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Aeromonas hydrophila suppresses complement pathways via degradation of complement C3 in bony fish by metalloprotease

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ABSTRACT

Aeromonas hydrophila is a pathogen that causes high mortality in the grass carp. The complement system, as a frontline defence of innate immunity, plays an important role in the immune response against pathogens. However, the immunity evasion mechanism of *A. hydrophila* against the complement system of grass carp remains unclear. In this study, we described an additional mechanism used by *A. hydrophila* GD18 to evade the complement system and survive in grass carp serum. First, *A. hydrophila* evaded the bactericidal activity of grass carp serum. Second, the haemolytic activity assays showed that *A. hydrophila* obviously suppressed the alternative pathway, which depended on preventing the formation or disabling the function of the membrane-attack complex (MAC). Further research indicated that *A. hydrophila* targeted complement C3, the central component of the three complement pathways, and degraded it in the grass carp serum, leading to the inhibition of the complement pathways, which resulted in the serum-resistance of *A. hydrophila.* Furthermore, cleavage analyses showed that extracellular proteases (ECPases) of *A. hydrophila* efficiently cleaved purified C3 as well as C3 in grass carp serum. Finally, protease inhibitor studies and mass spectrum analysis identified the secreted metalloprotease elastase (AhE), which was present in large amounts in crude ECPases, as the central molecule responsible for C3 cleavage. Compared to wild strain GD18, the AhE knockout, Δ*ahe* was dramatically reduced in the ability of serum resistance. Our findings suggested that *A. hydrophila* escaped serum-killing by suppressing the complement pathways via the degradation of complement C3 in bony fish, which was related to secreted metalloproteases.

1. Introduction

The grass carp, *Ctenopharyngodon idella*, is among economically important freshwater fish species in China [1]. However, high disease incidence and mortality have become a serious concern causing heavy economic losses. These are mainly caused by *Aeromonas hydrophila,* a Gram-negative bacterium that is widely distributed in aquatic environments and that can cause septicaemia in both fish and humans [2,3]. To successfully establish an infection, the bacterium must evade immune defences, including the complement system [4,5]. Previous studies have indicated that evasion of the complement system is critical for *A. hydrophila* to cause invasive disease in humans [6,7], which is reflected, in one aspect, in the observation that *A. hydrophila* can survive in host serum. Nevertheless, *A. hydrophila* survival and multiplication strategies against the host's complement system have not been fully elucidated in fish.

The complement system is a central component of innate immunity and is regarded as an important defender against pathogens. It consists of three activation pathways: the classical pathway (CP), lectin pathway (LP) and alternative pathway (AP) [8–10]. The three pathways converge at the cleavage of C3 to C3a and C3b, which leads to a series of events involving the cleavage of C5 and assembly of C5b, C6, C7, C8 and C9, leading to the formation of the membrane-attack complex (MAC), which damages the cytoplasmic membrane of the target [11]. To date, as represented by trout, carp and zebrafish data, it is now evident that almost all the homologues of the mammalian complement components are also present in the teleost system [12]. These findings have led to recognition that teleost complement system is equivalent or comparable to the mammalian system in both structural and functional aspects [13]. In addition, the complement system seems to play a more pivotal role in body defence in fish, whose adaptive immunity is considered to be at a relatively undeveloped state [14].

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Although the complement system works as a first line of defence and allows, in many cases, hosts to avoid infections, the evolution of the pathogens has resulted in the elaboration of evasion strategies against complement attack [15,16]. Complement evasion strategies of bacteria can be divided into three different groups: (1) the production of a capsule to prevent complement recognition, (2) the acquisition of host fluid phase complement regulatory proteins such as factor H (FH) and C4b-binding protein and (3) the secretion of proteases that inactivate complement molecules [17–19]. Among these secreted factors, we find a number of proteases that can cleave complement molecules. Interestingly, pathogen-derived proteases target a wide range of substrates, including proteins involved in cascade initiation, such as C1q and immunoglobulins, the central complement molecule C3 as well as terminal components, such as C5 [20]. An illustrative example of these proteases is *Pseudomonas*, which produces elastase (PaE) and alkaline protease (PaAP), which cleaves immunoglobulins and C1q, thus preventing activation [21]. Moreover, metalloprotease aureolysin from *Staphylococcus aureus* and surface-exposed autotransporter NalP from *Neisseria meningitides* cleave C3, producing two fragments (a shorter C3a-like and a longer C3b-like fragment) degraded by collaborating with host factors I and H and leading to decreased C3b deposition on the bacterial surface [22,23]. Staphylococcal superantigen-like protein 7 inhibits C5 conversion [24]. Recent studies have demonstrated that aquatic pathogens have evolved various tactics to prevent the killing of the complement system. For example, *Streptococcus agalactiae* utilises a metabolic trick to respond to plasma killing as a result of serum resistance [25], and high-molecular-weight O-antigens may protect *Vibrio* from serum killing by a dual act of avoiding initiating complement activation as well as sterically hindering the complement molecule from gaining access to and damaging the cell membrane [26]. Moreover, Sip1, a zinc metalloprotease of *Edwardsiella tarda*, is essential to serum resistance and host infection [27,28].

In this study, we examine the effect of *A. hydrophila* on serum complement activity and investigate the mechanism associated with serum resistance. We reveal that *A. hydrophila* resists serum attack by inhibiting the three complement pathways via degradation of complement C3 in grass carp, which may be related to the secreted metalloprotease elastase (AhE). This indicates a novel mechanism in the evasion of complement killing in bony fish.

2. Materials and methods

2.1. Proteins and antibodies

The rabbit anti-grass carp C3 (GenBank accession no.AAQ74974.1) polyclonal antibody was produced by immunising rabbits according to conventional methods by using recombinant C3 expressed in *E. coli.* The IgG fraction of the rabbit antiserum was purified using a HiTrap Protein G column (GE Healthcare). Thereafter, the specific pAbs were purified by affinity chromatography using NHS-activated Sepharose 4B (GE Healthcare) coupled with recombinant C3. Then, the native grass carp C3 was purified by affinity chromatography using NHS-activated Sepharose 4B (GE Healthcare, USA) coupled with anti-grass carp C3 polyclonal antibody, which was further confirmed by mass spectrum analysis (Spectrum zhonghe life technology co., LTD).

2.2. Bacterial strains and ECPases

Bacterial strains. *A. hydrophila* GD18, a virulent strain isolated from grass carp, which was confirmed by complete sequencing (Novogene Bioinformatics Technology) was cultured in tryptic soy broth (TSB) at 28 °C. *E. coli* DH5a, purchased from TransGen Biotech (Beijing, China), was cultured in Luria-Bertani broth (LB) medium at 37 °C.

Rough extraction of extracellular protease (ECPases). *A. hydrophila* GD18 was cultured overnight in TSB and subsequently diluted to an OD660 of 0.05 in fresh TSB, then shaked at 28 °C for 48 h, and supernatants were collected by centrifugation and passed through a 0.45-nm filter. Collected supernatants were concentrated 10 times using Amicon Ultra-10 filters (MWCO 10 kDa; Millipore), dialyzed against Tris-HCl (PH 8.0) and stored at −80 °C. The ECPases from *A. hydrophila* supernatant, were further purified by gel filtration on a superdex 75 column (GE Healthcare) using the AktaExplorer system (GE Healthcare), collecting components with protease activity. Proteolytic activity was checked by azocasein assay. Briefly, the protease were incubated with azocasein (5 mg/mL, in 50 mM Tris-HCl, PH 8.0) at 28 °C for 30 min, then incubated with 10%TCA at 0 °C for 30 min. Supernatant recovered by centrifugation was mixed with equal volume NaOH (1 M) and then measured absorbance at 442 nm. The ECPases were further analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrum analysis.

2.3. Serum survival assays

Blood was withdrawn from the caudal vein of grass carp by sterile injector and placed on ice immediately. The blood was allowed for clotting over night at 4 °C. For serum survival assay, the serum was treated with or without heating at 56 °C for 30 min. Besides, to prepare the activated serum, the grass carp was infected with *A. hydrophila* (10^6 CFU), then the serum was collected from the fish at 72 h post-bacterial infection. *A. hydrophila* GD18 and *E. coli* DH5a were cultured in TSB and LB medium to an OD_{600} of 0.8, respectively. The cells were washed and then resuspended to 2×10^6 CFU/ml in Hank's Balanced Salt Solution (HBSS) (Life technology, USA). The bacterial suspensions were mixed with the serum as follow: (1) Normal serum. (2) *A. hydrophila*-activated carp serum. (3) Heated inactivated serum. (4) 0.04 M ethylenediaminetetraacetic acid (EDTA) disodium salt treated serum. The control cells were mixed with HBSS. After incubation at 28 °C for 1 h, the mixture was serially diluted and plated in triplicate on TSA or LB agar plates. The plates were incubated at 28 °C for 36 h, and the colonies that appeared on the plates were enumerated. The genetic nature of the colonies was verified by PCR with primers specific to *A. hydrophila* GD18 and *E. coli* DH5a. The survival rate was calculated as follows: (number of cells that survived serum treatment/number of cells that survived control treatment) ×100%. The assay was performed three times independent.

2.4. Haemolytic activity assays

To prepare bacterial-incubated filtered serum, *A. hydrophila* and *E. coli* were cultured to an OD_{600} of 0.8, and the bacterial concentration was adjusted to 10^9 CFU/ml in DGVB (pH 7.4, consisting of 2.5 mM sodium barbiturate, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 50 mM NaCl, 0.1% gelatin, and 1% glucose), mixed with an equal volume grass carp serum. The mixture incubated at 28 °C for 1 h, after that, the mixture passed through a 0.22 μm filter to remove any bacterial cells. To prepare bacterial co-incubated serum, the serial diluted serum mixed with the bacteria above without filter. For haemolysis assay, sheep red blood cells (SRBC) (Guangzhou Future Technology, China) were washed and resuspended in DGVB, SRBC (10^8 cells) mixed with the above mentioned treatment diluted serum or the ECPase-treated serum, incubated at 28 °C for 30 min, then the supernatant was collected by centrifugation and determined for absorbance at 405 nm. Meanwhile, we also detected the survival rate of *A. hydrophila* and *E. coli* under the above condition.

2.5. Complement assays

C3 cleavage. (1) The diluted grass carp serum (2^2, 2^3 and 2^4 dilution) was incubated with *A. hydrophila* (10^9 CFU/ml) for 2 h at 28 °C in $HEPES^{++}$ (20 mM HEPES, 140 mM NaCl, 5 mM $CaCl_2$, 2.5 mM $MgCl_2$), or the 2^2 diluted grass carp serum was incubated with different concentrations of *A. hydrophila* (10^7, 10^8 and 10^9 CFU/ml). (2) The native

C3 (2 μg) was incubated with different concentrations of *A. hydrophila* (10^8 and 10^9 CFU/ml) for 2 h at 28 °C in $HEPES^{++}$. (3) The grass carp serum (2^3 dilution) or native C3 (2 μg) was incubated with the ECPase for 0, 10, 30, 60 min at 28 °C in $HEPES^{++}$. The reactions were stopped at by adding sample buffer containing DTT.

Western blotting. C3 cleavage analysis by Western blotting was performed as follow. The samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Bio-Rad Laboratories). The membranes were blocked for 1 h at room temperature in TBST buffer (25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20 [pH 7.5]) containing 5% nonfat dry milk, probed with the indicated primary Abs at an appropriate dilution overnight at 4 °C, washed three times with TBST, and then incubated with secondary Abs for 1 h at room temperature. After three additional washes with TBST, the membranes were stained with the Immobilon Western Chemiluminescent HRP Substrate (Millipore) and detected by using an ImageQuant LAS 4000 system (GE Healthcare). Abs were diluted as follows: anti-grass carp C3 at 1:1000, and HRP-conjugated anti-rabbit IgG (Thermo Fisher Scientific) at 1:5000. Results are representative of three independent experiments.

2.6. Proteolytic activity and inhibition assays

The chemical nature of the proteases was accessed by pre-incubation of the ECPases with inhibitors of serine, metallo-, or cysteine, (5 mmol/L Phenylmethylsulfonyl fluoride (PMSF), 5 mmol/L 1,10-phenanthroline, 28 μmol/L E−64, respectively) for 30 min. Proteolytic activity was checked by azocasein assay. The above treated ECPase were incubated with purified complement proteins (2 μg) for 5 min or serum for 1 h (amount corresponding to 1 μg of the complement protein studied) at 28 °C. The cleavage products were detected by means of Western blot.

2.7. Construction of the A. hydrophila GD18 ahe deletion mutant

The primers used in this study are listed in Table 1. The primers were designed according to the complete genome sequence of *A. hydrophila* GD18. Upstream and downstream flanking fragments of elastase (*ahe*) were amplified by PCR using primers F1/R1 and F2/R2 respectively. The fusion of the 2 fragments was amplified by overlap PCR using primers F1/R2. The fused segment (Δ*ahe*) was sequenced and then ligated into pRE112 at the KpnI sites. The resulting plasmid pΔahe was transformed into *E. coli* χ7213 for mobilization into *A. hydrophila* via conjugation. The transconjugants containing plasmid pΔ*ahe* integrated into WT strain chromosome by a single crossover event were selected on LA media containing chloramphenicol. Allelic exchange between the chromosomal gene and the mutagenized plasmidic copy was achieved by the second crossover event and was counter-selected on LB containing sucrose to determine the excision of pRE112 from the chromosome. The resultant strain, Δ*ahe* mutant, was selected by chloramphenicol sensitivity and sucrose resistance, and was verified by PCR using the primers F3/R3 and direct DNA sequencing of the mutation sites using genomic DNA preparations (TransGen Biotech) (see Table 1).

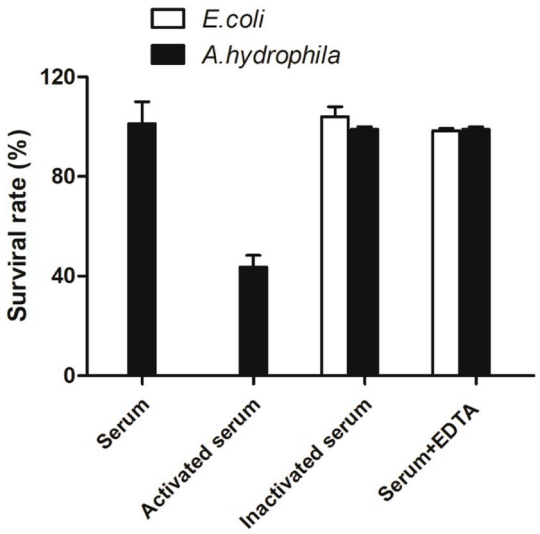

Fig. 1. Survival of *Aeromonas hydrophila* and *Escherichia coli* in fish serum. *A.hydrophila* GD18 and *E. coli* DH5a were incubated with normal serum, activated serum, heat-inactivated serum or EDTA-treated serum. The control cells were incubated without serum. After incubation, the survival rate of bacteria was determined by plate counting. The survival rate was calculated as follows: (number of cells that survived serum treatment/number of cells that survived control treatment) ×100%.

2.8. Statistical analysis

The statistical p values were calculated by one-way ANOVA with a Dunnett post hoc test (SPSS Statistics, version 19, IBM). Differences were considered significant at $p < 0.05$ and highly significant at $p < 0.01$.

3. Results

3.1. Survival of A. hydrophila in grass carp serum

As shown in Fig. 1, when *A. hydrophila* GD18 was incubated with grass carp normal serum, the survival rate of the bacteria was found to be 98.3%. In contrast, the survival rate of *Escherichia coli* DH5α, a serum-sensitive laboratory strain incubated under the same condition, was 0.14%. These results revealed that *A. hydrophila* GD18 resisted the bactericidal effect of grass carp serum. However, the survival rate of *A. hydrophila* GD18 in the infection-activated serum from grass carp post infected by *A. hydrophila* was obviously reduced (43.6%). In addition, when the EDTA was added to the serum or the serum was heat-inactivated, the survival rates of *E. coli* increased to the comparable levels of 100% and 98.7%, respectively, suggesting that the bactericidal effect in the serum is most likely mediated by the activated complement system.

Table 1
Primers used in this study.

Primer	Primer Sequence[a]	Application
F1	5′- CGGGGTACCCCCCTCGCCGCGGGCAAACTTCTCG-3′	Upstream fragment
R1	5′- CAACGGCCACTGGTGACCGTCTTTTCCTTAAGTCGATG -3′	
F2	5′- GACTTAAGGAAAAGACGGTCACCAGTGGCCGTTGACAA -3′	Downstream fragment
R2	5′- TATGAGGTACCTCTATCGCTCCCCCATCATAGCGGT-3′	
F3	5′- CCCCTCGCCGCGGGCAAACTTCTCG -3′	Mutant detection
R3	5′- GCAGGATCTGGGCCAGCCCCTGTTT-3′	

[a] Restriction sites for plasmid construction are underlined.

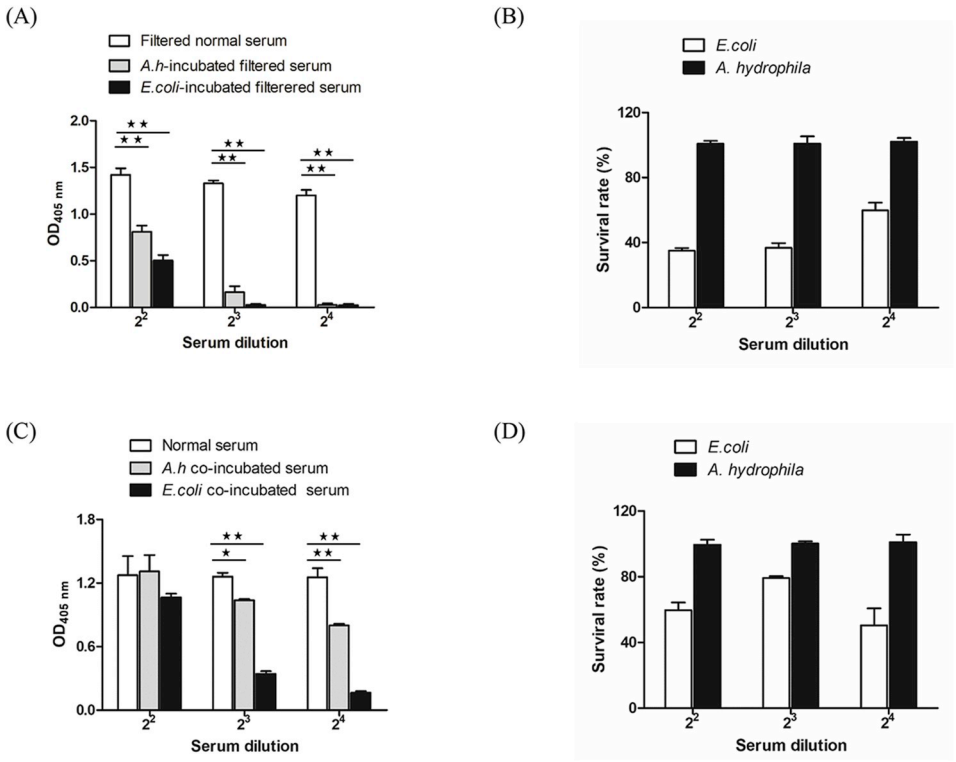

Fig. 2. Haemolytic activity and survival rate of bacteria-treated serum. (A) *A.hydrophila* GD18 and *E. coli* DH5a were incubated with the diluted serum for 1 h; the mixture was then passed through a 0.22-μm filter to remove bacterial cells, then incubated with SRBC, and determined for hemolysis. (B) *A. hydrophila* GD18 and *E. coli* DH5a were incubated with the diluted serum for 1 h. The survival rate was calculated as above. (C) *A.hydrophila* GD18 or *E. coli* DH5a was co-incubated with SRBC in the diluted grass carp serum for 1 h and then determined for hemolysis. (D) *A. hydrophila* GD18 or *E. coli* DH5a co-incubated with SRBC in the diluted grass carp serum for 1 h. The survival rate of the bacteria was calculated as above. The results are presented as the mean ± SEM of three fish. The *p* values were calculated by one-way analysis of variance with Dunnett's *post hoc* test. Differences were considered significant (shown as "*") at $p < 0.05$ and highly significant (shown as "**") at $p < 0.01$.

3.2. *A. hydrophila*-mediated inhibition of the alternative pathway

The serum was incubated with *A. hydrophila* GD18 or *E. coli* DH5α (for comparison), and then the co-incubated or leftover (filtered) complement activity of the serum were determined. The haemolytic activities of *A. hydrophila*-incubated filtered serum as well as those of *E. coli*-incubated filtered serum were lower than those of filtered normal serum to a significant extent (Fig. 2A). However, in *A. hydrophila*, unlike in *E. coli*, the complement system was consumed by pathogen lysis as the survival rate of *A. hydrophila* approached 100%, whereas those of *E. coli* were 36.72% and 59.78%, respectively (Fig. 2B). We further investigated whether inhibition of the alternative pathway by *A. hydrophila* occurred by complement activation suppression; the haemolytic activities of *A. hydrophila*-co-incubated serum were slightly lower than those of normal serum but still significantly higher than those of *E. coli*-incubated serum at 2^3 and 2^4 dilutions (Fig. 2C). Under this condition, the survival rate of *A. hydrophila* approached 100%, whereas those of *E. coli* were 78.3% and 50.5%, respectively (Fig. 2D). *A.hydrophila*-co-incubated serum retained a strong complement activity, which indicated that complement activation was not inhibited. These results suggested that inhibition of the alternative pathway of *A. hydrophila* depended on preventing the formation or disabling the function of MAC.

3.3. *A. hydrophila*-mediated C3 degradation

The AP serves as a vital line of defence due to the diversity and specificity of C3 [14], thus, we first analysed whether *A. hydrophila* affects the C3 molecules in the serum of grass carp. To investigate this hypothesis, *A. hydrophila* strains were incubated with serum samples at different dilutions, and the effect of *A. hydrophila* on complement C3 was determined by Western blot analysis. Before that, the C3 in grass carp and purified C3 were identified by SDS-PAGE and Western blot using rabbit anti-grass carp C3 polyclonal antibody (Fig. SA, B, C). Then, as shown in Fig. 3A, the amount of grass carp C3 in *A. hydrophila*-incubated serum was reduced, and the reduction was more evident in 2^4 dilutions than in 2^2 dilutions, which explained why *A. hydrophila*-incubated serum possessed low complement AP activity. Furthermore, the effect of different amounts of *A. hydrophila* on the C3 degradation was analysed (Fig. 3B). The tests revealed that the cleavage activity

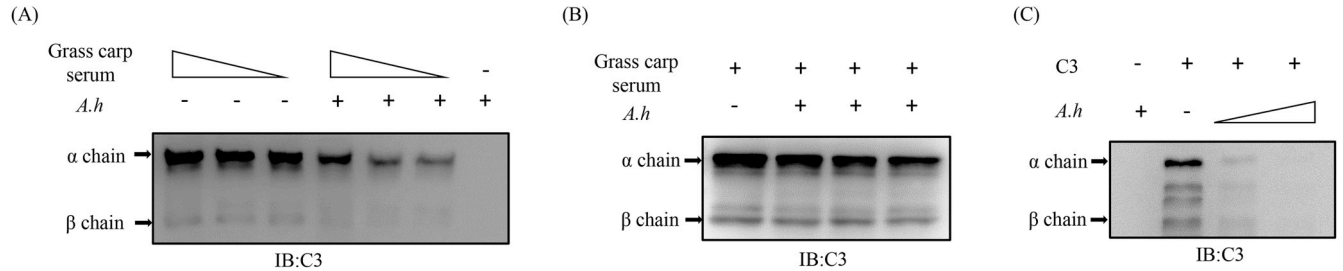

Fig. 3. *A. hydrophila*-mediated C3 degradation in grass carp serum. (A) *A. hydrophila* strains were incubated with the different diluted serum samples (2^2, 2^3 and 2^4 dilutions), and the effect of *A. hydrophila* (10^9 CFU/ml) on the complement C3 in grass carp serum was determined by Western blot analysis. (B) Different amounts of bacteria (10^7, 10^8 and 10^9 CFU/ml) were incubated with 2^2 diluted serum, and then the complement C3 in grass carp serum was determined by Western blot. (C) The native C3 was incubated with different concentrations of *A. hydrophila* (10^8 and 10^9 CFU/ml) for 2 h at 28 °C in $HEPES^{++}$, and then the effect of *A. hydrophila* on the purified C3 was determined by Western blot analysis The samples were detected by Western blot using anti-grass carp C3 poly polyclonal antibody. All experiments were repeated at least three times with similar results.

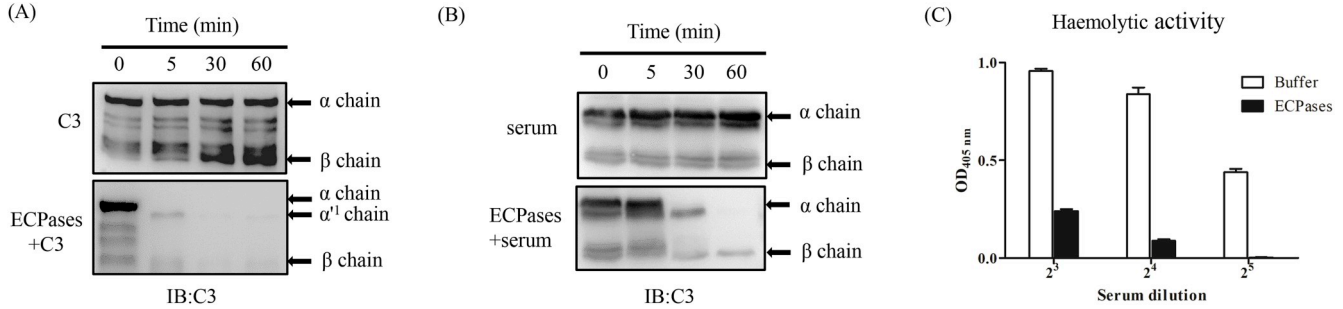

Fig. 4. ECPases secreted by *A. hydrophila* cleave C3. (A) Purified C3 (2 μg) was incubated with ECPase (0.4 μg), or (B) 2^3 diluted grass carp serum was incubated with ECPase (2 μg) for 0, 10, 30 and 60 min at 28 °C in $HEPES^{++}$; cleavage was analysed by Western blot. (C) Dilutions of grass carp serum were incubated with Tris-HCl buffer (50 mM, PH 8.0) or ECPases at 28 °C for 1 h, and then SRBC was mixed with the abovementioned treatment of diluted serum. Haemolytic activity was determined by measuring absorbance at 405 nm. All experiments were repeated at least three times.

increased with the amount of bacteria. To investigate whether the *A. hydrophila* directly target the key complement molecules C3, thereby promoting a cleavage, the proteolytic assays were performed with C3 and the *A. hydrophila* strain in $HEPES^{++}$ buffer. As shown in Fig. 3C, we observed that a complete degradation of C3 occurred in dose-dependent manner, suggesting that *A. hydrophila* directly cleaved C3 without synergy with complement regulatory factors in the grass carp.

3.4. Cleavage of C3 by ECPases secreted by A. hydrophila

Previous researches demonstrated that pathogen-derived proteases were able to inactivate complement molecules [29,30]. We incubated purified C3 with *A. hydrophila* extracellular proteases (ECPases) and analysed C3 cleavage by Western blot. After only 5 min of reaction, we observed that ECPases caused rapid cleavage of the C3 α-chain into a smaller fragment (α'^1 chain) of around 100 kDa (Fig. 4A). More pronounced cleavage occurred after 30 and 60 min of incubation, with less C3 α'^1 chain residue. Our data clearly demonstrated that ECPases were able to cleave purified complement molecules. However, we should consider that ECPases must also have activity in serum if they are to constitute an immune evasion strategy. To address this issue, we performed further proteolytic assays, which revealed that ECPases were able to cleave C3 in grass carp serum (Fig. 4B). However, compared with the cleavage with the purified complement protein, this cleavage in the serum was less effective. As shown in Fig. 4B, C3 α-chain cleavage occurred until 30 min of incubation, probably due to the variety of ions and inhibitors in the serum. In addition, the C3α'^1 chain was not detected in the serum, which was probably rapidly degraded by the proteases in the grass carp serum. Moreover, to determine whether ECPases block the complement cascades, we tested their activity by haemolytic assays (Fig. 4C). The ECPases-incubated serum possessed low haemolytic activity, in line with the above results (section 3.2) showing inhibition of the AP by *A. hydrophila*.

3.5. Complement pathway inhibition by ECPase depends on its proteolytic activity

The classes of proteases involved in the cleavage of the complement proteins were identified by inhibition assays. In these experiments, before the addition of the substrates, ECPases were treated with inhibitors of serine, metallo- and cysteine proteases, and then proteolytic activity was analysed by azocasein assay. The result showed that only 1,10-phenanthroline was able to inhibit the proteolytic activity (Fig. 5A). Subsequently, we tested whether this inhibitor-treated form

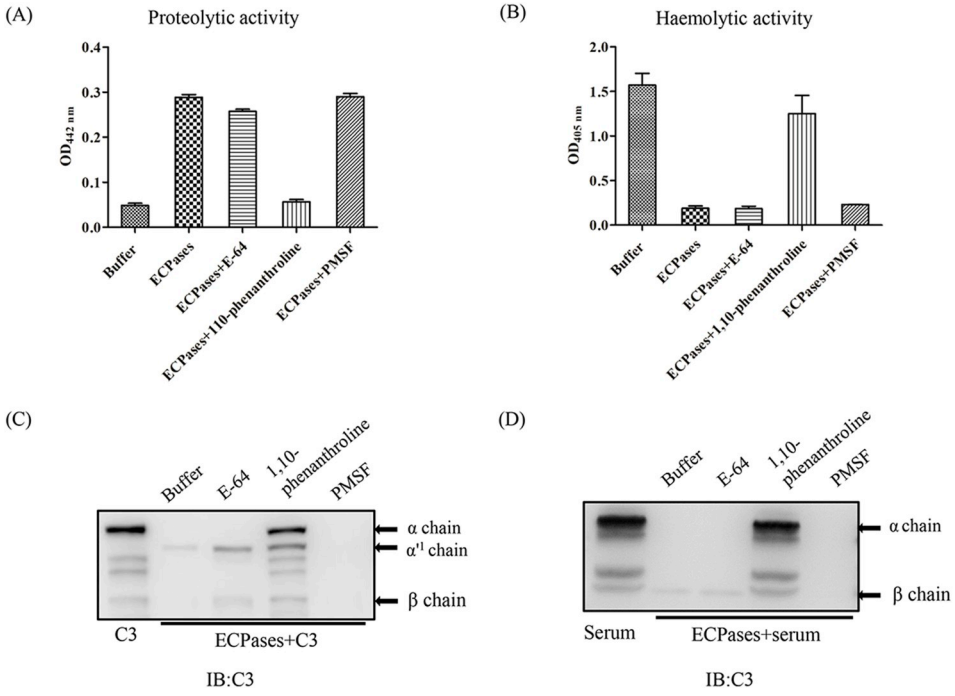

Fig. 5. Proteolytic activity of ECPases is inhibited by 1,10-phenanthroline. Pre-incubation of ECPase with inhibitors of serine, metallo- and cysteine proteases (5 mmol/L phenylmethylsulfonyl fluoride (PMSF), 5 mmol/L 1,10-phenanthroline and 28 μmol/L E−64, respectively) for 30 min. (A) Proteolytic activity was checked by azocasein assay. (B) Haemolytic activity was determined by measuring absorbance at 405 nm. The cleavage of purified C3 (C) and C3 in grass carp serum (D) was analysed by means of Western blot assays with polyclonal antibodies. All experiments were repeated at least three times.

of ECPase can still inhibit the haemolytic activity of the AP and degrade the purified C3 and C3 in the serum (Fig. 5B, C and 5D, respectively). We observed that only 1,10-phenanthroline was able to inhibit the haemolytic activity, which pointed toward the participation of metalloproteases in complement pathway inhibition.

3.6. Identification of metalloprotease of ECPases and characterization of its effect on serum resistance

The crude ECPases were analysed by SDS-PAGE and mass spectrum analysis. The result, in Fig. 6A, showed that a main 35-kDa band was detected in the crude ECPases. Further mass spectrum analysis and blast searches of the GenBank databases exhibited that five proteins (elastase, chitin binding protein, bacterial leucyl aminopeptidase, nitroreductase family protein and an uncharacterised protein (A0KI44)) were especially detected in the crude ECPases (Table 2). Combining with the above results in section 3.5, the result revealed zinc metalloprotease, elastase, was mainly responsible for complement degradation. Sequence analysis showed that elastase (AhE) of *A. hydrophila* GD18 consisted of 589 amino acid residues and had a theoretical molecular mass of 62.6 kDa, which was 97.8% identical to the AhyB of *A. hydrophila* AG2 [31]. However, when the protease of crude ECPases was visually checked, it was about 35 kDa. Then, the 35 kDa band was cut out of the SDS-PAGE gel and analysed the content by mass-spectrometry. Indeed, it was AhE (data not shown). To investigate the role of AhE on serum resistance, we examined the abilities of WT and Δ*ahe* strains survive in grass carp serum. As shown in Fig. 6B, following incubation with the serum, the survival rate of Δ*ahe* (64.8%) was significantly lower than that of the wild type AH GD18 (99.3%).

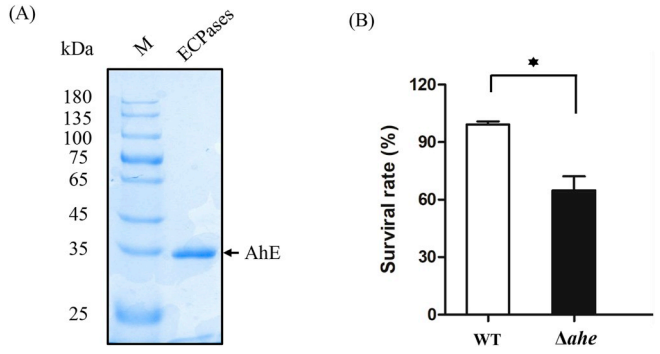

Fig. 6. Effect of *A. hydrophila* GD18 elastase (AhE) on bacterial resistance against serum damage. (A) Identification of *A. hydrophila* GD18 elastase (AhE) by SDS-PAGE analysis. (B) *A. hydrophila* GD18 (WT) and Δ*ahe* were treated with grass carp serum, and the survival rate was determined as above. Data are the means of three independent experiments and presented as means ± SEM. Values with different letters indicate significantly different ($P < 0.05$).

Table 2
Summary of the puried ECPases identified by mass spectrum analysis.

Serial number	NCBI no.	Protein name	Theoretical Mr (kDa)	Coverage (%)
1	A0KGK2	Elastase	62.6	48.1
2	A0KFW7	Chitin binding protein	53.6	4.5
3	A0KMQ2 A0KN73	Bacterial leucyl aminopeptidase	54.8	4.1
4	A0KI44	Nitroreductase family protein	22.6	3.9
5		Uncharacterised protein	70.8	2.1

4. Discussion

The complement system plays an important role in the immune response against pathogens in bony fish [32]. Meanwhile, aquatic pathogens have evolved various tactics to counter the bactericidal activity of the complement system [33,34]. Recently, studies have shown that *Streptococcus agalactiae* [25], *Vibrio* spp [26]. and *Edwardsiella tarda* [27,28], resist the bactericidal activity of the complement system. However, the role of the bony fish complement system in the defence against *A. hydrophila* is poorly understood. In this study, we examined the effect of *A. hydrophila* on serum complement activity and investigated the mechanism associated with serum resistance.

Upon infection, the complement cascade can be activated on the surface of a pathogen through three distinct pathways: AP, CP and LP [15]. The complement system of teleost fish, like that of higher vertebrates, can be activated through all three pathways [35,36]. However, in contrast to mammals, this system has its own unique characteristics in fish. For example, the alternative complement pathway in teleost is several orders of magnitude higher, because some complement components in fish are present in multiple isoforms, allowing for a wider recognition of foreign surfaces compared with that in mammals [37,38], suggesting that the AP plays a vital role in the fish complement system. In this study, we found that *A. hydrophila* was able to survive in grass carp serum. Given the importance of the AP, whether and how *A. hydrophila* resists the grass carp complement system by inhibiting the AP was further studied. The results suggested that *A. hydrophila*-incubated filtered serum possessed low complement activity and that the serum complement system remained largely unactivated or the MAC failed to confer protection. However, *A. hydrophila*-co-incubated filtered serum possessed strong complement activity, indicating that AP activation was not inhibited by *A. hydrophila*. Therefore, we inferred that *A. hydrophila* escaped the bactericidal activity of the serum by inhibiting the AP, which depends on preventing the formation or disabling the function of the MAC.

Microbes have developed several mechanisms to interfere with the complement system to survive in the host, including suppression of complement activation, utilisation of host complement regulators and production of proteases that degrade the complement molecule [16,39,40]. A previous study suggested that *Aeromonas* sp. with S-layers completely covering the LPS molecules was unable to activate the complement system in human serum [6]. However, the exposed molecules LPS were able to activate the complement system but were resistant to complement-mediated killing, because C3b was rapidly degraded and, therefore, the MAC was not formed [7]. In the current study, we showed that *A. hydrophila* used its proteases to dampen the complement response. Specifically, ECPases inactivated the central complement protein C3 in the serum of grass carp, despite containing a variety of ions and inhibitors [30], and blocked the important complement-dependent responses. The molecular mechanism by which ECPases inactivated C3 was surprising. In contrast to the *N. meningitidis* surface-exposed autotransporter (NalP) [23], *Enterococcus faecalis* gelatinase E (GelE) [41] exerted its function by cleaving C3 at one specific site, resulting in the cleavage of the C3 a-chain into C3b-like molecules, which were further degraded by the complement regulatory factors I and H [29]. The ECPases of *A. hydrophila* caused the cleavage of the C3 α-chain into a smaller fragment (α'^{1} chain) of around 100 kDa and then rapidly and fully degraded purified C3 without the need for auxiliary complement regulatory factors. The fact that ECPases inactivated C3 was analogous with the mechanism described for the proteases of other bacteria [29,30]. Nevertheless, no research has been done regarding the cleavage sites of C3 by an analysis of C3 fragmentation. Hence, further studies are needed.

Another interesting finding in this study was that the ECPases involved in complement inactivation were probably metalloproteases, which are considered virulence factors for diverse pathogens [18,42]. Studies have shown that *Vibrio tubiashii* and *Vibrio aestuarianus*, pathogens of the Pacific oyster, secrete zinc metalloproteases that are toxic to the host, and *S. aureus* produces metalloprotease aureolysin that inhibits all pathways of

complement activation, thereby degrading complement components [22,43]. Further mass spectrometric analysis suggested that the elastase of *A. hydrophila* GD18 could be one of the proteases responsible for the effects previously observed with the crude ECPases, which shared high similarity with AhyB of *A. hydrophila* AG2 [31]. AhyB produced from *A. hydrophila* is released extracellularly as a zymogen (pro-AhyB) with a molecular mass of 62.7 kDa, which is further processed into a mature protease and a C-terminal propeptide with 38 kDa by the action of AhpA serine protease or AhpB protease itself [31]. According to this, we suggested that the elastase of *A. hydrophila* GD18 might be cleaved or processed into a mature form by a similar mechanism. Hence, a smaller-molecule-mass elastase was expected (Fig. 6). Several lines of evidence suggest that elastase may be important for the degradation of host tissues, allowing bacteria to obtain nutrients and to invade host tissues [44]. Although several pathogenicity factors of elastase protease have been well characterised, in this work, we demonstrated that AhE cleaved a complement core protein C3 and that this cleavage inhibited the complement system. In line with this observation, Δ*ahe* display dramatically reduced the survival rate in grass carp serum. These results indicated that AhE was essential to serum resistance of *A. hydrophila*.

In conclusion, the current study suggests that *A. hydrophila* resists serum attack by inhibiting the three complement pathways via degradation of complement C3 in grass carp. Further, the secreted metalloprotease elastase (AhE), present in large amounts in crude ECPases, may be the central molecule responsible for C3 cleavage. Thus, our findings reveal the novel immune evasion mechanism of *A. hydrophila* against the complement system of grass carp.

Conflicts of interest statement

The authors have no conflicting commercial or financial interest in publishing this paper.

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Appendix A. Supplementary data

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