Acquisition of iron from host sources by mesophilic Aeromonas species

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The mesophilic Aeromonas species are opportunistic pathogens that produce either of the siderophores amonabactin or enterobactin. Acquisition of iron for growth from Fe-transferrin in serum was dependent on the siderophore amonabactin; 50 of 54 amonabactin-producing isolates grew in heat-inactivated serum, whereas none of 30 enterobactin-producing strains were able to grow. Most isolates (regardless of siderophore produced) used haem as a sole source of iron for growth; all of 33 isolates grew with either haematin or haemoglobin and 30 of these used haemoglobin when complexed to human haptoglobin. Mutants unable to synthesize a siderophore used iron from haem, suggesting that this capacity was unrelated to siderophore production. Some members of the mesophilic Aeromonas species have evolved both siderophore-dependent and -independent mechanisms for acquisition of iron from a host.

Introduction

Competition for iron between a vertebrate host and an invading micro-organism is one of the points on which the outcome of an infection is balanced. The absolute nutritional requirement of most bacteria for the metal means that the microbe cannot establish itself without overcoming the host’s iron-witholding defences. Transferrin is responsible for the iron-limiting bacteriostatic activity of serum, although some pathogenic bacteria use Fe-transferrin as a source of the metal. Certain siderophores (microbial iron transport cofactors) are able to chelate iron from Fe-transferrin. Production of the siderophores aerobactin and anguibactin is crucial for virulence of pathogenic Escherichia coli and Vibrio anguillarum, respectively (Crosa, 1984; Carbonetti et al., 1986). Because they produce cell surface transferrin receptors, the pathogenic Neisseria species can derive iron directly from Fe-transferrin without intervention of a siderophore (West & Sparling, 1985).

Haem, primarily in the form of haemoglobin, is another potential source of iron to an invading microbe. To access iron in haem, cytolytic (haemolytic) destruction of host cells may be required. This may account in part for the importance of haemolysins in virulence (Marre et al., 1986; Chakraborty et al., 1987; Kathariou et al., 1987). Many haemolysins are regulated by iron, their synthesis being increased by iron starvation (Riddle et al., 1981; Lebeck & Gruenig, 1985; Stoebner & Payne, 1988). Free haemoglobin and haem should be scarce in the sera of vertebrates because these compounds are complexed by the serum proteins haptoglobin and haemopexin, respectively (Muller-Eberhard, 1970; Sutton, 1970). Association of haemoglobin with haptoglobin prevents use of haem iron by E. coli (Eaton et al., 1982) suggesting an iron-withholding defence function for haptoglobin, although certain other bacteria readily acquire iron from haemoglobin–haptoglobin complexes (Francis et al., 1985; Dyer et al., 1987; Stull, 1987; Zakaria-Meehan et al., 1988). Haemopexin and albumin bind haem, preventing use of haem iron by species of Neisseria (Dyer et al., 1987) but not by Haemophilus influenzae (Stull, 1987).

The mesophilic Aeromonas species are opportunistic Gram-negative pathogens that cause diseases in hosts ranging from fish to man (Khardori & Fainstein, 1988). The haemolysin(s) of A. hydrophila have been shown to be iron regulated (Riddle et al., 1981) and to be important in virulence (Chakraborty et al., 1987). Most isolates produce one of two siderophores, either enterobactin or amonabactin; a few produce no siderophore (Barghouthi et al., 1989a; Zywno et al., 1989). The mechanism by which these extracellular pathogens obtain iron from a vertebrate host is not known; therefore, the purpose of the present study was to determine the capacities of the mesophilic aeromonads to use Fe-transferrin and various haem-proteins as sole iron sources.

Abbreviation: EDDA, ethylenediamine-di(3-hydroxyphenylacetic acid).
Methods

Bacteria. Strains of Aeromonas hydrophila, A. sobria and A. caviae (isolated from clinical and environmental sources) were obtained from C. Lobb, J. Bertolini and S. Stuart of this institution. S. Zywno, USFDA Laboratory, Dauphin Island, Alabama, L. Pickering, University of Texas Health Science Center, Houston, and L. Ford, Louisiana State University, Baton Rouge, also supplied strains. An amonabactin mutant, A. hydrophila SB22, was supplied by S. Barghouthi of this institution. It was constructed by marker exchange mutagenesis of wild-type A. hydrophila 495A2 with the transposon-inactivated amonabactin biosynthetic gene AmoA::Tn5 (Barghouthi et al., 1989c). An enterobactin mutant, A. hydrophila GM89, was generated by chemical mutagenesis and isolated as previously described (Barghouthi et al., 1989b). Cultures were kept at −70 °C in 20% (v/v) glycerol in nutrient broth (Difco). The type of siderophore produced by these strains was reported previously (Zywno, 1989).

Sources and treatment of sera. Pooled human serum (obtained from Mississippi Blood Products, Jackson) and pooled channel catfish (ictalurus punctatus) serum (collected by venipuncture) were heat-inactivated at 56 °C for 30 min (to destroy complement activity) and were then filter sterilized (Millipak 40, 0.22 μm pore size, Millipore). Human complement serum was purchased from Sigma. Channel catfish complement serum was prepared from blood collected by venipuncture of healthy fish and was filter sterilized (Millex-GV, 0.22 μm pore size, Millipore). All sera were stored at −70 °C, before use they were slowly thawed and unused portions were discarded.

Assay for growth in heat-inactivated serum. Overnight cultures of the test strains in nutrient broth were diluted in potassium phosphate buffered saline (10 mm-potassium phosphate, 0.15 m-NaCl, pH 7.4) and were used to inoculate 1 ml of serum at an initial level of 10^3 c.f.u. ml⁻¹. After incubating this culture overnight at 37 °C, OD₆₀₀ was determined. An OD₆₀₀ ≥ 0.2 was considered positive for growth. For some determinations, iron (5 μg ml⁻¹) or amonabactin (1 μg ml⁻¹) was added to the cultures. Viable cell counts were determined by plating dilutions of the cultures onto Luria agar.

Preparation of host haem compounds. Haematin, human haemoglobin and human haptoglobin (phenotypes 1, 2, and 2-1) were purchased from Sigma. Channel catfish haemoglobin was obtained from freshly collected blood. The erythrocytes were separated from the plasma by centrifugation and washed three times with phosphate-buffered saline. The erythrocytes were lysed and the amount of haemoglobin in the lysate was determined spectrophotometrically from the A₄₁₄ by comparison with known concentrations of human haemoglobin. Haemoglobin–haptoglobin complexes were formed by incubating 2 ml human haemoglobin (0.25 mg ml⁻¹) with 1 ml haptoglobin.

Host haem compound utilization assay. To determine which host haem compounds could be utilized as iron sources by isolates of the Aeromonas species, the following assay was employed. Iron deficient L-EDDA agar was prepared by adding EDDA [ethylenediamine-di (o-hydroxyphenylacetic acid); Sigma] at a final concentration of 0.5 mg ml⁻¹ to Luria agar. Overnight cultures were inoculated into molten L-EDDA agar at a density of 10⁶ c.f.u. ml⁻¹. Compounds to be tested were impregnated on paper disks (that had been washed with 25 mM-EDTA and water) which were placed on the seeded agar plates. Plates were incubated and examined at 24 and 48 h for zones of growth surrounding the disks.

Assay for resistance to the bactericidal activity of complement sera. A modification of the microcolorimetric serum resistance assay of Moll et al. (1979) was used to measure resistance of the isolates to complement lysis. This assay is based on the fact that lysis by complement is immediately bactericidal, abolishing a micro-organism’s capacity to metabolize a substrate such as glucose. Therefore, resistance to complement lysis may be assessed by determining the capacity to metabolize glucose in the presence of serum. An overnight culture of each isolate in nutrient broth was diluted (1:10) into 200 μl nutrient broth in the well of a microtitre plate (Linbro, 12 × 8 flat bottom, Flow Laboratories). After 3 h incubation, this exponential-phase culture was diluted (1:10) into microtiter wells containing 200 μl PGB medium [1% (w/v) peptone, 1% (w/v) glucose and 0.0075% bromocresol purple], and PGB medium containing 30% (v/v) serum (PGBS). After incubating for 4-5 h, the A414 of each well was determined with a microtitre plate reader (Titertek, Flow Laboratories). Using bromocresol purple instead of the previously recommended bromothymol blue (Moll et al., 1979) prevented interference from culture turbidity, allowing A414 to represent colour change and not increase in culture turbidity. When heat-inactivated serum was used in this assay, all of the 198 strains responded as if resistant to lysis, confirming that the assay was an accurate measure of the sensitivity of a strain to complement lysis.

Results

Utilization of Fe-transferrin in serum

The capacity of Aeromonas species to use Fe-transferrin as a sole iron supply was determined by measuring growth in heat-inactivated serum. Of 54 amonabactin-producing isolates tested, 50 grew in heat-inactivated human serum, whereas none of 30 enterobactin-producing strains were able to grow (Table 1). Adding 5 μg iron ml⁻¹ to the heat-inactivated serum permitted growth of all isolates, demonstrating that inhibition was due to iron restriction. Moreover, the heat-inactivated serum was only bacteriostatic for those isolates unable to grow, because plate counts revealed no significant loss of viable cells at 24 h after inoculation (initially, 3.0 × 10⁷ c.f.u. ml⁻¹; at 24 h, 6.9 × 10³ c.f.u. ml⁻¹). The four amonabactin-producing isolates that did not grow in serum belonged to the species A. caviae (Table 1). An amonabactin mutant, A. hydrophila SB22, did not grow in heat-inactivated human or catfish serum; however, supple-

<table>
<thead>
<tr>
<th>Species</th>
<th>Siderophore produced:</th>
<th>Growth in heat-inactivated serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hydrophila</td>
<td>Amonabactin</td>
<td>32/32</td>
</tr>
<tr>
<td>A. sobria</td>
<td>Enterobactin</td>
<td>0/5</td>
</tr>
<tr>
<td>A. caviae</td>
<td></td>
<td>5/5</td>
</tr>
<tr>
<td>Totals</td>
<td></td>
<td>50/54</td>
</tr>
</tbody>
</table>

* No isolates were available when assays were done.

Table 1. Utilization of human Fe-transferrin as a sole iron source by mesophilic Aeromonas species

Growth assays were done twice and are expressed as no. isolates positive/no. isolates tested.
Table 2. Requirement for iron or amonabactin for growth in heat-inactivated serum

Representative data are shown; three experiments were performed for both human and catfish serum.

<table>
<thead>
<tr>
<th>A. hydrophila strain</th>
<th>No supplement</th>
<th>Iron</th>
<th>Amonabactin</th>
</tr>
</thead>
<tbody>
<tr>
<td>495A2</td>
<td>1.02</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SB22</td>
<td>0.00</td>
<td>2.66</td>
<td>0.471</td>
</tr>
</tbody>
</table>

ND, Not determined.

* Iron (5 μg ml⁻¹) was added as FeCl₃; amonabactin was added at 1 μg ml⁻¹.

Utilization of host haem compounds as sole iron sources

To determine if isolates of the mesophilic *Aeromonas* species could use various host haem compounds, we assayed the capacity of these compounds to reverse growth inhibition due to iron starvation. All of 33 isolates tested (representing both amonabactin- and enterobactin-producing strains) used either haematin or haemoglobin (human or catfish) as a source of iron (Table 3). Because human serum contains the haemoglobin-binding protein haptoglobin, complexes of haemoglobin with the three human haptoglobin phenotypes were also tested. Most isolates used haemoglobin associated with all three haptoglobin types, although one isolate of each species was unable to use any of the phenotypes. One strain (*A. hydrophila* 131) used types 1 and 2 but failed to grow with the heterozygous type 2-1 (Table 3).

Fig. 1. Use of haemoglobin or haemoglobin–haptoglobin by the amonabactin mutant *A. hydrophila* SB22 (a) and the enterobactin mutant *A. hydrophila* GM89 (b) for growth in Luria broth containing 0.1 mg EDDA ml⁻¹ with no addition (○); 50 μg haemoglobin ml⁻¹ (△); or haemoglobin-haptoglobin complexes containing 50 μg haemoglobin ml⁻¹ and 100 μg haptoglobin ml⁻¹ (□). The data shown are representative growth curves from replicate experiments.

To assess the role of siderophores in the use of haemproteins as iron sources, the siderophore mutants (*A. hydrophila* SB22, an amonabactin mutant; and *A. hydrophila* GM89, an enterobactin mutant) were tested for their ability to use these iron sources in an iron-deficient rich medium (Luria broth supplemented with EDDA). Both mutants were able to grow only when supplemented with either haemoglobin or haemoglobin–haptoglobin complexes (Fig. 1).

Resistance to serum complement bactericidal activity

Of 88 amonabactin-producing isolates, 70 (80%) were

Table 3. Utilization of host haem compounds as sole iron sources by mesophilic *Aeromonas* species

Growth assays were done three times and are expressed as the number of isolates able to grow on each haem source.

<table>
<thead>
<tr>
<th>Species</th>
<th>Total no. of isolates</th>
<th>Haematin</th>
<th>Haemoglobin*</th>
<th>Human haptoglobin type:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><em>A. hydrophila</em></td>
<td>21</td>
<td>21</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td><em>A. sobria</em></td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td><em>A. caviae</em></td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

* Human or catfish haemoglobin.
Table 4. Association of siderophore type and resistance to the bactericidal action of human complement serum

Numbers in parentheses are no. of resistant isolates/no. of isolates tested.

<table>
<thead>
<tr>
<th>Species</th>
<th>Amonabactin</th>
<th>Enterobactin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. hydrophila</td>
<td>90 (56/62)</td>
<td>15 (2/13)</td>
</tr>
<tr>
<td>A. sobria</td>
<td>80 (4/5)</td>
<td>49 (42/86)</td>
</tr>
<tr>
<td>A. caviae</td>
<td>47 (10/21)</td>
<td>- *</td>
</tr>
<tr>
<td>All isolates</td>
<td>80 (70/88)</td>
<td>44 (44/99)</td>
</tr>
</tbody>
</table>

* No isolates were available when assays were done.
† P < 0-00001 by chi-square test comparing resistance of enterobactin and amonabactin producers.

Discussion

Vertebrate serum is a complex material containing the respectively microbiostatic and microbicidal components transferrin and complement. The capacity of a micro-organism to use Fe-transferrin as an iron supply can be measured in a defined bacteriological medium to which purified Fe-transferrin is added, but the results may be misleading. Some siderophores may be inactivated by components of vertebrate serum. For example, serum albumin binds to the siderophore enterobactin and renders it nonfunctional; the siderophore aerobactin is unaffected by albumin (Konopka & Neilands, 1984). To approximate more closely to the situation in an animal host, we determined microbial use of Fe-transferrin as an iron source by measuring bacterial growth in human serum heated to destroy its complement activity. Growth of the Aeromonas species in heat-inactivated human serum was absolutely dependent on amonabactin production, suggesting that amonabactin mediated the use of Fe-transferrin. The fact that the enterobactin-producing strains were unable to grow in heat-inactivated serum confirms the finding that enterobactin is not functional in serum (Konopka & Neilands, 1984). The mesophilic aeromonads probably lack a system for the direct use (without a siderophore) of Fe-transferrin, because the amonabactin mutant A. hydrophila SB22 required added amonabactin or iron for growth in heat-inactivated serum. Aeromonas species were also able to use a variety of haem compounds as sources of iron. The finding that both the amonabactin and enterobactin mutants were able to use haem compounds indicated that use of haem as an iron source was siderophore-independent. Binding of haemoglobin by haptoglobin protected the haem moiety from utilization by a few isolates, supporting the proposition that haptoglobin may have bacteriostatic activity for some microbes (Eaton et al., 1982). It can be predicted that an isolate producing enterobactin must rely on its ability to use haem as an iron source during an infection. The cytolysins/haemolysins necessary for the release of haem compounds from host cells may also be crucial for the virulence of enterobactin-producing strains.

In addition to the iron-limiting bacteriostatic activity imposed by transferrin, vertebrate serum also contains complement which has an immediate bactericidal lytic action on many bacteria. For the strains that we tested, the group that produce amonabactin were statistically (P < 0-00001) more resistant to complement lysis than the enterobactin-producing strains. Resistance of Aeromonas species to complement lysis has been related to a protein surface array (S-layer) and to lipopolysaccharide (Munn et al., 1982; Dooley & Trust, 1988).

In summary, certain members of the mesophilic Aeromonas species have evolved two separate systems for acquisition of host iron. One of these is siderophore-mediated and accesses iron in Fe-transferrin; however, of the two siderophores made by different isolates of the aeromonads, only amonabactin functions in the serum environment. Most isolates also use (by a siderophore-independent process) various haem compounds as sole iron sources. Whether either or both of these alternative methods of iron acquisition are used during an infection remains unknown.

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References


