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-withholding 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(*o*-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 *et al.*, 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 $\mu\text{g ml}^{-1}$) or amonabactin (1 $\mu\text{g ml}^{-1}$) 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 A_{540} 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 mg 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 10^4 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 $\times$ 8 flat bottom, Flow Laboratories). After 3 h incubation, this exponential-phase culture was diluted (1:10) into microtitre 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 A_{414} 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 A_{414} 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, 3.0×10^3 c.f.u. ml^{-1} ; at 24 h, 6.9×10^3 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 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.

Species	Growth in heat-inactivated serum	
	Siderophore produced:	
	Amonabactin	Enterobactin
<i>A. hydrophila</i>	32/32	0/5
<i>A. sobria</i>	5/5	0/25
<i>A. caviae</i>	13/17	—*
Totals	50/54	0/30

* No isolates were available when assays were done.

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.

<i>A. hydrophila</i> strain	Growth (OD ₆₀₀) in heat-inactivated serum supplemented with:*		
	No supplement	Iron	Amonabactin
495A2	1.02	ND	ND
SB22	0.00	2.66	0.471

ND, Not determined.

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

mentation with 1 µg amonabactin ml⁻¹ or with 5 µg iron ml⁻¹ allowed growth (Table 2).

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 haem-proteins 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.

Species	Total no. of isolates	Growth on haem source indicated				
		Haematin	Haemoglobin*	Haemoglobin-haptoglobin		
				Human haptoglobin type:		
				1	2-1	2
<i>A. hydrophila</i>	21	21	21	20	19	20
<i>A. sobria</i>	9	9	9	8	8	8
<i>A. caviae</i>	3	3	3	2	2	2

* 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.

Species	Resistant isolates (%)	
	Siderophore produced:	
	Amonabactin	Enterobactin
<i>A. hydrophila</i>	90 (56/62)	15 (2/13)
<i>A. sobria</i>	80 (4/5)	49 (42/86)
<i>A. caviae</i>	47 (10/21)	—*
All isolates†	80 (70/88)	44 (44/99)

* No isolates were available when assays were done.

† $P < 0.00001$ by chi-square test comparing resistance of enterobactin and amonabactin producers.

resistant to the immediate bactericidal action of complement while only 44% of 99 enterobactin-producing isolates were similarly resistant (Table 4). Chi-square analysis revealed that this difference was statistically significant ($P < 0.00001$), suggesting that the probably independent traits of amonabactin synthesis and complement resistance tend to occur together. Like the parent strain, the amonabactin mutant *A. hydrophila* SB22 retained resistance to complement lysis. Human complement serum and channel catfish complement serum gave identical results with all strains.

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 entero-

bactin 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

- BARGHOUTH, S., YOUNG, R., OLSON, M. O. J., ARCEAUX, J. E. L., CLEM, L. W. & BYERS, B. R. (1989a). Amonabactin, a novel tryptophan- or phenylalanine-containing phenolate siderophore in *Aeromonas hydrophila*. *Journal of Bacteriology* **171**, 1811–1816.

- BARGHOUTH, S., YOUNG, R., ARCENEUX, J. E. L. & BYERS, B. R. (1989b). Physiological control of amonabactin biosynthesis in *Aeromonas hydrophila*. *Biology of Metals* **2**, 155–160.
- BARGHOUTH, S., ARCENEUX, J. & BYERS, R. (1989c). Cloning and transposon mutagenesis of a siderophore (amonabactin) gene from *Aeromonas hydrophila*. In *Abstracts of the Annual Meeting of the American Society for Microbiology* D214, p. 118.
- CARBONETTI, N. H., BOONCHAI, S., PARRY, S. H., VAISANEN-RHEN, V., KORHONEN, T. K. & WILLIAMS, P. H. (1986). Aerobactin-mediated iron uptake by *Escherichia coli* isolates from human extraintestinal infections. *Infection and Immunity* **51**, 966–968.
- CHAKRABORTY, T., HUHLE, B., HOF, H., BERGBAUER, H. & GOEBEL, W. (1987). Marker exchange mutagenesis of the aerolysin determinant in *Aeromonas hydrophila* demonstrates the role of aerolysin in *A. hydrophila*-associated systemic infections. *Infection and Immunity* **55**, 2274–2280.
- CROSA, J. H. (1984). The relationship of plasmid-mediated iron transport and bacterial virulence. *Annual Review of Microbiology* **38**, 68–89.
- DOOLEY, J. S. G. & TRUST, T. J. (1988). Surface protein composition of *Aeromonas hydrophila* strains virulent for fish: identification of a surface array protein. *Journal of Bacteriology* **170**, 499–506.
- DYER, D. W., WEST, E. P. & SPARLING, P. F. (1987). Effects of serum carrier proteins on the growth of pathogenic *Neisseriae* with heme-bound iron. *Infection and Immunity* **55**, 2171–2175.
- EATON, J. W., BRANDT, P., MAHONEY, J. R. & LEE, J. T. (1982). Haptoglobin: a natural bacteriostat. *Science* **215**, 691–693.
- FRANCIS, R. T., JR, BOOTH, J. W. & BECKER, R. R. (1985). Uptake of iron from hemoglobin and the hemoglobin-haptoglobin complex by hemolytic bacteria. *Journal of Biochemistry* **17**, 767–773.
- KATHARIOU, S., METZ, P., HOF, H. & GOEBEL, W. (1987). Tn916-induced mutations in the hemolysin determinant affecting virulence of *Listeria monocytogenes*. *Journal of Bacteriology* **169**, 1291–1297.
- KHARDORI, N. S. & FAINSTEIN, V. (1988). *Aeromonas* and *Plesiomonas* as etiological agents. *Annual Review of Microbiology* **42**, 395–419.
- KONOPKA, K. & NEILANDS, J. B. (1984). Effect of serum albumin on siderophore-mediated utilization of transferrin iron. *Biochemistry* **23**, 2122–2127.
- LEBECK, G. & GRUENIG, H. (1985). Relation between the hemolytic property and iron metabolism in *Escherichia coli*. *Infection and Immunity* **50**, 682–686.
- MARRE, R., HACKER, J., HENKEL, W. & GOEBEL, W. (1986). Contribution of cloned virulence factors from uropathogenic *Escherichia coli* to nephropathogenicity in an experimental rat pyelonephritis model. *Infection and Immunity* **54**, 761–767.
- MOLL, A., CABELLO, F. & TIMMIS, K. N. (1979). Rapid assay for the determination of bacterial resistance to the lethal activity of serum. *FEMS Microbiology Letters* **6**, 273–276.
- MULLER-EBERHARD, U. (1970). Hemopexin. *New England Journal of Medicine* **283**, 1090–1094.
- MUNN, C. B., ISHIGURO, E. E., KAY, W. W. & TRUST, T. J. (1982). Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. *Infection and Immunity* **36**, 1069–1075.
- RIDDLE, L. M., GRAHAM, T. E. & AMBORSKI, R. L. (1981). Medium for the accumulation of extracellular hemolysin and protease by *Aeromonas hydrophila*. *Infection and Immunity* **33**, 728–733.
- STOEBNER, J. A. & PAYNE, S. M. (1988). Iron-regulated hemolysin production and utilization of heme and hemoglobin by *Vibrio cholerae*. *Infection and Immunity* **56**, 2891–2895.
- STULL, T. L. (1987). Protein sources of heme for *Haemophilus influenzae*. *Infection and Immunity* **55**, 148–153.
- SUTTON, H. E. (1970). The haptoglobins. *Progress in Medicine and Genetics* **7**, 163–215.
- WEST, S. E. H. & SPARLING, P. F. (1985). The response of *Neisseria gonorrhoeae* to iron limitation: alterations in the expression of membrane proteins without apparent siderophore production. *Infection and Immunity* **47**, 388–394.
- ZAKARIA-MEEHAN, Z., MASSAD, G., SIMPSON, L. M., TRAVIS, J. C. & OLIVER, J. D. (1988). Ability of *Vibrio vulnificus* to obtain iron from hemoglobin-haptoglobin complexes. *Infection and Immunity* **56**, 275–277.
- ZYWNO, S., BAILEY, C., ARCENEUX, J. & BYERS, R. (1989). Siderophore distribution in isolates of the *Aeromonas* species. In *Abstracts of the Annual Meeting of the American Society for Microbiology* D91, p. 97.