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Heme Requirement for Growth of Fastidious Atypical Strains of *Aeromonas salmonicida*

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The growth of fastidious atypical strains of the fish pathogen *Aeromonas salmonicida* on both solid and liquid media was dependent specifically on a source of heme which was apparently required for initiation of growth at low inoculum densities. Thus, hemin enhanced the plating efficiencies of such strains on solid medium and significantly reduced their inoculum-size-dependent lag times in broth. The heme requirement could also be satisfied by hematoporphyrin and, less effectively, by hemoglobin. Since the requirement was a stable property of all 17 strains tested, it may prove to be another taxonomic criterion by which the atypical strains can be differentiated from the typical strains of *A. salmonicida*.

Aeromonas salmonicida is the causative agent of the fish disease known as furunculosis (4). The disease caused by typical strains is normally systemic and is generally restricted to salmonid species. On the other hand, strains designated as atypical *A. salmonicida* cause an ulcerative form of furunculosis which occurs in a wider variety of fish species. The atypical strains also differ from typical strains in several taxonomic criteria. For example, most atypical strains do not form the brown, water-soluble pigment which is characteristic of typical strains. In addition, the two groups have notable differences in antibiotic resistance patterns and physiological properties. Despite the differences, there can be no doubt that the atypical and typical strains are closely related. This conclusion is based on serological studies (5), phage sensitivity patterns (5, 8), and DNA base composition and homology (3). The differences exhibited by the atypical strains in terms of host range, disease symptoms, and taxonomic properties have probably caused mistaken identifications on more than one occasion. For example, strains previously designated as *Haemophilus piscium* are now considered to be atypical strains of *A. salmonicida* (5, 8).

Atypical *A. salmonicida* strains are fastidious, slow-growing organisms. The primary isolation of these organisms from diseased fish apparently cannot be achieved on tryptic soy agar (TSA), a medium commonly used for the culture of typical strains, and media enriched with blood or serum appear to be required for this purpose (6). The difficulties encountered in the isolation of atypical *A. salmonicida* strains have prompted the suggestion that the incidence of this pathogen may actually be underestimated (6). The purpose of this study was to investigate the basis for the fastidious growth of these organisms.

Blood agar (TSA-blood: TSA from Difco Laboratories supplemented with 5% human blood) proved to be the best medium for growing the atypical strains in our collection. We compared the growth of our strains on TSA-blood and on TSA. Bacteria grown on TSA-blood were suspended in sterile saline to a density of approximately 10^9 cells per ml. A 10-fold serial dilution of the suspension was prepared, and 0.1-ml amounts of each dilution were plated on TSA and TSA-blood. The plating efficiencies on these media were determined by comparing the viable cell counts obtained. Table 1 shows typical results obtained with one of the best-growing strains in our collection, strain A400. The counts obtained on TSA were 2,000-fold lower than the counts on TSA-blood. It should also be noted that the colonies on TSA were the size of pinpoints and did not progress beyond this state even after several weeks of incubation, clearly indicating that TSA was deficient in an essential growth factor(s). The addition of hemin (10 μg/ml) increased the plating efficiency on TSA to a value close to that obtained with TSA-blood. As shown in Table 2, the plating efficiencies of all atypical *A. salmonicida* strains in our collection on TSA were enhanced by the addition of hemin, and the minimum concentrations required for this purpose varied from 0.2 to 3 μg/ml. The colonies of atypical *A. salmonicida* were dark brown on TSA plus hemin (TSA-hemin) and resembled those of wild-type Pgm^+ *Yersinia pestis* grown in the presence of hemin (7). The pigmentation may reflect the hemin-binding capability of the cells.

Other compounds were tested for growth factor activity by a filter paper disk assay. TSA plates were inoculated with about 10^5 to 10^6 cells of the test strain, and 6-mm-diameter Whatman 3MM filter paper disks saturated with solutions of test compounds at the indicated concentrations or with the solvents alone were placed on the lawn. The relative amounts of growth around the disks were recorded after 2 to 5 days of incubation at 25°C depending on the strain. A typical result of a test with hemin is shown in Fig. 1. Unless indicated otherwise, all test compounds were obtained from Sigma Chemical Co., St. Louis, Mo. Of those tested, only 2 other substances had growth-enhancing activity, and the essential results summarized in Table 2 suggest that the growth requirement was specific for heme. Hematoporphyrin (8 mg/ml) was as effective in this assay as hemin (10 mg/ml) in stimulating growth of all strains. On the other

TABLE 1. Effects of plating media on the determination of the viable cell count of a culture of strain A400[a]

Medium	No. of viable cells/ml	Relative plating efficiency
TSA-blood	8.2×10^8	1.0
TSA	4.0×10^5	0.0005
TSA-hemin	5.2×10^8	0.6

[a] A 10-fold serial dilution of a culture of strain A400 was plated on each medium, and the viable counts obtained were compared.

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TABLE 2. Heme requirements of atypical *A. salmonicida* strains

Strain	Source[a]	Relative growth stimulation[b]		
		Hemin	Hematoporphyrin	Hemoglobin
A400	T. J. Trust, goldfish, Australia	+++ (2)	+++	+
A401	T. J. Trust, goldfish, Australia	+++ (ND)	+++	+
A402	T. J. Trust, goldfish, Australia	+++ (0.2)	+++	+
A403	T. J. Trust, goldfish, Australia	+++ (3)	+++	+
A404	T. J. Trust, goldfish, Australia	+++ (3)	+++	−
A405	T. J. Trust, goldfish, Australia	+++ (ND)	+++	−
A406	T. J. Trust, goldfish, Australia	+++ (ND)	+++	+
A419	E. B. Shotts, Jr., strain Ozark, goldfish, United States	+++ (2)	+++	+
A430[c]	*H. piscium* ATCC 10801	+ (ND)	+	−
A442[c]	*H. piscium* ATCC 15711	+ (ND)	+	−
A444[c]	*H. piscium* ATCC 14361	++ (3)	++	−
A445[c]	*H. piscium* ATCC 14362	++ (3)	++	−
A452	R. Bootsma, strain V76/134, carp, The Netherlands	++ (2)	+	+
A460	W. D. Paterson, strain SL1, Atlantic salmon, Canada	+++ (ND)	+++	−
A461	W. D. Paterson, strain SK2, Atlantic salmon, Canada	+++ (ND)	+++	−
A462	W. D. Paterson, strain SK3, Atlantic salmon, Canada	+++ (0.2)	+++	−
A463	W. D. Paterson, strain PL, Atlantic salmon, Canada	+++ (ND)	+++	−

[a] T. J. Trust, this laboratory; E. B. Shotts, Jr., Department of Medical Microbiology, University of Georgia, Athens; ATCC, American Type Culture Collection, Rockville, Md.; R. Bootsma, Department of Pathology, University of Utrecht, Utrecht, The Netherlands; W. D. Paterson, Connaught Laboratories, Toronto, Canada.

[b] +++, Good growth; ++, moderate growth; +, poor growth; −, no growth. No strains grew in medium with protoporphyrin. The numbers in parentheses under hemin refer to minimum concentrations (micrograms per milliliter) required for optimum growth stimulation. ND, not determined.

[c] These strains are currently listed in the American Type Culture Collection Catalogue as *A. salmonicida* subsp. *achromogenes*.

hand, protoporphyrin IX (10 mg/ml) was completely inactive, indicating that the iron atom was essential. This iron requirement could not be satisfied with $FeCl_3$ (50 μM), $FeCl_2$ (50 μM), or ferric citrate (100 μM). Hemoglobin (10 mg/ml) stimulated growth of only a few strains and was notably less effective than hemin or hematoporphyrin in these cases. Myoglobin (10 mg/ml), another heme protein, was completely inactive. This may mean that molecular size or structure is important for growth factor activity. Other inactive substances included human transferrin (10 mg/ml), human milk lactoferrin (10 mg/ml), mucin (10 mg/ml), and *Aeromonas hydrophila* 495A2 siderophore (100 μg/ml; the kind gift of B. R. Byers, University of Mississippi Medical Center).

It should be noted that in addition to a source of heme, blood apparently contained at least one other unidentified factor necessary for optimum growth on TSA. Although the plating efficiencies of all atypical strains were restored by the

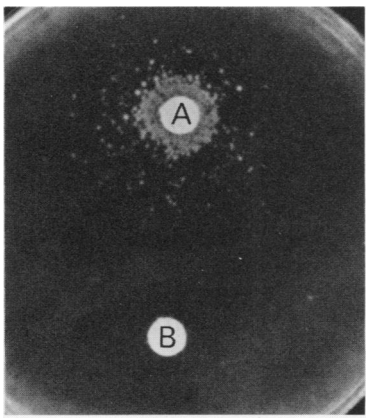

FIG. 1. Effect of hemin on growth of strain A403. Filter paper disks saturated with a 10-mg/ml solution of hemin (A) and with solvent alone (B) were placed on TSA inoculated with about 10^6 cells.

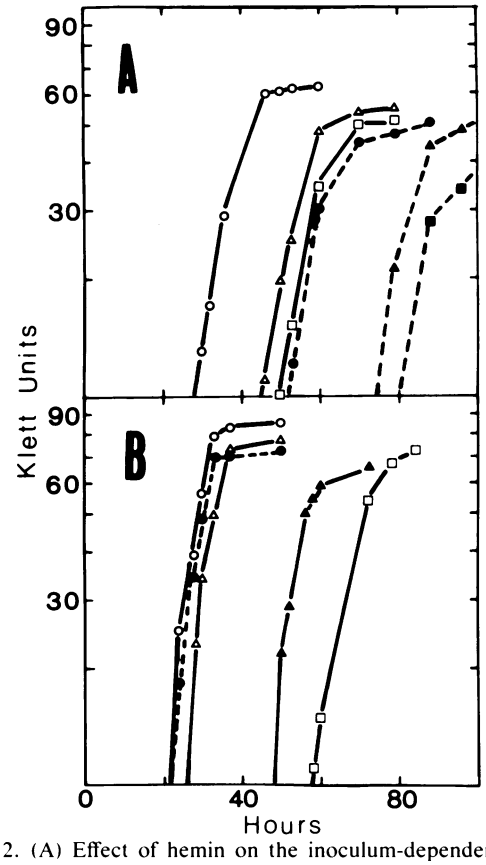

FIG. 2. (A) Effect of hemin on the inoculum-dependent lag of strain A403. Duplicate sets of TSB cultures were inoculated to densities of 2.0×10^7 cells per ml (○, ●), 4.0×10^6 cells per ml (△, ▲), and 2.0×10^5 cells per ml (□, ■). Hemin was added to one set of cultures (○, △, □) at 10 μg/ml. The cultures were incubated in a 25°C water bath shaker, and the culture turbidities were determined with a Klett-Summerson colorimeter with a green filter. (B) Effect of hemin concentration on growth of strain A403. TSB cultures inoculated with 3.5×10^7 cells per ml were supplemented with hemin at 0 (□), 1 (▲), 5 (△), 10 (●), and 20 (○) μg/ml.

addition of hemin, the colonies formed on TSA-hemin were notably smaller and slower in development than those on TSA-blood. There were also clear indications of strain differences with respect to this additional growth requirement. Thus, some strains (e.g., A400 and A401) grew obviously better than other strains (e.g., A452 and the strains previously designated as *H. piscium*) on TSA-hemin. This could mean that the requirement is not the same for all strains, or it could simply reflect strain differences in physiological capabilities.

The effect of hemin on growth in tryptic soy broth (TSB) was investigated. Two strains, A400 and A403, were used, and both gave similar results. Neither strain grew in TSB without hemin when the inoculum density was less than 5×10^4 cells per ml. However, hemin-independent growth occurred with higher inoculum densities. Figure 2A shows that such growth was preceded by an inoculum-size-dependent lag which was significantly reduced by the addition of hemin (10 μg/ml). Figure 2B shows that the reduction in lag time was dependent on hemin concentration. The hemin concentration required for optimum stimulation of strain A403 was threefold higher in TSB than on TSA (Table 2), i.e., 10 and 3 μg/ml, respectively. The following points are also noteworthy. (i) Exhaustive efforts to isolate hemin-independent mutants from populations which developed in the absence of hemin were unsuccessful. The large number of isolates obtained from TSB cultures grown without hemin still exhibited hemin dependence upon subsequent subculture. We also failed to find hemin-independent mutants by screening colonies appearing on TSA in the experiment described in Table 1. These results indicate that the hemin requirement in these strains was a stable characteristic, but at high inoculum densities at least some of the cells could initiate hemin-independent growth. (ii) The growth yields obtained in TSB and TSB plus hemin with strains A400 and A403 were only about 30% of the yields obtained with typical *A. salmonicida* strains in these media. This probably reflects the lack of the additional growth factor(s) required by atypical strains in TSB as noted above. (iii) Hemin usually did not significantly increase the final cell yield in TSB, and the only notable effect was therefore the reduction in the inoculum-dependent lag. In this respect, the effect of heme on these organisms strikingly resembles the growth-initiating effect of the iron-chelating hydroxamic acid, schizokinen, on *Bacillus megaterium* (1, 2). However, the functional role of hemin remains to be determined.

In summary, the growth of atypical *A. salmonicida* strains on solid and liquid media was dependent specifically on a source of heme which was apparently required to initiate growth at low inoculum densities. Thus, hemin enhanced the plating efficiencies of such strains on solid media and reduced their inoculum-dependent lag times in broth. It is premature to comment on the significance of these findings with respect to the virulence mechanisms of this fish pathogen. However, we consider it significant that all of the atypical strains tested exhibited a heme requirement. This fact, coupled with the apparent stability of the property, suggests that a heme requirement may represent still another useful taxonomic criterion to differentiate the atypical group from the typical group.

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