Contribution of nuclease to the pathogenesis of *Aeromonas hydrophila*

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*Aeromonas hydrophila* is a gram-negative bacterium that is widely distributed in aquatic environments and can cause septicemia in both fish and humans. However, the underlying mechanisms leading to severe infection are not well understood. In this study, an *A. hydrophila* nuclease (*ahn*) deletion mutant was constructed to investigate its contribution to pathogenesis. This mutant did not differ from the wild-type strain in terms of its growth or hemolytic phenotype. However, the *ahn*-deficient mutant was more susceptible to being killed by fish macrophages and mouse blood in vitro. Furthermore, evidence obtained using both fish and murine infection models strongly indicated that the inactivation of Ahn impaired the ability of *A. hydrophila* to evade innate immune clearance in vivo. More importantly, the virulence of the mutant was attenuated in both fish and mice, with reductions in dissemination capacities and mortality rates. These findings implicate Ahn in *A. hydrophila* virulence, with important functions in evading innate immune defenses.

**Introduction**

*Aeromonas hydrophila* is a gram-negative, facultative anaerobic organism and an autochthonous inhabitant of freshwater environments.1 It causes a variety of diseases in different animal species, from aquatic to terrestrial animals, including humans. In fish, it is responsible for fatal aeromonad septicemia, which causes significant economic losses to aquaculture worldwide each year.2 In humans, the clinical manifestation of *A. hydrophila* infection ranges from gastroenteritis to extraintestinal infections including soft tissue infections, meningitis, and septicemia.3–5

The pathogenesis of *A. hydrophila* is known to involve multiple determinants, including adhesins, enterotoxins, hemolysins, aerolysin, and proteases.6,7 Despite increasing research in recent years, knowledge of the mechanisms employed by *A. hydrophila* in the development of septicemia is still limited. To cause septicemia, pathogenic bacteria have to successfully evade innate immune defenses and maintain a certain level of bacteremia.8 Phagocytes recruited to sites of infection play important roles in the elimination of invading pathogens. In addition to phagocytosis, the release of extracellular traps (ETs) is a recently described mechanism of the innate immune response to infection.9 This mechanism was initially intensely investigated in neutrophils and was found to have an antimicrobial effect. Recent research has found that other immune cell types, such as heterophils, eosinophils, and macrophages/monocytes are capable of releasing ETs.10–12 Regardless of the cell types from which ETs originate, they share several common features, including a DNA backbone with embedded proteases, histones, and antimicrobial peptides.13 In fish, neutrophil ETs were first characterized in fathead minnows.14 During *A. hydrophila* infection, the formation of ETs by the head-kidney increases, demonstrating its contribution to defense against *A. hydrophila* infection.15 Many bacteria produce nuclease20 to break down the DNA backbone of ETs, which allows the bacteria to rapidly escape from the infection site. Experimental evidence suggests that these enzymes increase bacterial invasiveness.16 For instance, group A *Streptococcus* (GAS) secrete DNases to escape being killed, suggesting the existence of a novel immune evasion strategy.17,18 The nuclease activities of 4 virulent *A. hydrophila* isolates obtained from a 2010 disease outbreak in West Alabama have been found to be significantly higher than that of a reference strain.20 However, it remains unknown whether nucleases mediate pathogenesis of *A. hydrophila*.

In this study, the role of a nuclease in the pathogenesis of *A. hydrophila* was explored. We constructed *A. hydrophila* nuclease (*ahn*) mutant and complement strains. The growth rates, hemolytic phenotypes and morphologies of the different strains were compared. Furthermore, we employed both murine and fish infection models to investigate the contribution of Ahn during *A. hydrophila* infection.
Results

Construction and characterization of the A. hydrophila ahn mutant

To investigate the role of Ahn in the pathogenesis of A. hydrophila, an ahn deletion mutant was constructed by allelic replacement in a wild-type (WT) strain (Fig. 1A). The complement strain was constructed by allelic replacement in a Δahn mutant strain of truncated Ahn with full-length Ahn. The resulting knockout mutant Δahn and complement strain CΔahn were verified by PCR and direct sequencing. The phenotypes of the Δahn and CΔahn strains were compared with that of the parental strain under normal culture conditions, revealing that they had similar growth rates (Fig. 1B), hemolytic activities (Fig. 1C), and cell morphologies, which were visualized by scanning electron microscopy (Fig. 1D).

Ahn promotes bacterial survival in fish macrophage and mouse blood killing assays

To investigate the role of ahn in A. hydrophila resistance against innate immune responses, we examined the abilities of the WT, Δahn, and CΔahn strains to survive in a fish macrophage killing assay and in whole mouse blood. After 1 h of incubation with freshly isolated head-kidney macrophages from blunt snout bream (Megalobrama amblycephala), the WT strain was significantly more resistant to macrophage killing than the isogenic Δahn mutant (P < 0.05) (Fig. 2A). After being cultured in whole mouse blood, the number of Δahn mutant was reduced by 68.4% after 1 h, showing a significantly reduced survival rate compared with the WT strain (44.7% reduction) (P < 0.05) (Fig. 2B). The complementary strain behaved similarly to the WT strain in the bactericidal assay (P > 0.05).

ahn knockout reduces A. hydrophila survival in vivo

In A. hydrophila-infected fish, severe ascites was observed (Fig. 3A), and the bacterial load in the ascites was monitored. The results showed that 24 h post-inoculation, the bacterial load in the ascites was significantly lower (P < 0.05) in the Δahn-infected group compared with the WT-infected group (Fig. 3B), suggesting that the mutant could not survive effectively in vivo. To test this idea, we performed competitive growth assays in both fish abdominal cavity and murine air sac infection model. An approximately 1:1 mixture of WT and Δahn bacteria was inoculated i.p. into blunt snout bream or subcutaneously into mice with 0.8 mL air. Twenty-four hours later, ascites or lavage samples were collected after the fish or mice were euthanized. Bacterial cells recovered from the ascites and lavages were analyzed by colony PCR to determine the competitive indices. Both the ascites and air sac lavage samples had competitive indices of approximately 0.7 (Fig. 3C), which confirmed that the ahn mutation impaired the ability of A. hydrophila to evade innate immune clearance in vivo.

Contribution of ahn to dissemination

To compare the dissemination abilities of the WT and Δahn mutant strains, mice and fish infection models were utilized. Equal numbers of WT and Δahn mutant bacteria were inoculated i.p. into blunt snout bream or subcutaneously into mice with 0.8 mL air. Twenty-four hours later, ascites or lavage samples were collected after the fish or mice were euthanized. Bacterial cells recovered from the ascites and lavages were analyzed by colony PCR to determine the competitive indices. Both the ascites and air sac lavage samples had competitive indices of approximately 0.7 (Fig. 3C), which confirmed that the ahn mutation impaired the ability of A. hydrophila to evade innate immune clearance in vivo.

Figure 1. Construction and phenotypic characterization of the Δahn mutant and the complement strain, CΔahn. (A) Strategy for the deletion of ahn by homologous recombination. (B) Growth curves of the WT, Δahn and CΔahn strains. (C) β-Hemolytic phenotype of the strains grown on sheep’s blood agar. (D) Scanning electron microscopic pictures of different strains (scale bar = 1 μm).
significantly higher than those of the Δahn strain (P < 0.05). In addition, pathological examination of the kidneys revealed no obvious changes in the Δahn-infected fish, while renal tubular cell swelling and inflammatory cell infiltration were observed in the WT-infected fish (Fig. 4D). These results provide convincing evidence that Ahn contributes to the systemic dissemination of *A. hydrophila*.

Ahn contributes to *A. hydrophila* virulence in both fish and mice infections

A blunt snout bream infection model was used to determine the LD$_{50}$ values of the parental and mutant strains. The LD$_{50}$ of the WT strain was $1.15 \times 10^8$ CFUs, while that of the Δahn mutant was $6.96 \times 10^8$ CFUs, which was a 6-fold increase compared to the parental strain (Table 2). For survival assay, fish were infected with an equal dose ($8.0 \times 10^8$ CFUs) of the WT or Δahn mutant strain. The mortality rate was 100% for the WT-infected fish, and death occurred largely during the first 2 days post-infection (Fig. 5A). Most dying fish showed typical clinical signs of hemorrhagic septicemia. The mortality rate was 40% for the fish infected with the mutant strain, and no evident external lesions were observed in the surviving fish (Fig. 5A).

To further study the effect of the ahn deletion on the virulence of *A. hydrophila*, the virulence of the WT and Δahn strains were assessed in a BALB/c mouse infection model. Mice were infected with an equal dose ($1.6 \times 10^7$ CFUs) of the WT or Δahn mutant strain. Almost all mice in the WT group presented with severe clinical signs, such as a rough hair coat, prostration depression, and weakness, and 4 out of 10 mice died during the first 2 days post-infection (Fig. 5B). In contrast, all of the mice infected with the Δahn strain survived and did not present any severe clinical signs. Taken together, these results indicate that Ahn significantly contributed to the virulence of *A. hydrophila*.

Discussion

The fish innate immune system consists of a variety of immune defense mechanisms and serves as the first line of defense against pathogens. The ability of *Aeromonas hydrophila* to cause a wide range of diseases, including diarrhea, necrotizing fasciitis, and septicemia in humans and motile aeromonad septicemia in fish, underlines its potential to successfully evade the innate immune system.

The pathogenesis of *A. hydrophila* appears to involve interplay between multiple virulence factors, including the S-layer, O-antigens, capsular polysaccharides, exotoxins, and a repertoire of
exoenzymes. In addition, several new virulence factors have been characterized over the past decade. For example, a functional T3SS and 2 T3SS effectors have been identified in aeromonads and have been found to cause host cell death. Likewise, the T6SS effector hemolysin co-regulated protein reduces *A. hydrophila* uptake by macrophages and interferes with macrophage activation and maturation by inhibiting proinflammatory cytokines and inducing immunosuppressive cytokines. An outer membrane endopeptidase of *A. hydrophila* activates big endothelin-3 and is required to induce pathognomonic skin ulcers. Further, the repeat in toxin A (RtxA) of *A. hydrophila* possesses a functional actin cross-linking domain that induces host cell rounding and apoptosis. In addition, a flagellum is required for *A. hydrophila* survival in the macrophages of its host. However, although numerous studies have identified factors reportedly associated with *A. hydrophila* virulence, further studies of the mechanisms by which it evades the immune system are still necessary.

The discovery of neutrophil extracellular traps (NETs) has forced a reappraisal of the principal means by which neutrophils function in innate immune defense against invading pathogens. In recent years, ET-based bacterial killing has also been demonstrated for mast cells, eosinophils, chicken heterophils, and macrophages/monocytes. Cells from not only humans and mice but also a variety of other animals, including cats, oxen, horses, fish, and even invertebrates, have been demonstrated to be capable of releasing ETs. During inflammation, ETs have evolved to control infections by preventing pathogen dissemination, inactivating virulence factors, and exterminating microbes by antimicrobial activity. ETs are also likely to alert the immune system by activating DNA receptors, such as TLR-9, which aid in immune cell recruitment and the mounting of an acquired immune response or in reducing inflammation.

Successful microbial pathogens have in turn evolved complex and efficient methods to overcome immune mechanisms. Multiple virulence factors that counteract ETs have been identified. These mechanisms include the degradation of ET backbones by nucleases, enabling the liberation of bacteria. In early studies, extracellular nucleases were thought to be involved in nutrient acquisition and the elimination of exogenous DNA. Their contribution to innate immune evasion by pathogenic bacteria was first illustrated in GAS. The GAS DNase Sda1 has been demonstrated to be both necessary and sufficient to promote GAS neutrophil resistance and virulence in a murine model of infection.

| Table 1. Primers used in this study |
|------------------------------------|
| Primer | sequence (5’–3’) | Product size (bp) | Target gene |
|--------|-----------------|-----------------|-------------|
| P1     | CGCTCTAGACGCTTTAGGTCTGGTCTGATA | 540 | Upstream fragment |
| P2     | GAGACTATCTTGGCTGACCGCACGGGGTCGCTTCT |  | |
| P3     | AAGGAACACGTCGCTGGTACGCCAACGATTAGTTC | 531 | Downstream fragment |
| P4     | CCCGTTACCGTTGGCTCCCTTTTTGCTT | 2216 (WT)/527 (Δahn) | Fragment containing ahn |
| P5     | CCAGCGGATCTCAATCTTCC |  | |
| P6     | TCTGAAGCGATGGATGTGC |  | |

*The underlined sequences are restriction sites.*
necrotizing fasciitis. Furthermore, the escape of GAS from NETs as facilitated by Sda1 serves as a selective force for hypervirulent bacterial variants. Moreover, pneumococcus and Staphylococcus aureus encode endonucleases that liberate them from NETs, which allows for the invasion of deeper organs. A. hydrophila, a nuclease has been identified as one of 23 proteins in 9 toxic extracellular protein fractions of highly virulent strains by mass spectrometry. The activities of this nuclease in 4 virulent isolates have been found to be significantly higher than that of an A. hydrophila reference strain. Furthermore, the activity of this nuclease has been detected in supernatant from A. hydrophila. However, whether it mediates pathogenesis in A. hydrophila is still not well known.

In this report, to evaluate the role of a nuclease, Ahn, in A. hydrophila pathogenesis, Δahn mutant and complement strains with no antibiotic resistance markers were constructed through homologous recombination. The growth curves for the WT, Δahn and ΔΔahn strains were similar (Fig. 1B), indicating that this nuclease was not required for nutrient acquisition under culture conditions in vitro. Moreover, the mutant and WT strains did not differ in their morphologies or hemolytic phenotypes (Fig. 1C, D). To investigate the resistance of host killing as mediated by Ahn, bactericidal assay was conducted using both fish macrophages and murine blood. The results showed that the inactivation of Ahn significantly reduced the ability of A. hydrophila to escape killing by macrophages and blood (Fig. 2), indicating an important role of this nuclease in vivo. To verify this speculation, the WT and Δahn strains were inoculated i.p. into blunt snout bream. The bacterial load recovered from the ascites of Δahn-infected fish was significantly lower than that from WT-infected fish (Fig. 3B), showing that the survival of the Δahn mutant was impaired compared with that of the WT strain (Fig. 3C). Pathological examination revealed that the extent of kidney injury in the WT-infected fish was more severe than that in the Δahn-infected fish (Fig. 4D). Finally, the virulence of the Δahn mutant was impaired compared with that of the WT strain (Fig. 5). Therefore, Ahn is critical for the systemic dissemination of A. hydrophila in both fish and murine infection models and is an important virulence factor for A. hydrophila.

In summary, the present study revealed that the nuclease Ahn is required for A. hydrophila resistance against innate immune elimination and that it contributes to the systemic dissemination and virulence of this bacterium. More importantly, this study is the first demonstration, to our knowledge, of the involvement of this nuclease in A. hydrophila pathogenesis in both murine and fish models, indicating that it is involved in a general mechanism of invasion that is used in varying hosts.

### Materials and Methods

#### Declaration of ethical approval

All animal experimental procedures were strictly carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of Hubei Province, China. The animal experiment protocol was approved by the Laboratory Animal Monitoring Committee of Huazhong Agricultural University. All efforts were made to minimize suffering.

#### Bacterial strains and growth

The A. hydrophila strain J-1 was used as the wild type (WT) strain in this study. This strain was isolated from dead cultured cyprinoid fish in eastern China in 1989 and was used as a vaccine strain in China. A. hydrophila was cultured in Luria broth (LB) or on Luria agar (LA) plates (Difco, USA) at 28°C, supplemented with chloramphenicol (50 μg/mL) (Sigma, USA) and 10% sucrose (Sigma, USA) when required. Escherichia coli
\( \chi 7213 \) strain was cultured in LB broth at 37°C, supplemented with diaminopimelic acid (50 \( \mu \)g/mL) (Sigma, USA).

**Construction of the A. hydrophila \( \text{ahn} \) deletion mutant and phenotype characterization**

The primers used in this study are listed in Table 1. The primers were designed according to the gene sequence of \( \text{AHA}_3441 \), referring to the complete genome sequence of \( \text{Aeromonas hydrophila} \) strain ATCC 7966. Upstream and downstream flanking fragments of \( \text{ahn} \) were amplified by PCR using primers P1/P2 and P3/P4 respectively. The fusion of the 2 fragments was amplified by overlap PCR using primers P1/P4. The fused segment (\( \Delta \text{ahn} \)) was sequenced and then ligated into pRE112 at the XbaI/KpnI sites. The resulting plasmid p\( \Delta \text{ahn} \) was transformed into \( E. \text{coli} \chi 7213 \) for mobilization into \( A. \text{hydrophila} \) via conjugation. The transconjugants containing plasmid p\( \Delta \text{ahn} \) integrated into WT strain chromosome by a single crossover event were selected on LA media containing chloramphenicol. Alleric 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, \( \Delta \text{ahn} \) mutant, was selected by chloramphenicol sensitivity and sucrose resistance, and was verified by PCR using 2 pair primers P5/P6 and direct DNA sequencing of the mutation sites using genomic DNA preparations. For gene complementation, fragment was amplified from WT strain with primers P1/P4. The PCR product was cloned into pRE112 at the XbaI/KpnI sites, producing p\( \text{Ca}hn \). The resulting plasmid was transconjugated into \( \Delta \text{ahn} \) mutant to screen the complement strain C\( \Delta \text{ahn} \) as described above.

To determine the growth kinetics of different strains, 1:100 diluted overnight cultures were cultured in LB medium at 28°C with shaking at 200 rpm. Samples were taken hourly, and the optical densities were measured at 600 nm (OD\( 600 \)). \( \beta \)-hemolytic phenotype of different strains were confirmed on sheep’s blood agar (BD, USA). For scanning electron microscopy, log-phase bacteria were collected and fixed with 2.5% glutaraldehyde, washed with water and post-fixed with 0.5% osmium tetroxide, followed by 1% tannic acid. Subsequently, the samples were dehydrated with a graded ethanol series (30%, 50%, 70%, 80%, 90%, 100%), critical point dried with liquid CO\(_2\) and coated with 5-nm platinum. The samples were examined using a scanning electron microscope (Hitachi SU8010, Japan).

**Bactericidal assay**

To investigate role of \( \text{Ahn} \) in the evasion of immune cell-mediated killing in \( A. \text{hydrophila} \), bactericidal assays were conducted to compare the growth of WT and \( \Delta \text{ahn} \) mutant strains co-cultured with head-kidney macrophages and murine blood. Head-kidney macrophages from blunt snout bream (\( \text{Megalobrama amblycephala} \)) were isolated as described previously. \(^{38}\) Briefly, the head-kidney tissue was dissected from anesthetized blunt snout bream and forced through a 100 \( \mu \)m mesh filter (Falcon) to produce a single cell suspension in Leibovitz medium (L-15) (Invitrogen, USA) containing 2% fetal bovine serum (FBS). The head-kidney cell suspension was layered onto a 34%/51% Percoll (Pharmacia, USA) density gradient and centrifuged at 400 \( \times \) g for 30 min in an angle-head rotor. After centrifugation, the macrophage-enriched interface was transferred to a separate tube and washed twice with L-15. A suspension of \( 1 \times 10^7 \) cells/mL in L-15 containing 10% FBS was plated in 24-well tissue culture plates. Macrophages were incubated with log-phase \( A. \text{hydrophila} \) strains to reach an MOI of 10:1. The plates were incubated at 28°C for 1 hour. Then, colonies were counted, and the percentage of surviving bacteria was calculated with the following equation: (CFUs with macrophages/CFUs without macrophages) \( \times \) 100%.

Blood killing assay was performed as previously described. \(^{39}\) Diluted cultures of log-phase \( A. \text{hydrophila} \) strains (50 \( \mu \)L) were combined with 450 \( \mu \)L heparinized mouse blood, and the mixtures were incubated at 37°C for 1 hour. The percentage of live bacteria was subsequently calculated as follows: (CFUs after co-incubation/CFUs in original inoculum) \( \times \) 100%.

**Bacterial infection**

Blunt snout bream (100–130 g) were obtained from the fish base of Huazhong Agricultural University (Wuhan, China). The fish were maintained at 25–26°C in a recirculating freshwater system at the College of Fisheries and acclimatized for 2 weeks before experiments were performed. The fish were fed twice daily with commercial bream feed (Haid Company, China).

To compare the dissemination abilities of \( A. \text{hydrophila} \) strains in fish, blunt snout breams (5 fish/group) were challenged i.p. with a WT or \( \Delta \text{ahn} \) mutant strain (at a dose of \( 2 \times 10^7 \) CFUs/fish). At 24 h following injection, the fish were euthanized, and liver, kidney and ascites samples were collected. The bacterial loads in these samples were analyzed by plating. The collected kidney samples were prepared for histological examination. The \( LD_{50} \) values of the \( A. \text{hydrophila} \) strains in the blunt snout breams were determined. One hundred blunt snout breams were randomly divided into 10 groups. The fish were injected i.p. with 1.0 mL of a 4-fold serially diluted suspension containing \( 1.3 \times 10^7 \) to \( 3.2 \times 10^9 \) CFUs of bacteria in sterile Dulbecco’s phosphate-buffered saline (DPBS). The fish were observed for 14 days to determine the survival rate. Surviving fish were sacrificed on day 14 post-infection. \( LD_{50} \) values were calculated according to Karber’s methods. For virulence comparisons,
another 20 fish were randomly divided into 2 groups and were inoculated i.p. with 8.0 × 10^6 CFUs in 1 mL DPBS of the WT or Δahn mutant strain. The survival rate was monitored for 14 days. Surviving animals were sacrificed on day 14 post-infection.

To assess the dissemination abilities of the A. hydrophila strains in mice, female BALB/c mice (4–6 weeks old, 5 mice/group) (Hubei CDC, Wuhan, China) were challenged i.p. with the WT or Δahn mutant strain (at a dose of 2 × 10^6 CFUs/mouse). At 24 h following injection, the mice were euthanized for the collection of blood samples. The bacterial load in the blood was determined by plating. For virulence comparisons, another 20 BALB/c mice were randomly divided into 2 groups and were inoculated i.p. with 1.6 × 10^7 CFUs in 200 μL DPBS of the WT or Δahn mutant strain. Survival time and clinical signs were monitored for 14 days. Surviving animals were sacrificed on day 14 post-infection.

**Competitive growth assay**

The competitive assay was performed as previously described.3,4,11 Ten blunt snout brems were i.p. injected with 1:1 Δahn mutant – WT mixture (1.0 mL/fish). Fish were euthanized at 24 h after inoculation and the ascites samples were recovered. The ascites samples were plated onto LB agar plates. A mixture of 1:1 Δahn mutant – WT were injected subcutaneously (0.2 mL/mice) into 10 female BALB/c mice (4–6 weeks old) with 0.8 mL air. Mice were euthanized at 24 h after inoculation and the air sac was lavaged with 1 mL DPBS. The lavage samples were plated onto LB agar plates. The percentage of Δahn colonies for above samples was determined by analyzing 50 colonies of each fish sample by PCR using primers P5/P6 (Table 1). The competitive index was determined as the Δahn:WT ratio in recovered sample/the ratio in the inoculum.

**Histopathological studies**

To examine differences in pathological changes, kidney samples were collected from blunt snout brems infected by WT and Δahn strains. The kidney samples were fixed in 10% formalin (buffer PBS; pH 7.2) for 24 h. Following fixation, the samples were dehydrated with ethanol, cleared with xylene, and infiltrated with paraffin. After paraffin embedding, blocks were processed to obtain 4 μm sections, which were stained with a standard hematoxylin and eosin method. Stained samples were examined by light microscopy (Nikon, Japan).

**Statistical analysis**

Statistical analysis was performed by Prism software program (GraphPad Soft-ware, Inc.). Survival data were analyzed with the log-rank (Mantel-Cox) test. Except for survival study, the P values in other experiments were obtained by using the 2-tailed Mann-Whitney t test.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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