⁹Fe³⁺ bound to EDDA (Fig. 2). Strain A449 showed immediate uptake of ⁵⁹Fe³⁺, regardless of the previous culture conditions. In contrast, cells of strain A450 which had been grown in the Fe³⁺-sufficient medium were unable to immediately take up ⁵⁹Fe³⁺. These noninduced cells experienced a 90-min lag before taking up substantial ⁵⁹Fe³⁺.

Siderophore production. The ability to produce a low-molecular-weight soluble siderophore(s) was determined by measuring ⁵⁹Fe³⁺ uptake from ⁵⁹Fe³⁺-lactoferrin across a dialysis membrane. When a broth-EDDA culture of strain A450 was separated from the lactoferrin-bound ⁵⁹Fe³⁺, a preformed soluble siderophore(s) in the culture medium allowed cells of A450 to acquire ⁵⁹Fe³⁺ (Table 2). Predictably, a broth culture of strain A450 was unable to acquire ⁵⁹Fe³⁺ because of the absence of a preformed siderophore(s) in the culture medium. Cells of A449 were unable to obtain ⁵⁹Fe³⁺ from lacto-

FIG. 1. ⁵⁹Fe³⁺ uptake from ⁵⁹Fe³⁺-EDDA by cells of *A. salmonicida* A450. Cells were grown to exponential phase (10⁶ cells per ml) in 10 ml of broth plus 0.28 mM EDDA (- - - ) or in broth (---). 1 μM ⁵⁹Fe³⁺ was added to the growth medium, and cell-associated radioactivity was determined.

FIG. 2. Inducibility of iron uptake mechanisms of *A. salmonicida*. Cells were grown to mid-exponential phase (10⁶ cells per ml) in 10 ml of broth (□) or broth plus 0.28 mM EDDA (○), harvested by centrifugation, and resuspended in 10 ml of broth plus 0.28 mM EDDA with 1 μM bound Fe³⁺. Cell-associated radioactivities of strain A449 (- - - ) and strain A450 (---) were determined.
ferrin-bound $^{59}$Fe$^{3+}$ across a dialysis membrane regardless of whether they are growing in the presence or absence of EDDA; however, this strain was able to use the siderophore(s) produced by strain A450 to obtain glycoprotein-bound Fe$^{3+}$ across a dialysis membrane (Table 2).

Procedures used to detect phenolate siderophores in E. coli (20, 21) were then applied to EDDA-culture supernatants of strains A449, A450, and four other typical strains. All failed to give the positive Arnow reactions (4) obtained with control supernatants of S. typhimurium LT2. Furthermore, the Csáky assay (9) and various modifications (10, 12) failed to detect a siderophore of the hydroxamate class.

The ability of the siderophore(s) produced by strain A450 to cross-feed atypical A. salmonicida strain A406 was also tested. This strain displayed no growth in medium containing low Fe$^{3+}$, but when it was provided with excess Fe$^{3+}$ or broth-EDDA culture supernatant from strain A450 (30-h culture), normal growth was displayed (Fig. 3). Other experiments showed that the extent of A406 growth was directly related to the amount of A450 supernatant added and that broth-EDDA culture supernatants from strains A438 and A470 also cross-fed atypical strain A406 (data not shown). Growth of A406 in the presence of EDDA was promoted to a lesser extent by cell-free supernatants from broth-EDDA cultures of S. typhimurium LT2 (Fig. 3). Cultures supernatants from EDDA-grown A449 failed to facilitate growth of A406, as did culture supernatants from strains A450, A438, and A470 cultured in plain broth (data not shown). In these experiments, DNase and RNase were added to the various supernatants to minimize the possibility that genetic transfer, such as of plasmid DNA, might be involved in subsequent growth of A406.

Inhibition of Fe$^{3+}$ uptake. Differences in the mechanisms of aerobic uptake by strains A449 and A450 were shown with the metabolic inhibi-
in the presence and absence of EDDA were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, several proteins of high apparent molecular weight were seen in cells grown under Fe limitation. These proteins were, however, poorly resolved (Fig. 4A) because they ran in the same region of the electrophoretogram as the high-molecular-weight fraction of *A. salmonicida* lipopolysaccharide (17). This interference was avoided by using an A-layer-deficient derivative of A450 which lacked this high-molecular-weight lipopolysaccharide fraction (17). This deep rough lipopolysaccharide mutant, A450-3R, was indistinguishable from A450 in its ability to grow under conditions of iron limitation. The results in Fig. 4B show that under Fe limitation, three outer membrane polypeptides with apparent molecular weights of 83,200, 77,700 and 76,600 were induced.

**DISCUSSION**

*A. salmonicida* is a highly efficient pathogen of fish (11). Intramuscular injection of as few as 10^2 virulent cells can produce disease within 96 h. Similarly, asymptomatic carrier fish subjected to environmental stress can die within 24 h with fulminant furunculosis. In both cases, postmortem examination has revealed the presence of large numbers of bacteria in the tissues, implying that the organism is readily able to grow in the low-Fe\(^{3+}\) environment found in vivo. This study suggests that one important contributor to this ability to grow rapidly in fish is the efficiency with which typical strains of *A. salmonicida* can sequester iron. In this property, it is similar to the other systemic pathogen of fish, *V. anguillarum*, in which a high-affinity iron-sequestering mechanism has been demonstrated to be an important virulence attribute (6). Unlike *V. anguillarum*, however, virulent strains of *A. salmonicida* have an additional mechanistic problem to overcome in the form of a regular surface protein array. This layer protects the cell from bacteriophage (13) and serum complement (17), as well as from several proteolytic enzymes (unpublished data). Although the exclusion limit of this layer is not currently known, studies with other surface arrays suggest that these structures act as barriers for molecules with molecular weights larger than ca. 3,000 (28). If this is the case with the A-layer, then iron-carrying glycoproteins such as lactoferrin or transferrin with a molecular weight of 90,000 would certainly be excluded from direct access to the outer membrane.

This study has shown that typical strains of *A. salmonicida* utilize two distinct strategies to sequester iron, one involving production of a soluble low-molecular-weight siderophore(s) and one which does not involve soluble siderophore production. Three of the four typical strains tested produced the siderophore(s). In the case of *A. salmonicida* A450, the siderophore(s) gave neither positive Arnow nor positive Csáky reactions, suggesting that it may not belong to either phenolate or hydroxamate classes. The siderophore(s) clearly differs from those produced by *Aeromonas hydrophila*, which are detectable by the Arnow test (unpublished data) and have been reported to be indistinguishable from enterobactin (C. R. Andrus and S. M. Payne, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, D13, p. 61). The iron-sequestering system of strain A449 was noninducible and appeared not to involve the production of a soluble siderophore(s). This strain appeared to require cell contact with iron-chelator complexes such as the glycoprotein lactoferrin and was able to directly strip the iron from this chelator. In this regard, it resembles the strains of *N. meningitidis* studied by Simonson et al. (26, 27), which also required cell contact with the iron chelator. As was the case when *N. meningitidis* acquired Fe\(^{3+}\) from transferrin, the iron-sequestering activity of *A. salmonicida* strain A449 was energy dependent, with the A449 system being strongly inhibited by the proton gradient uncoupler 2,4-DNP. Although apparently unable to produce soluble siderophores, *A. salmonicida* strain A449 was able to utilize the siderophore(s) produced by

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**FIG. 4.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the outer membrane fraction isolated from cells of *A. salmonicida* grown in the presence of 0.28 mM EDDA or 0.28 mM EDDA + 0.54 mM FeCl\(_3\). Proteins were stained with Coomassie blue, and molecular weight standards (×1,000 daltons) are given on the left of the gels. (A) Strain A449 grown with EDDA (lane 1) and EDDA + Fe\(^{3+}\) (lane 2) and strain A450 grown with EDDA (lane 3) and EDDA + Fe\(^{3+}\) (lane 4). (B) Strain A450-3R grown with (lane 1) and without EDDA (lane 2). Induced outer membrane proteins are indicated by the arrow.
strain A450. This ability readily distinguished strain A449 from the direct-iron-stripping strains of Neisseria meningitidis, which were unable to use Fe³⁺ from four common siderophores.

As is the case with other bacterial species, A. salmonicida displays iron-repressible outer membrane proteins. The role of these outer membrane proteins in A. salmonicida is unclear. However, in the case of E. coli, some of the low-Fe³⁺-induced proteins are involved in the binding of Fe³⁺-siderophore complexes (20, 24). Owing to the size (ca. 700 daltons) and the hydrophilic nature of ferric enterobactin, E. coli requires an outer membrane component to transport the iron ligand into the cell. The first step in this transport of ferric enterobactin involves the binding of the ligand to an outer membrane receptor, the fenA gene 81,000-dalton product (20). Since the low-Fe³⁺-induced outer membrane proteins of A. salmonicida are in the same subunit size range as this iron chelator receptor of E. coli and also of other bacteria, they may well have the same function.

The atypical strains of A. salmonicida were perceptibly poorer in their ability to sequester iron under conditions of iron limitation. However, like typical strain A449, atypical strain A406 was able to utilize the siderophore(s) in strain A450 supernatant fluid. Indeed, the generally poorer growth of these atypical strains even in the presence of soluble siderophores may indicate that they require their Fe³⁺ in a different form altogether. Perry and Brubaker (23) have reported that Yersinia pestis is able to obtain iron from hemin without the benefit of excreted siderophores, and preliminary evidence suggests that this may be the case with atypical strains of A. salmonicida (Ishiguro et al., unpublished data). This apparently restricted ability of atypical strains of A. salmonicida to acquire Fe³⁺ may also be reflected in their pathogenesis. The atypical strains commonly produce a slowly progressing infection with surface lesions and little or no systemic involvement until late in the disease process.

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LITERATURE CITED