Asian Fisheries Science 10(1998):317-321
Asian Fisheries Society, Manila, Philippines
https://doi.org/10.33997/j.afs.1998.10.4.004

The Role of *Aeromonas hydrophila* Protease in the Utilization of Fish Serum Iron in vitro

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Abstract

Some authors argue that pathogenic bacteria could compete against serum transferrin for iron by their siderophore. However, *Aeromonas hydrophila* CRI 14, the causative agent of goldfish (*Carassius auratus*) hemorrhage disease, could not produce detectable siderophore when incubated at iron limited medium and analyzed by the chrome azurol S method. This strain produced clear orange halos around its colonies on blue agar only after 2 weeks of incubation, whereas it reaches the plateau phase after 36 h of incubation in goldfish serum. Incubating 1 ml of fish serum with the extracellular proteins of 1 ml culture of CRI 14 at 37 for 30 min., the transferrin-bounded iron contents decreased from $1.5 g \cdot ml^{-1}$ to $0.9 g \cdot ml^{-1}$ and $1.3\ g \cdot ml^{-1}$ to $0.45 g \cdot ml^{-1}$ for goldfish and bighead (*Aristichthys nobilis*), respectively, and the iron binding capacity also decreased from $2.3 g \cdot ml^{-1}$ to $1.8 g \cdot ml^{-1}$ and $2.7 g \cdot ml^{-1}$ to $1.0 g \cdot ml^{-1}$ for goldfish and bighead, respectively. An in situ iron-binding experiment after polyacrylamide gel electrophoresis (PAGE) further showed that both iron-binding and free transferrin have been digested by *A. hydrophila* protease. These results showed that *A. hydrophila* protease can compensate its inadequacy of siderophore for iron.

Introduction

Because of the electronic structure of its atom, which can undergo reversible changes in its oxidation state, iron is involved in so many essential processes inside cells, ranging from respiration to ribonucleotide synthesis. Therefore, every organism has its strategy for the acquisition of iron.

In the animal body, most iron is contained intracellularly in heme, iron-sulfur proteins and the iron-storage compound ferritin. Extracellular iron is mostly complex to carrier glycoproteins such as transferrin in serum. To survive in the host, many pathogenic bacteria have evolved several means of iron- transport. A strategy for iron acquisition by pathogens is the synthesis of high-affinity, iron-sequestering Fe^{3+}- ligands called siderophores. In many cases, siderophore contributes to virulence by enabling the bacteria to sequester transferrin iron, and can be thought as one of the criteria of pathogenic bacteria (Martinez 1990).

Aeromonas hydrophila has also been shown to produce siderophores (Gierer *et al.* 1992). However, previous experiments showed that the pathogenicity of *A. hydrophila* was not always accompanied by the presence of siderophores (Tang *et al.* 1995). Some pathogenic *A. hydrophila* could not produce detectable siderophore.

This investigation was carried out to determine the iron-utilizing apparatus of *A. hydrophila.*

Materials and methods

Strain, reagents and media

A. hydrophila CRI 14 was isolated from intestinal walls of diseased goldfish and identified in the lab (Tang and Zhu 1995). Chrome azurol S (CAS) analyze solution was prepared following the Schwyn and Neilands (1987) method, with a modified 0. 1 mol/L acetic acid buffer. Hank's solution was prepared as in Zhou (1986). Nitroso-R solution was prepared as follows: 0. 5 g Nitroso- R, 1g hydroxyamine hydrochloride, 2.7 g $NaAoC3H_2O$ and 1. 5 ml acetic acid were dissolved in 100 ml water. The basal medium for CAS assay was M70 medium reported by Farmer *et al.* (1992). Siderophore detection was carried out as in Schwyn and Neilands (1987).

Preparation of fish sera and bacterial protease

Fish blood were collected from caudal artery with a syringe which was rinsed by heparin sodium (10,000um$\cdot l^{-1}$). It was then centrifuged at 3000 rpm for 3 min, after which the supernatant was saved as serum. The bacteria were incubated in LB overnight and the supernatant was collected. The supernatant was precipitated with 55% saturation of ammonium sulphate. The precipitates then were resuspended with 20 mmol/L Tris.Cl pH 7.5 buffer and dialyzed against the same buffer overnight as bacterial protease sample.

Digestion of fish sera with bacterial protease

The serum sample was mixed with 1/10 vol of bacterial protease and digested at 35 for 30 minutes. After digestion, the samples were subjected to polyacrylamide gel electrophoresis (PAGE) and underwent electrophoresis.

Electrophoresis and staining of transferrin

The acrylamide concentration which was used in the present experiment was 10%. After PAGE, the gel was rinsed in Nitroso- R solution and destained in water/methanol/acetic acid (5/5/1) solution (Mueller *et al.* 1962). Then ferric ammonium citrate was added to the destaining solution. The transferrin zones became green in polyacrylamide gel within 2-3 minutes.

Results and Discussion

The influence of iron on A. hydrophila

Using nitrilotriacetic acid (NTA)and 8-hydroxyquinoline (8-HQ) to chelate the Fe in LB medium, the wet weight and total extracellular proteins were compared. When grown in LB medium, the wet weight and proteins per ml of culture were 1.1 mg and 1.8 g, respectively. In LB+0.1 mM NTA, these two parameters were 1.5 mg and 1.2 g, respectively. In LB+0.1 mM 8-HQ, 1.4 mg

and 1.1 g respectively. When the iron was chelated, the proteins per unit of wet weight decreased.

When grown in M70 that has been depleted of iron, some outer membrane proteins were induced. Fig. 1 shows the results.

Besides, there were more effects of iron on *A. hydrophila*. When grown in LB medium, *A. hydrophila* CRI 14 took 8 hours to grow and 16 hours to reach plateau phase. However, when this strain was grown in the sera of goldfish and common carp, the required times for tow stages were 24 h and 44 h, respectively

These results showed that iron was an important factor for the growth of *A. hydrophila*.

A. hydrophila CRI 14 produces just a little quantity of siderophore

In many pathogens, siderophore can sequester iron from transferrin. However, *A. hydrophila* CRI 14 produce only a little amount of siderophore. When incubated overnight in M70-Fe medium, there was no detectable siderophore in the culture supernatant. When streaked in CAS plates, CRI 14 produced just a faint halo after 2 days. There were clear halos after 2 weeks of incubation (Fig. 2).

A. hydrophila proteases can compensate for its inadequacy in siderophore for iron and can contend with fish transferrin in iron acquistion in vitro

When CRI 14 was incubated at M70-Fe medium, its extracellular protease cannot be detected. The apparent hemolysin activity in the culture was only 30% of that of control (M70). This means that protease is crucial for *A. hydrophila* to cause disease.

Like bacteria, fishes also need iron. Transferrin is an important protein in acquiring iron for fishes. The primary Chinese freshwater fishes all have several transferrins. Furthermore, the polymorphism of transferrin is very complex among primary cultivated Chinese carps (Fig. 3).

Among 30 common carps (*Cyprinus carpio*) and goldfishes, 8 different transferrin combinations and 6 different transferrin combinations have been detected. Among 30 silver carps (*Hypophthalmichthys molitrix*), which have less breeds than common carp and goldfish, there were only 3 different combinations.

Because of transferrins, most of the iron in serum was bounded by transferrin. In common carp, the concentration of transferrin-bounded iron is 1.58g/ml, which accounts for 44% of the bounded iron in serum. In goldfish, the transferrin-bounded concentration is 1. 54g/ml, which accounts for 66% of the total bounded iron in goldfish serum. This shows that transferrin is efficient in bounding iron and is the principal iron-bounding protein in serum.

Digestion of fish sera by bacterial proteases was determined by PAGE. The results showed that most fish transferrins were digested by bacterial proteases (Fig.4).

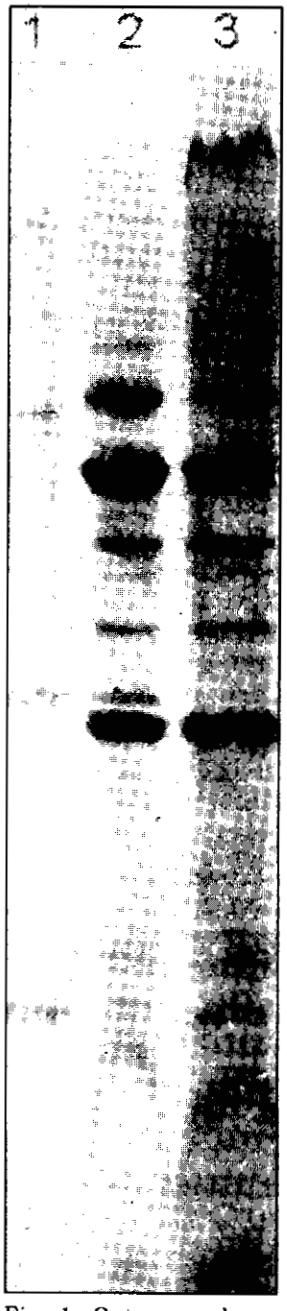

Fig. 1. Outer membrane proteins of *A. hydrophila*. The molecular concentration is 12%.
1. Molecular weight standards: Rabbit muscle phosphorylase b, 97.4kD; Bovine serum album 66.2kD; Hen egg white ovalbumin 42kD; Bovine carbonic anhydrase 31kD.
2. CRI 14 in M70-Fe.
3. CRI 14 in M70.

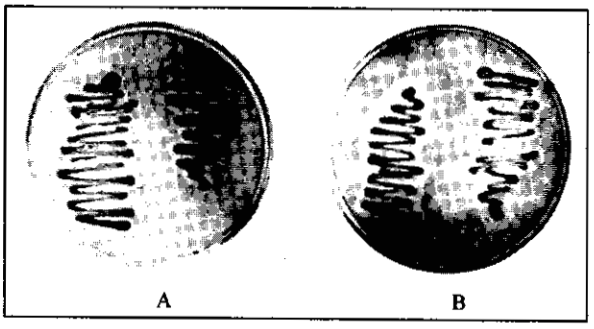

Fig. 2. Siderophore production of CRI 14 in CAS medium.
A: two days
B: two weeks.

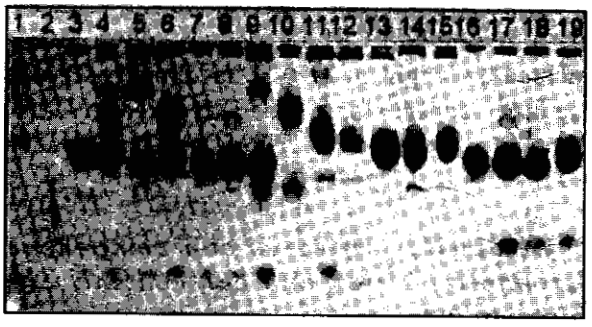

Fig. 3. Transferrin polymorphism of common carp, silver carp and bighead.
1. human Tf.
2. bovine Hb.
3-10. serum samples of different common carps.
11-16. serum samples of various goldfishes. 17-19 silver carp samples.

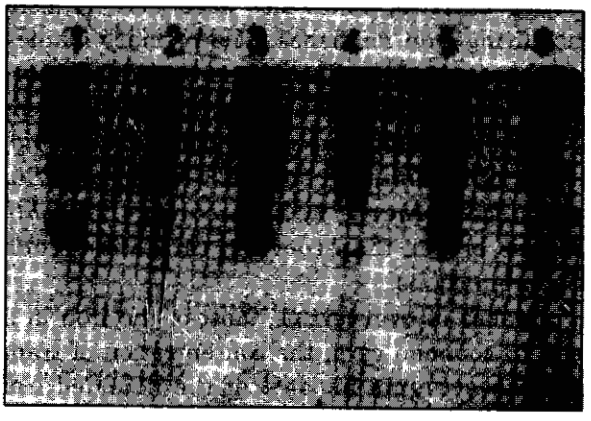

Fig. 4. Release of iron from transferrin by *A. hydrophila* protease.
1,3,5: different goldfish serum samples.
2,4,6: are the same samples, digested by bacterial protease.

Determination of the transferrin-bound iron concentration before and after bacterial protease digestion also revealed that *A. hydrophila* protease could release iron from fish transferrin. Incubating 1 ml of fish serum with the extracellular proteins of 1 ml culture of CRI 14 at 37 for 30 min., the transferrin bound iron contents decreased from $1.5g \cdot ml^{-1}$ to $0.9g \cdot ml^{-1}$ and $1.3g \cdot ml^{-1}$ to $0.45g \cdot ml^{-1}$ for goldfish and bighead, respectively. Iron-binding capacity also decreased from $2.3g \cdot ml^{-1}$ to $1.8g \cdot ml^{-1}$ and $2.7g \cdot ml^{-1}$ to $1.0g \cdot ml^{-1}$ for goldfish and bighead, respectively.

Although *A. hydrophila* CRI 14 produced siderophores, production speed was not in accordance with its growth in fish sera. Therefore, there are probably other routes of iron acquisition in *A. hydrophila*. The results of this paper showed that protease of *A. hydrophila* could digest most of the transferrin zones and release transferrin-bounded and other ferric proteins bounded by iron. Furthermore, it showed that *A. hydrophila* plays an important role in iron acquisition.

Acknowledgment

This work was funded by the 8th Five-Year Plan key project on biotechnology of the Ministry of Agriculture, China.

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Manuscript received 20 June 1996; accepted 6 November 1997.