Melanization and Pathogenicity in the Insect, *Tenebrio molitor*, and the Crustacean, *Pacifastacus leniusculus*, by *Aeromonas hydrophila* AH-3

Chadanat Noonin1, Pikul Jiravanichpaisal1,2, Irene Söderhäll1, Susana Merino2, Juan M. Tomás3, Kenneth Söderhäll1*

1 Department of Comparative Physiology, Uppsala University, Uppsala, Sweden, 2 National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok, Thailand, 3 Departamento de Microbiología, Facultad de Biología, Universidad de Barcelona, Barcelona, Spain

Abstract

*Aeromonas hydrophila* is the most common *Aeromonas* species causing infections in human and other animals such as amphibians, reptiles, fish and crustaceans. Pathogenesis of *Aeromonas* species have been reported to be associated with virulence factors such as lipopolysaccharides (LPS), bacterial toxins, bacterial secretion systems, flagella, and other surface molecules. Several mutant strains of *A. hydrophila* AH-3 were initially used to study their virulence in two animal species, *Pacifastacus leniusculus* (crayfish) and *Tenebrio molitor* larvae (mealworm). The AH-3 strains used in this study have mutations in genes involving the synthesis of flagella, LPS structures, secretion systems, and some other factors, which have been reported to be involved in *A. hydrophila* pathogenicity. Our study shows that the LPS (O-antigen and external core) is the most determinant *A. hydrophila* AH-3 virulence factor in both animals. Furthermore, we studied the immune responses of these hosts to infection of virulent or non-virulent strains of *A. hydrophila* AH-3. The AH-3 wild type (WT) containing the complete LPS core is highly virulent and this bacterium strongly stimulated the prophenoloxidase activating system resulting in melanization in both crayfish and mealworm. In contrast, the ΔwaaE mutant which has LPS without O-antigen and external core was non-virulent and lost ability to stimulate this system and melanization in these two animals. The high phenoloxidase activity found in WT infected crayfish appears to result from a low expression of pacifastin, a prophenoloxidase activating enzyme inhibitor, and this gene expression was not changed in the ΔwaaE mutant infected animal and consequently phenoloxidase activity was not altered as compared to non-infected animals. Therefore we show that the virulence factors of *A. hydrophila* are the same regardless whether an insect or a crustacean is infected and the O-antigen and external core is essential for activation of the proPO system and as virulence factors for this bacterium.

Introduction

*Aeromonas hydrophila* is a Gram-negative bacterium living in aquatic environments. It can be found in freshwater, seawater, and also chlorinated drinking water. This bacterium has been considered as a food-borne pathogen since it is found in many food products, for example sea food, shrimp cocktail, ground meat and raw vegetables [1–3]. *A. hydrophila* is the most common species of *Aeromonas* that causes infections in human and other animals such as amphibian, reptile, fish and crayfish [4–6]. Infection of this bacterium is also a major problem in carp aquaculture in India [7]. Furthermore, *A. hydrophila* was isolated from several rainbow trout farms and found resistant to antibiotics used in aquaculture in Australia [8], and recently it was also isolated from freshwater crayfish (*Pacifastacus leniusculus*) and was found to be highly virulent to this animal [5].

The pathological conditions found in fish infected with *A. hydrophila* are usually hemorrhagic septicemias (reddish eyes, skin, gills, and fins) and tail and fin rot [9]. Catfish infected with this bacterium exhibited hemorrhagic fins and had larger spleen, kidney and liver [10]. Crayfish infected with *A. hydrophila* showed necrotic injury in gill, heart and hepatopancreas [5]. The pathogenesis of *Aeromonas* species have been reported to be associated with virulence factors such as lipopolysaccharides (LPS), bacterial toxins, bacterial secretory system, flagella and capsules. These factors are believed to be important in both resistance of bacteria to host immune responses and bacterial virulence [3,6,11–15]. Some other factors, such as siderophores (high-affinity iron chelating molecules) and porins (pore-forming proteins), are also reported to be involved in bacterial growth and *A. hydrophila* pathogenesis [16–18].

LPS have been widely studied and reported to contribute an important role in resistance of *Aeromonas* spp. to the host immune system as well as in inducing harmful effects to the host [19–21]. The O-antigen, LPS core (external and internal), and lipid A, are assembled to form a complete LPS structure and are considered to play a role in bacterial pathogenesis [20,22]. Several genes of *A.
**A. hydrophila** involved in LPS biogenesis have been studied. For example, the *waaL* gene encodes a ligase protein required for ligation of the O-antigen to the LPS core, *wzz* gene is responsible for the length of the O-antigen, *waaE* gene plays a role in LPS core synthesis, and *msbB* gene is involved in lipid A biosynthesis. These genes are believed to be associated with virulence of *A. hydrophila* [22–24]. The chemical structure of the most relevant LPS structure from *A. hydrophila* AH-3 strain and mutants is shown in Figure 1.

Previously, crayfish has been used as an experimental model to study the host immune response after *A. hydrophila* challenge and melanization, e.g. phenoloxidase activity was found to be important for crayfish immune defense against *A. hydrophila* infection [5,25]. The details of prophenoloxidase (proPO) activating cascade have been studied in *Manduca sexta* and *Tenebrio molitor*, and the induction of melanization in *Tenebrio* larvae can be easily observed after microbial infection [26,27]. In this study we used *A. hydrophila* AH-3 wild type strain and several mutants on crayfish and *Tenebrio* experimental models to study the *A. hydrophila* most determinant virulence factor of in these animal models. Furthermore, we also studied the immune responses of the host to infection with *A. hydrophila* AH-3 virulent or non-virulent strains in both animal models.

**Results**

1. **Virulence of *A. hydrophila* AH-3 strains**

   To investigate how surface molecules on bacteria or secretion systems influence pathogenicity, the virulence of *A. hydrophila* AH-3 wild type and several mutant strains was studied in two animal species, *P. leniusculus* (Table 1) and *T. molitor* (Table 2). These bacterial strains have been mutated in genes involved in the synthesis of cell surface structures and molecules, or secretion systems. No major differences in growth curves could be observed among the mutants when they grow in LB or in minimal Davies medium with glucose as a single carbon and energy source. In this study, *A. hydrophila* B1 was used as a positive control, since this strain was previously reported as a highly virulent bacterium to freshwater crayfish [5]. Since the bacterial species used in this study had mutations in genes encoding some component of the cell walls, this in some cases had effects on the character of the bacterial mutants, which made it difficult to adjust the bacterial dose for injection to be exactly the same for each mutant species. As a consequence, there are some variations in injected dose or CFU between different bacterial mutant strains as shown in Table 1 and Table 2. The virulence of all bacterial strains can be determined from the injected CFU and the time of death after bacteria injection. Similar results for the different strains were obtained in crayfish and mealworm. *A. hydrophila* B1 used as a positive control was the most virulent strain causing death within 4 h and 21 h in crayfish and mealworm, respectively. The *A. hydrophila* AH-3 WT was less virulent than *A. hydrophila* B1, and this wild-type strain caused death within 25 h in both crayfish and mealworm. *A. hydrophila* AH-3 which lacks a Type VI secretion system (T6SS) and the AH-3 strain which lacks lateral flagella [28] showed similar virulence as the *A. hydrophila* AH-3 WT. In contrast, the mutant strains AH-3 ΔwaaL and Δwzz [24] seemed to be more virulent than the WT in crayfish since all challenged

![Figure 1. LPS structures of *A. hydrophila* AH-3 wild type (a), AH-3 ΔwaaL mutant (b), and AH-3 ΔwaaE mutant (c).](doi:10.1371/journal.pone.0015728.g001)
animals died especially if a lower CFU of these two mutants was used for challenge. However, injection with AH-3 Δuge or Δwzz mutants took approximately 60 h or 45 h to cause crayfish or mealworm death, respectively. This is about two-fold longer time when compared to the WT. Therefore, A. hydrophila AH-3 which lacks a Type III secretion system (T3SS) [29], the strain which lacks polar flagellum [30] and all other mutant strains, excluding the ΔwaaE (O:34 antigen and external core negative strain) [23], were all virulent but to a lesser extent than the A. hydrophila AH-3 WT. The ΔwaaE was not virulent at all and could not kill any animals within the experimental period in this study. Moreover, it is important to notice that AH-3 ΔwaaE [23] mutant showed a decrease in virulence but not to the same extent as that of the AH-3 ΔwaaE mutant. Therefore, the virulent A. hydrophila AH-3 WT and the non-virulent ΔwaaE were selected for further detailed studies. The AH-3 ΔwaaE mutant showed a generation time in rich or minimal medium never superior to 20% of the corresponding wild type strain AH-3.

2. Cytotoxicity of extracellular products of bacteria

To investigate whether the toxins or extracellular products produced from the highly virulent A. hydrophila WT and the non-virulent ΔwaaE play a role in pathogenicity the bacterial extracellular products were prepared from these two strains and were used for cytotoxicity test using the crayfish hematopoietic cells (Figure 2). Extracellular products from both bacterial strains exhibited cytotoxic effects to the cells. Hematopoietic cells were completely lysed within 30 and 60 min after incubation with the extracellular products from the A. hydrophila WT and the ΔwaaE, respectively.

3. Bacterial clearance in mealworms

The results in Figure 3 clearly show that mealworm could not eliminate the virulent AH-3 WT from their hemolymph and the bacteria grew very fast at 12 h after injection, from 0.4–0.6 x 10^8 CFU/animal at the time of injection to an average of 2 x 10^7 CFU/ml hemolymph (Figure 3a). In contrast, the worms completely cleared the non-virulent ΔwaaE mutant within 12 h after injection, and no bacterial colonies were observed after 12 h injection of 1.8–2.9 x 10^6 CFU/animal (Figure 3b).

4. Effect of bacterial challenge on the expression of crayfish antimicrobial peptides

The virulent and non-virulent A. hydrophila strains were tested to see whether they have any effect on the expression of antimicrobial peptide genes in crayfish. The expression levels of crayfish antimicrobial peptide genes were determined 6 h after injection of A. hydrophila WT and the ΔwaaE mutant. The results in Figure 4 show that there was no change in expression level of Picroustin1 in hepatopancreas or hemocytes after injection with WT, but the expression of this gene was increased in both tissues of crayfish injected with the ΔwaaE mutant strain. Picroustin2 expression was not changed after injection with WT or the ΔwaaE mutant. In the case of Picroustin3, expression of this gene in hepatopancreas was decreased after injection with WT, but the expression was not changed after injection with ΔwaaE mutant. The expression level of LGBP did not seem to be changed after either injection of WT or ΔwaaE mutant. The ALF gene expression was obviously decreased at least in hemocytes following AH-3 WT injection, but not changed after ΔwaaE mutant injection while the expression of astacidin2 was not changed after injection with either AH-3 WT or ΔwaaE mutant.

5. Induction of melanization and activation of the proPO system

The results presented in Figure 5 clearly show that melanin was formed in mealworms injected with the virulent AH-3 WT. On the other hand, melanin formation could not be observed in mealworms injected with control buffer or the non-virulent ΔwaaE mutant strain. Activation of the proPO system in crayfish after injection with the AH-3 WT or the ΔwaaE mutant was studied and the results are shown in Figure 6. Activity of PO in a hemocyte lysate prepared from crayfish injected with AH-3 WT was significantly

| Table 1. Virulence of different A. hydrophila AH-3 strains in P. leniusculus (crayfish). |
|-----------------------------------------------|
| Bacterial strain | Injection CFU/crayfish (x10^6) | Mortality (No. dead/No. tested) | Time of dead after injection(h) |
|------------------|---------------------------------|----------------------------------|---------------------------------|
| A. hydrophila B1 | 7.2±0.5                         | 4/4                              | 3.8±0.3                         |
| A. hydrophila AH-3 wild type | 4.2±0.7                         | 6/9                              | 25.5±8.4                        |
| AH-3 TSS5 negative | 3.0±0.2                         | 3/4                              | 46.0±11.8                       |
| AH-3 TSS5 negative | 8.8±0.3                         | 4/4                              | 133±3.6                         |
| AH-3 ΔwaaE O:34 LPS-antigen negative | 2.8±0.2                         | 2/7                              | 90.0±2.0                        |
| AH-3 polar flagellum negative | 2.2±0.3                         | 5/9                              | 57.8±17.9                       |
| AH-3 lateral flagella negative | 6.2±0.6                         | 7/9                              | 25.3±6.4                        |
| AH-3 ΔwaaE O:34 antigen and LPS core negative | 7.9±0.5                         | 0/7                              | -                               |
| AH-3 ΔmsbB lipid alteration | 3.0±0.7                         | 3/4                              | 373±17.0                        |
| AH-3 major porin negative | 3.0±0.4                         | 3/7                              | 97.7±24.3                       |
| AH-3 siderophore negative | 1.4±0.1                         | 3/4                              | 423±16.5                        |
| AH-3 uge lacks capsule | 2.3±0.0                         | 4/4                              | 59.6±16.3                       |
| AH-3 wzz lacks some repetitions of the O:34 antigen LPS | 1.4±0.3                         | 4/4                              | 59.8±11.0                       |

Mutant strains used:

AH-3 lateral flagella negative: AH-3 ΔflrA [28], AH-3 TSS5 negative: A3::flrA [29], AH-3 TSS5 negative: A3 ΔwaaE (this work), AH-3 ΔwaaE, [23], AH-3 polar flagellum negative: A3ΔflrA [30], AH-3 ΔwaaE [23], AH-3 ΔmsbB (this work), AH-3 major porin negative: AH-3Δ330 [17], AH-3 siderophore negative: AH-3: CirA (this work), AH-3 lacking capsule: A3: uge (this work), A3::wzz [24].

doi:10.1371/journal.pone.0015728.t001
Increased compared to the buffer injected control group ($P < 0.05$). However, activity of PO was increased to a low extent in crayfish injected with the $D_{waAE}$ mutant. The proPO and pacifastin, a proPO activating enzyme inhibitor [31], transcripts were examined (Figure 7). The proPO and pacifastin light chain (proteinase subunit) transcripts were decreased in WT infected crayfish but there were no obvious changes in the $D_{waAE}$ mutant infected animals. However, both AH-3 WT and $D_{waAE}$ mutant showed a similar effect on the expression of the pacifastin heavy chain (transferrin subunit) [31] when compared to the CFS control animals.

**Table 2. Virulence of different A. hydrophila AH-3 strains in T. molitor larvae (mealworm).**

| Bacterial strain                       | Injection CFU/mealworm ($\times 10^4$) | Mortality (No. dead/No. tested) | Time of dead after injection(h) |
|----------------------------------------|----------------------------------------|--------------------------------|---------------------------------|
| Ringer's solution                      | -                                      | 7/40                            | 7 days                          |
| A. hydrophila B1                       | 1.3                                    | 10/10                           | 16–21                           |
| A. hydrophila AH-3 wild type           | 1                                      | 20/20                           | 21–25                           |
| AH-3 T3SS negative                     | 1.1                                    | 10/10                           | 41                              |
| AH-3 T6SS negative                     | 1.8                                    | 10/10                           | 21                              |
| AH-3 $\Delta_{waOE}$ O:34 LPS-antigen negative | 0.8                                    | 10/10                           | 4–7 days                        |
| AH-3 polar flagellum negative          | 0.4                                    | 10/10                           | 20–21                           |
| AH-3 lateral flagella negative         | 0.7                                    | 10/10                           | 20                              |
| AH-3 $\Delta_{waOE}$ O:34 antigen and LPS core negative | 1.7                                    | 2/20                            | 2–3 days                        |
| AH-3 $\Delta_{msbB}$ lipid alteration  | 0.4                                    | 10/10                           | 42                              |
| AH-3 major porin negative              | 0.6                                    | 10/10                           | 46                              |
| AH-3 siderophore negative              | 0.7                                    | 10/10                           | 46                              |
| AH-3 uge lacks capsule                 | 1                                      | 10/10                           | 46                              |
| AH-3 $\Delta_{waOE}$ uge lacks some repetitions of the O:34 antigen LPS | 0.7                                    | 10/10                           | 45                              |

Mutant strains used: AH-3 lateral flagella negative: AH-3:lafK [28], AH-3 T3SS negative: A3::axsA [29], AH-3 T6SS negative: AH3 $\Delta_{vasH}$ (this work), AH-3 $\Delta_{waOE}$ [23], AH-3 polar flagellum negative: A3::flrA [30], AH-3 $\Delta_{waOE}$ [23], AH-3 $\Delta_{msbB}$ (this work), AH-3 major porin negative: AH-330 [17], AH-3 siderophore negative: AH-3::CirA (this work), AH-3 lacking capsule: A3::uge (this work), A3::wzz [24].

doi:10.1371/journal.pone.0015728.t002

**Discussion**

*Aeromonas hydrophila* is a pathogenic bacterium for both terrestrial and aquatic animals. Several mutant strains of *A. hydrophila* AH-3 were used to study their virulence in two different animal species, *Pacifastacus leniusculus* (crayfish) and *Tenebrio molitor* larvae (mealworm). The *A. hydrophila* AH-3 strains used in this study have mutations in genes involved in the synthesis of flagella, LPS structures, secretion systems, and some other factors, which have been reported to be involved in *A. hydrophila* pathogenicity [6,11–15].

![Figure 2. Cytotoxicity of extracellular products of A. hydrophila AH-3 wild type and $\Delta_{waAE}$ mutant.](https://doi.org/10.1371/journal.pone.0015728.g002)
flagella, siderophores, porin, and bacterial capsules have been previously reported to have correlations with *A. hydrophila* pathogenesis, the mutation of these factors in this study did not show any major effects on the virulence of *A. hydrophila* AH-3 in *P. leniusculus* or in *T. molitor* larvae.

This study also used *A. hydrophila* AH-3 strains with mutations in several LPS genes and found that the bacterium completely lost its virulence when the *waaE* gene was mutated. The *ΔwaaE* mutant did not cause death in *P. leniusculus* at all and only two *T. molitor* larva died after injection with this mutant strain. The death of *T. molitor* larva, however, could be an effect of injection since some animals also died after being injected with buffer control. The results are correlated with a study performed in mice which showed the LD$_{50}$ for intraperitoneal injection of AH-3*ΔwaaE* mutant was approximately 20-fold higher than that of the AH-3 wild type, while the AH-3*ΔwaaE* mutant was completely avirulent (data not shown). The results, therefore, indicate that the LPS structure is important for *A. hydrophila* AH-3 virulence especially in the invertebrate models used in this study, and the most important part of this structure is the O:34 antigen LPS and the external core (Figure 1 shows the relevant LPS chemical structures).

Some secretion systems are involved in the release of bacterial toxins and effector proteins, some of which have been reported to play a role in bacterial virulence [6,13–15,35,36]. One of the secretion systems which have been widely studied is a type III secretion system (T3SS). This secretion system is present in both clinical and environmental strains of *A. hydrophila* [35]. Mutation of some genes involved in the function of T3SS reduced the virulence of *A. hydrophila* SSU [35]. Mutation of these factors in this study did not alter the virulence of *A. hydrophila* AH-3 in the experimental models used in this study because mutation of these two secretion systems did not alter the virulence of *A. hydrophila* AH-3 infection in *P. leniusculus* or in *T. molitor* larvae as the AH-3 *ΔwaaE* mutant did.

Several toxins like aerolysins, phospholipases, β-hemolysin, HlyA-like hemolysin have been reported to be important for virulence of *A. hydrophila*. Phospholipase and hemolysin genes are present in *A. hydrophila* and their products were reported to have the toxic effects to rainbow trout, crayfish and mice [5,12,38]. The *A. hydrophila* AH-3 used in this study has at least the hemolysin genes. β-hemolysin and HlyA-like hemolysin genes present in both the WT and the *ΔwaaE* mutant. The hemolytic, cytotoxic, and caseinolytic extracellular activities showed similar values for the AH-3 *ΔwaaE* mutant and the wild type strain (data not shown). Although the WT is virulent whereas the *ΔwaaE* mutant is non-virulent in *in vivo* systems, the extracellular products obtained from these two strains showed cytotoxic effects to crayfish hematopoietic cells even though the extracellular products obtained from the *waaE* negative strain showed a slightly lower effect. This indicates that the toxins produced by *A. hydrophila* do not seem to play a major role in *A. hydrophila* virulence *in vivo* in our models. Considering the expression of other virulence factors which might be affected by *waaE* mutation, the AH-3 *ΔwaaE* was able to express either polar or lateral flagellum like the AH-3 WT (data not shown). The WaaL LPS mutation in *A. hydrophila* AH-3 downregulated the expression of T3SS [29]. A similar effect was observed for the AH-3*ΔwaaE* (data not shown). This indicated that

**Figure 3. Bacterial clearance in mealworm.** *A. hydrophila* AH-3 WT (a) or *ΔwaaE* mutant (b) were injected into the worm at amounts of 0.4–0.6×10$^4$ CFU or 1.8–2.9×10$^5$ CFU, respectively. Hemolymph samples were collected at 1 h (n = 3 for each group) and 12 h (n = 7 for each group) after injection for determining CFU of bacteria. This experiment was repeated 2 times. * represents CFU of each individual. – represents mean CFU at each time point. * P<0.05, significant difference when compared to mean CFU at 1 h. Statistical analysis was performed using T-test. doi:10.1371/journal.pone.0015728.g003

Polar flagellum and lateral flagella are factors suspected to be involved in *Aeromonas* spp. virulence. Many studies show that mutation in genes involved in polar flagellum and lateral flagella synthesis lead to reduction in bacterial motility and adhesion of *Aeromonas* spp. to Hep-2 cell [11,28,30]. There is at least 50% of *Aeromonas* spp. which have two flagella systems. These flagella systems have been mentioned to be associated with bacterial pathogenesis by increasing host cell adhesion ability and swarming motility of *A. hydrophila* [32–34]. Moreover, some other structures of *A. hydrophila* such as siderophores, porins, and capsules are also believed to be associated with bacterial pathogenesis. The genes encoding siderophores, high-affinity iron chelating molecules, are present in the *A. hydrophila* ATCC 7966 genome. These molecules have also been reported to affect *Aeromonas* spp. pathogenesis and growth [12,16,18]. A pore-forming protein, porin, on the outer membrane of *A. hydrophila* has also been shown to correlate with bacterial resistance to the host immune system. Lack of this protein in O-antigen depleted *A. hydrophila* strains (serum sensitive) increases bacterial serum resistant ability and this results in induction of bacterial survival in human serum [17]. Although the
LPS in some cases has a role in regulation of T3SS system [29]. However, T3SS mutation alone has no drastic effects on virulence of \( A.\ hydrophila \) AH-3 in our study.

The study on bacterial clearance in \( T.\ molitor \) larvae showed different capacity of the worms in handling infection with virulent or non-virulent bacterial strains. The WT continuously grew inside the worms with a statistically significant increase of CFU at 12 h after injection (\( P<0.05 \)) and finally caused death of the worms whereas the non-virulent \( \Delta waaE \) mutant was completely eliminated by \( T.\ molitor \) larvae within 12 h. The difference between these two bacterial strains is mainly in their LPS structure. The WT has complete LPS, but the \( \Delta waaE \) mutant which is non-pathogenic lacks the O-antigen and the external core (Figure 1). This indicates that both components of the LPS molecule are important for \( A.\ hydrophila \) AH-3 to escape from the host defense system. The WT has complete LPS, but the \( \Delta waaE \) mutant which is non-pathogenic lacks the O-antigen and the external core (Figure 1). This indicates that both components of the LPS molecule are important for \( A.\ hydrophila \) AH-3 to escape from the host defense system. This assumption is supported by some previous studies which showed that serum-resistant strains of \( A.\ hydrophila \) with complete LPS had high percent survival in non-immune human serum and low binding capacity to complement components [17,19].

In addition to having different immune-resistant capacity, WT and \( \Delta waaE \) mutant induced different host immune responses. Expression of antimicrobial peptide (AMP) transcripts was investigated in crayfish after injection with WT and \( \Delta waaE \) mutant, and only minor changes in AMP expression was detected. Expression of \( Pl\ crustin3 \) and ALF (anti-lipopolysacharide factor) decreased after WT infection but no change in other AMP expressions could be observed. Upregulation of \( Pl\ crustin1 \) was observed after \( \Delta waaE \) mutant infection. Expression of this gene have been reported to be upregulated after infection with either pathogenic or non-pathogenic bacteria [39] but an interesting observation in this study is that the upregulation of this gene was observed only following an infection with the non-pathogenic \( \Delta waaE \) mutant.

The AH-3 \( \Delta waaE \) had an ability to induce PO activity in crayfish at a low level but could not activate melanization in \( T.\ molitor \) larvae or it might activate melanization to a very low extent which could not be observed. In contrast, the AH-3 WT significantly induced PO activity (\( P<0.05 \)) and subsequent melanization \( \text{in vivo} \). The AH-3 WT has a complete LPS with O-antigen to protect itself from host defense system [17,19,21] whereas \( \Delta waaE \) mutant lacks its defense structure (LPS devoid of O-antigen and external core) and it was cleared very fast from

![Figure 4. Expression of AMP genes in crayfish hepatopancreas and hemolymph after bacterial challenge.](doi:10.1371/journal.pone.0015728.g004)

Figure 4. Expression of AMP genes in crayfish hepatopancreas and hemolymph after bacterial challenge. Hepatopancreas and hemolymph were taken out from the crayfish at 6 h after bacterial injection. Five hundred and 100 ng of total RNA from hepatopancreas and hemocytes, respectively, were used for RT-PCR. The PCR product of each gene was detected at the following cycles: 25 cycles for 40S; 30 and 25 cycles for \( Pl\ crustin1 \) in hepatopancreas and hemocyte, respectively; 28 and 22 cycles for \( Pl\ crustin2 \) in hepatopancreas and hemocyte, respectively; 30 cycles for \( Pl\ crustin3 \), LGBP, ALF and astacin2. Each lane shows gene expression in each crayfish.

doi:10.1371/journal.pone.0015728.g004

![Figure 5. Melanization in mealworm.](doi:10.1371/journal.pone.0015728.g005)

Figure 5. Melanization in mealworm. Mealworms were injected with \( A.\ hydrophila \) AH-3 WT (1×10^4 CFU) or \( \Delta waaE \) mutant (1.7×10^4 CFU) (10 animals for each group), and melanin formation was observed for 7 days or until the animal died.

doi:10.1371/journal.pone.0015728.g005
The expression of proPO was slightly down-regulated in WT infected crayfish, although these animals had a high PO activity, and as expected there was no obvious change in expression of this gene or the PO activity in ΔwaaE mutant infected crayfish. This higher activity of PO seems to be a result of that the pacifastin transcripts were decreased in the WT infected animals. Pacifastin is an efficient inhibitor of ppA (prophenoloxidase-activating enzyme) and this inhibitor prevents activation of the proPO system. Then if there is no pacifastin, activation of proPO can occur. In a study by Liu et al., [25], knockdown of pacifastin resulted in high PO activity and low bacterial number in crayfish hemolymph at 3 h after bacterial infection. Low expression of pacifastin in WT infected crayfish and high induction of PO activity in this study correlates well with this previous study. The reason for the slight down-regulation of proPO might occur because the proPO system can be activated continuously in the absence of pacifastin and the production of toxic products by PO may have reached levels in which those products are toxic to the animals and as a consequence the proPO transcripts were down-regulated.

Pacifastin of crayfish consist of two subunits containing a proteinase inhibitor light chain and a transferrin heavy chain. These two subunits are encoded by different mRNA transcripts [31], and as described above the expression level of the light chain transcript was decreased in WT infected crayfish but not in ΔwaaE mutant infected crayfish. In contrast, the heavy chain subunit was down regulated in crayfish infected with either WT or ΔwaaE mutant. The crayfish pacifastin heavy chain consists of three transferrin lobes of which two can bind iron [31]. Iron is essential for bacterial metabolism and growth and A. hydrophila has iron chelating molecules called siderophores for iron acquisition from host iron-binding molecules such as transferrin or lactoferrin [18]. A recent study in fish showed a reduction in serum iron and saturated transferrin following a bacterial infection [40]. Therefore, the down regulation of transferrin subunits of pacifastin might be explained by the low iron level caused by WT or ΔwaaE mutant AH-3 infection.

In conclusion, mutation in several known virulent factors in an A. hydrophila strain in this study provides us a clear picture that O-antigen and external core of the LPS molecule are the A. hydrophila AH-3 most important factor for virulence in our animal models. A. hydrophila AH-3 WT, which contains a complete LPS core, strongly stimulated the proPO-activating system and melanization in P. leniusculus and T. molitor larvae, respectively. LPS, therefore,

**Figure 6. Activation of the proPO system in crayfish after bacterial challenge.** Crayfish was injected with A. hydrophila AH-3 WT (5.8×10^6 CFU) or ΔwaaE mutant (10.1×10^6 CFU) or CFS (control) (5 crayfish were used for each group). Six hours after injection, hemolymph was collected and used for HLS preparation and PO activity assay. The experiments were performed 2 times with a number of 10 crayfish for each experimental group. The results shown are mean of the 2 separate experiments. The bars represent SE of the data. * P<0.05, significant difference when compared to CFS. Statistical analysis was performed using one-way ANOVA followed by Turkey’s multiple comparison test. doi:10.1371/journal.pone.0015728.g006

**Figure 7. Expression of proPO and pacifastin transcripts.** Hemolymph was taken out from the crayfish at 6 h after CFS or bacterial injection. One hundred of total RNA from hemocytes was used for RT-PCR. The PCR product of each gene was detected at the following cycles: 25 cycles for 40S and proPO, 30 cycles for pacifastin light chain, and 38 cycles for pacifastin heavy chain. Each lane shows gene expression in each crayfish. doi:10.1371/journal.pone.0015728.g007
triggers host immune responses and protects bacteria against host defense process which then results in continuous growth of bacteria in the host circulating system and finally leads to death of the host.

**Materials and Methods**

1. Animals

*Procambarus clarkia* (crayfish) was maintained in aquarium at 10°C with aerated running fresh water. Intermolt crayfish with weights ranging from 30–40 g were selected for the experiments. *Tenebrio multicorn* larvae (mealworm) were maintained at room temperature (20–22°C) in beakers placed on the laboratory bench and they were fed with wheat bran. Mealworms with sizes from 2.5–3.5 cm were used for the experiments.

2. *A. hydrophila* AH-3 mutant construction

An inner DNA fragment of *vasH*, *mshB*, *CirA*, and *uge* was independently obtained from AH-3 chromosomal DNA using appropriate primers and subcloned in the *pir* replication dependent plasmid pSF100 [41] through initial cloning in pGEM-T plasmid. These plasmid constructs (pSF-VasH, pSF-MshB, pSF-CirA, pSF-Uge, respectively) were used to obtain *vasH*, *mshB*, *CirA*, *uge* deficient mutants from *A. hydrophila* strain AH-3 by a single recombination event leading to the generation of two incomplete copies of wild type genes in the chromosome of these mutants, as previously described [41]. Plasmids pSF-VasH, pSF-MshB, pSF-CirA, and pSF-Uge, were independently isolated, transformed into *E. coli* SM10 (pir) [41], and transferred by conjugation from *E. coli* SM10 to the AH-3 rifampin-resistant (Rifr) mutant as previously described [17]. KmR Rifr transconjugants arising from the different conjugations using pSF-VasH, pSF-MshB, pSF-CirA, and pSF-Uge, should contain the mobilized plasmid integrated onto the chromosome by homologous recombination between the wild type gene screened and the plasmid, leading to two incomplete copies of the wild type gene studied (defined insertion mutant). Chromosomal DNA from 10 transconjugants obtained were independently obtained from AH-3 chromosomal DNA using appropriate primers and subcloned in the appropriate *E. coli* plasmid (pSF100). Then, 10 transconjugants obtained were independently analyzed by Southern blot hybridization with appropriate DNA probes to obtain the following defined insertion mutants: AH3 *vasH* (T6SS negative), AH3 *mshB* (lipid alteration deficient), AH3 *CirA* (siderophore negative), and AH3 *uge* (lacking capsule), as previously described [17].

3. Virulence study

This study was performed in both crayfish and mealworm. All mutant and wild type strains of *A. hydrophila* AH-3 were grown at 28°C in tryptic soy broth (Dhuka, 20 μg/ml of kanamycin was required for growing all mutant strains except for the T3SS negative one) until they reached a bacteria suspension with an optical density of 0.5–0.6 at 600 nm. The bacterial pellets were then collected and washed 3 times with 0.85% NaCl, and resuspended and diluted in buffer CFS (0.2 M NaCl, 5.4 mM KCl, 10 mM CaCl2, 2 mM MgCl2, 10 mM NaH2PO4, pH 6.8) or in insect Ringer solution (128 mM NaCl, 18 mM CaCl2, 1.3 mM KCl, 2.3 mM NaHCO3, pH 7) for injection in crayfish or mealworm, respectively. Cell concentration for injection was determined and adjusted by performing viable plate counts and is reported as mean colony-forming unit (CFU) ± SE.

The bacterial suspension (100 μl) was injected into the base of a crayfish walking leg, and 10 μl of the bacterial suspension or insect Ringer solution was injected using Ultra-Fine needle, 31G×8 mm, (BD Micro-Fine) into the abdominal part of *Tenebrio* larvae at the position between the third and the second segments. After injection, animals were normally maintained and fed, and were observed for 7 days. The time that animals died after the injection was recorded and reported as mean ± SE. The mortality of the animal is given as number of animals dead/total number of animals tested (No. dead/No. tested).

4. Cytotoxicity of extracellular products of *A. hydrophila* AH-3 WT and Δ*vasHE*

Two bacterial strains, *A. hydrophila* AH-3 WT (WT) and *A. hydrophila* AH-3 Δ*vasHE* mutant (Δ*vasHE* mutant) were chosen for further study because the WT is highly virulent whereas the Δ*vasHE* mutant completely lost its virulence. Extracellular products of the bacteria were prepared fresh as described by Jiravanichpaisal et al. [5]. Briefly, bacteria, WT and Δ*vasHE* mutant, were cultured overnight in 10 ml TSB, and then the culture medium was centrifuged at 8000 g for 10 min at 4°C. The supernatant was collected and sterile filtered through 0.22-μm-pore membranes (Millipore). Then, 10 μl of sterile extracellular products of bacteria or 10 μl of TSB was incubated at room temperature (20–22°C) with freshly isolated crayfish hemopoietic (hpt) cells, which had been prepared as previously described by Soderhall et al. [42]. Briefly, the hpt was dissected out from crayfish and digested with 0.1% of collagenase type I and type IV at 37°C in RPMI (10 mM Na2HPO4, 10 mM KH2PO4, 0.15 M NaCl, 10 μM CaCl2, 10 μM MgCl2, 2.7 μM KCl, pH 6.8). The cells were finally resuspended in modified L-15 culture medium [43] and seeded into 96-well plate with the amount of 3×104 cells/well. During the experiments, the morphology of hpt cells was observed every 15 min for 2 h. This experiment was repeated 3 times.

5. Bacterial clearance

This experiment was performed in mealworm, *A. hydrophila*, WT (0.4–0.6×10⁵ CFU/animal) or Δ*vasHE* mutant (1.8–2.9×10⁴ CFU/animal) were injected into mealworm (n = 3–7) as described above. At 1 h and 12 h after injection, the mealworms were bled, and 10 μl of hemolymph was collected from each individual worm to perform viable plate counts to determine CFU of bacteria in each mealworm. This experiment was repeated 3 times.

6. Expression of crayfish antimicrobial peptides (AMPs) and some other immune-related genes after bacterial challenge

Crayfish (n = 3 for each experimental group) was injected with bacteria WT (3.3×10⁴ CFU/animal) or Δ*vasHE* mutant (1.8×10⁵ CFU/animal), and 6 h after injection hepatopancreas and hemolymph were collected and were kept separately. Total RNA was isolated from hemocytes and hepatopancreas using Trizol reagent (Gibco BRL) following the manufacturer’s instruction. Each RNA sample was then treated with 2 units of RNase-free DNase I (Ambion) at room temperature (20–22°C) in beakers placed on the laboratory bench and then collected and washed 3 times with 0.85% NaCl, and resuspended and diluted in buffer CFS (0.2 M NaCl, 5.4 mM KCl, 10 mM CaCl2, 2 mM MgCl2, 10 mM NaH2PO4, pH 6.8) or in insect Ringer solution (128 mM NaCl, 18 mM CaCl2, 1.3 mM KCl, 2.3 mM NaHCO3, pH 7) for injection in crayfish or mealworm, respectively. Cell concentration for injection was determined and adjusted by performing viable plate counts and is reported as mean colony-forming unit (CFU) ± SE.

The bacterial suspension (100 μl) was injected into the base of a crayfish walking leg, and 10 μl of the bacterial suspension or insect Ringer solution was injected using Ultra-Fine needle, 31G×8 mm, (BD Micro-Fine) into the abdominal part of *Tenebrio* larvae at the position between the third and the second segments. After injection, animals were normally maintained and fed, and were observed for 7 days. The time that animals died after the injection was recorded and reported as mean ± SE. The mortality of the animal is given as number of animals dead/total number of animals tested (No. dead/No. tested).

First strand cDNA synthesis was performed using Oligo (dT)₁₂₀ primer and all other reagents were from ThermoScript-PCR kit (Invitrogen) according to manufacturer’s instruction. One microgram of RNA obtained from hepatopancreas or hemolymph was used as starting material. cDNAs were then subjected to PCR using the primers shown in Table 3. The 40S ribosomal protein gene was used as an internal loading control for RT-PCR analysis. PCR was performed using the following condition: 95°C for 2 min, 22–38 cycles (see figure 4 and 7 legends for cycles of each gene) of 95°C for 20 s, 58°C for 20 s,
72°C for 30 s, and followed by a cycle of 72°C final extension for 3 min. The PCR products were then subjected to 1.5% agarose gel electrophoresis, followed by ethidium bromide staining and visualized under ultraviolet light.

7. Induction of melanization in mealworms, T. molitor
To study the effect of a bacterial infection on melanization, the experiments were performed in mealworm because melanin formation could be seen easily in this animal. A. hydrophila WT (1×10⁴ CFU/animal) or ΔwaaE mutant (1.7×10⁴ CFU/animal) were injected into mealworms (n = 10 each), and melanin formation was observed for 7 days or until the animals died.

8. Induction of prophenoloxidase activating system
A study of the effect on the prophenoloxidase (proPO) activating system after bacterial challenge was performed in crayfish because it was more convenient to get enough hemolymph from crayfish for the study when compared to that from mealworm. Activation of the proPO system in crayfish after bacterial challenge was done by preparing hemocyte lysate (HLS) and then determined LPS-activated PO activity in the HLS samples. A. hydrophila WT (5.8×10⁸ CFU/animal) or ΔwaaE mutant (10.1×10⁸ CFU/animal) or CFS was injected into 5 crayfishes each. Six hours after injection, crayfishes were bled and 10 drops of hemolymph from each individual were collected and pooled together in one experimental group and this sample was then centrifuged at 800×g for 20 min at 4°C. The obtained supernatant was HLS and was used for PO activity assay. The assay was performed by incubating 50 μl of HLS, 50 μl of 1 mg/ml LPS (E. coli 005:B5; Sigma), and 50 μl of 3 mg/ml L-3,4-dihydroxyphenylalanine (L-DOPA) (Sigma) at room temperature (20–22°C) for 10 min. As a control reaction, sterile distilled water was used instead of LPS. PO activity was determined by measuring the absorbance of dopachrome at 490 nm. The protein content in HLS was determined and PO activity was reported as ΔA490/mg protein/10 min. This experiment was repeated 2 times with a total amount of 10 crayfishes for each experimental group.

9. Statistical analysis
All experiments were repeated 2–3 times with 3–10 animals (for each experimental group) used as indicated in each experiment. All animals used in this study were independent from each other, and each animal was used for the measurement or for our study only one time. The T-test was performed when two experimental groups were compared, and One-way ANOVA followed by Turkey’s multiple comparison was used for multiple comparisons. The P-value <0.05 was considered as a statistically significant difference.

**Author Contributions**
Conceived and designed the experiments: CN PJ IS JMT KS. Performed the experiments: CN PJ SM. Analyzed the data: CN PJ IS SM JMT KS. Contributed reagents/materials/analysis tools: SM JT. Wrote the paper: CN PJ IS JMT KS.

**Table 3. Primers used for RT-PCR.**

| Gene                  | Primer (5’-3’)                  | Product size (bp) | Accession No. | References       |
|-----------------------|---------------------------------|-------------------|---------------|-----------------|
| Picrustin1            | GGTACCACTGGTACCTGATC (F)        | 368               | EF523612      | [39]            |
|                       | TGTAAATGGTAGGCCCATTC (R)        |                   |               |                 |
| Picrustin2            | CTGAGAAGCGGGCTGACAG (F)         | 358               | EF523613      | [39]            |
|                       | GCAAAACGCAAGCTGAGG (R)          |                   |               |                 |
| Picrustin3            | AGGGAGGGTACGTGGGTGTA (F)        | 417               | EF523614      | [39]            |
|                       | GCGAGGGTCCGCTTGTTT (R)          |                   |               |                 |
| Astacin2              | CCTTACAACACCCACACTGCC (F)       | 140               | DQ822206      | [39]            |
|                       | CTGCCGAGCTGGTGTTGAGT (R)        |                   |               |                 |
| ALF                   | TCGGAAATCCTGCAACC (F)           | 451               | EF523760      | [44]            |
|                       | TGGAAAGATCCTGGAACACTAGGA (R)    |                   |               |                 |
| LGBP                  | TCATGAGGCGCAAGCTCACC (F)        | 529               | AJ250128      | [45]            |
|                       | AAGTACCATTTGTTGCACCGCC (R)      |                   |               |                 |
| 40S                   | CCGACCCCTTTCTTTCTT (F)          | 360               | CF542417      | Unpublished data|
|                       | GAACATGCTGGCCACCGG (R)          |                   |               |                 |
| proPO                 | TGGCAGCCTGACCTGGTTTAC (F)       | 406               | X83494        | [46]            |
|                       | TCCCTGGCTTCTGTGCATTGAC (R)      |                   |               |                 |
| Pacifastin light chain| TGACAAAGGCTGGCTTGTGG (F)        | 536               | U81825        | [31]            |
|                       | TGGAGCCCATGACGAACCT (R)         |                   |               |                 |
| Pacifastin heavy chain| TGAGGTGGCCGTTTCTGGC (F)         | 540               | U81824        | [31]            |
|                       | GACCTTGCCGACACCTGACT (R)        |                   |               |                 |

**References**
1. Daskalov H (2006) The importance of Aeromonas hydrophila in food safety. Food Control 17: 474–483.
2. Edberg S, Browne F, Allen M (2007) Issues for microbial regulation: Aeromonas as a model. Crit Rev Microbiol 33: 89–100.
3. Janda JM, Abbott SL (2010) The genus Aeromonas: taxonomy, pathogenicity and infection. Clin Microbiol Rev 23: 35–73.
4. Erova T, Sha J, Horneman A, Borchardt M, Khajanchi B, et al. (2007) Identification of a new hemolysin from diarrheal isolate SSU of Aeromonas hydrophila. FEMS Microbiol Lett 275: 301–311.
5. Jiravanichpaisal P, Roos S, Edman L, Liu H, Stoderhall K (2009) A highly virulent pathogen, Aeromonas hydrophila, from the freshwater crayfish Pacifastacus leniusculus. J Invertebr Pathol 101: 56–66.
6. Suarez G, Sierra J, Wang S, Erova T, et al. (2008) Molecular characterization of a functional type VI secretion system from a clinical isolate of *Aeromonas hydrophila*. Microb Pathog 44: 344–361.

7. Sahoo P, Das Mahapatra K, Saha J, Barat A, Sahoo M, et al. (2008) Family associations between immune parameters and resistance to *Aeromonas hydrophila* infection in the Indian major carp, *Labeo rohita*. Fish Shellfish Immunol 25: 163–169.

8. Akinbowale A, Peng H, Grant P, Barton M (2007) Antibiotic and heavy metal resistance of *Aeromonas* in montiporid species. *Facts and facts* 137: 237–243 from rainbow trout (*Oncorhynchus mykiss*) farms in Australia. *Int Antimicrob Agents* 30: 177–182.

9. Austin B, Austin DA (2007) Bacterial Fish Pathogens: Diseases of Farmed and Wild Fish, 4th ed. Chichester, UK: Sprinker-Praxis books in aquatic and marine science, Praxis Publishing Ltd.

10. Alagappan KM, Deivasigamani B, Kumaran S, Sakthivel M (2009) Histopathological alternations in estuarine catfish (*Arius mulatus*; Thunberg, 1792) due to *Aeromonas hydrophila* infection. *World Journal of Fish and Marine Sciences*: 1: 1245–1249.

11. Rabaan A, Gryllos I, Tomas J, Shaw J (2001) Motility and the polar flagellum of *Aeromonas caviae* adherence to HEp-2 cells. *Infect Immun* 69: 4257–4267.

12. Seshadri R, Joseph S, Chopra A, Sha J, Shaw J, et al. (2006) Genome sequence of *Aeromonas hydrophila* ATCC 7960*: July of all the trades. *J Bacteriol* 188: 8272–8282.

13. Sha J, Pillai L, Fadil A, Galindo C, Erova T, et al. (2005) The type III secretion system and cytotoxic enterotoxin alter the virulence of *Aeromonas hydrophila*. *Infect Immun* 73: 6346–6357.

14. Sha J, Wang S, Suarez G, Sierra J, Fadil A, et al. (2007) Further characterization of a type III secretion system from *Aeromonas hydrophila* - Part II. *Microb Pathog* 43: 147–160.

15. Sierra J, Suarez G, Sha J, Folt S, Popov V, et al. (2007) Biological characterization of a new type III secretion system effector from a clinical isolate of *Aeromonas hydrophila*. *J Biol Inorg Chem* 5: 57–66.

16. Massad G, Areceaneus J, Byers B (1994) Acquisition of iron from host sources by mesophilic *Aeromonas* species. *Facts and facts* 137: 237–243 from rainbow trout (*Oncorhynchus mykiss*) farms in Australia. *Int Antimicrob Agents* 30: 177–182.

17. Nogueras M, Merino S, Aguilar A, Benedi V, Tomas J (2000) Cloning, molecular analysis of three *Aeromonas salmonicida* serum resistance of virulent *Aeromonas hydrophila*. *FEMS Microbiol Lett* 190: 3176–3184.

18. Stintzi A, Raymond K, Lee H, Vilches S, Merino S, et al. (2004) A type III secretion system is required for *Aeromonas hydrophila* AH-1 pathogenesis. *Infect Immun* 72: 1248–1256.

19. Silver AC, Kikuchi Y, Fadil AA, Sha J, Chopra AK, et al. (2007) Interactions between innate immune cells and a bacterial type III secretion system in mutualistic and pathogenic associations. *Proc Natl Acad Sci USA* 104: 9481–9486.

20. Merino S, Aguilar A, Nogueras M, Regue M, Swift S, et al. (1999) Cloning, sequencing, and role in virulence of two phospholipases (A1 and C) from mesophilic *Aeromonas sp* serogroup O:34. *Infect Immun* 67: 4000–4013.

21. Jiravanichpaisal P, Kim YA, Andrews T, Soderhall K (2007) Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Procambarus clarkii*. *Characterization and expression pattern*. *Dev Comp Immunol* 31: 321–330.

22. Sahoo P, Das Mahapatra K, Saha J, Barat A, Sahoo M, et al. (2008) Family associations between immune parameters and resistance to *Aeromonas hydrophila* infection in the Indian major carp, *Labeo rohita*. *Fish Shellfish Immunol* 25: 163–169.

23. Jime†nez N, Canals R, Lacasta A, Kondakova A, Lindner B, et al. (2008) Molecular characterization of a functional type VI secretion system from *Aeromonas hydrophila*. *J Biol Chem* 283: 23355–23363.

24. Kan H, Kim CH, Kwon HM, Park JW, Roh KB, et al. (2008) Molecular control of phospholipase A2-induced melanin synthesis in an insect. *J Biol Chem* 283: 23355–23363.

25. Liu H, Jiravanichpaisal P, Lee SY, Soderhall K (2005) An ancient 155-kDa heterodimeric proteinase inhibitor containing a unique glucan-binding protein as a negative regulatory component of phenoloxidase-induced melanin synthesis. *J Biol Chem* 280: 24744–24751.

26. Canals R, Altarriba M, Vilches S, Hornburgh G, Shavg J, et al. (2006) Analysis of the lateral flagellar gene system of *Aeromonas hydrophila* AH-3. *J Bacteriol* 188: 852–862.

27. Zhao M, Soderhall I, Park JW, Ma YG, Osaki T, et al. (2003) A novel 43-kDa protein as a negative regulatory component of phospholipaseA2-induced melanin synthesis. *J Biol Chem* 278: 24744–24751.

28. Canals R, Altarriba M, Vilches S, Hornburgh G, Shavg J, et al. (2006) Polar flagellum biogenesis in *Aeromonas hydrophila*. *J Bacteriol* 188: 542–553.

29. Liang Z, Soutrap-Jensen L, Aspin A, Hall M, Soderhall K (1997) Pacifastin, a novel 155-kDa heterodimeric proteinase inhibitor containing a unique transferrin chain. *Proc Natl Acad Sci USA* 94: 6682–6687.

30. Kirov S, Tassell B, Semmler A, Donovan L, Rabaan A, et al. (2002) Lateral flagella and swimming motility in *Aeromonas species*. *J Bacteriol* 184: 547–553.

31. Kirov S (2003) Bacteria that express lateral flagella enable dissection of the multifunctional roles of flagella in pathogenesis. *FEBS Microbiol Lett* 224: 151–159.

32. van der Marel M, Schroers V, Neuhau J, Steinhaugen D (2008) Chemotaxis toward, adhesion to, and growth in gut mucus of two *Aeromonas hydrophila* strains with different pathogenicity for common carp, *Cyprinus carpio*. *In Fish Dis* 31: 321–330.

33. van der Marel M, Schroers V, Neuhau J, Steinhaugen D (2008) Chemotaxis toward, adhesion to, and growth in gut mucus of two *Aeromonas hydrophila* strains with different pathogenicity for common carp, *Cyprinus carpio*. *In Fish Dis* 31: 321–330.

34. van der Marel M, Schroers V, Neuhau J, Steinhaugen D (2008) Chemotaxis toward, adhesion to, and growth in gut mucus of two *Aeromonas hydrophila* strains with different pathogenicity for common carp, *Cyprinus carpio*. *In Fish Dis* 31: 321–330.

35. van der Marel M, Schroers V, Neuhau J, Steinhaugen D (2008) Chemotaxis toward, adhesion to, and growth in gut mucus of two *Aeromonas hydrophila* strains with different pathogenicity for common carp, *Cyprinus carpio*. *In Fish Dis* 31: 321–330.