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ORIGINAL ARTICLE

Attenuation of a virulent *Aeromonas hydrophila* with novobiocin and pathogenic characterization of the novobiocin-resistant strain

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Abstract

Aim: To determine whether novobiocin resistance strategy could be used to attenuate a virulent *Aeromonas hydrophila* AH11P strain and to characterize the growth and pathogenic differences between the novobiocin-resistant strain and its virulent parent strain AH11P.
Methods and Results: A novobiocin-resistant strain AH11NOVO was obtained from a virulent *Aer. hydrophila* strain AH11P through selection of resistance to novobiocin. AH11NOVO was found to be avirulent to channel catfish (*Ictalurus punctatus*), whereas AH11P was virulent. When AH11NOVO vaccinated channel catfish were challenged with AH11P at 14 days postvaccination, relative per cent of survival of vaccinated fish was 100%. The cell proliferation rate of AH11NOVO was found to be significantly ($P < 0{\cdot}05$) less than that of AH11P. *In vitro* motility assay revealed that AH11NOVO was nonmotile, whereas AH11P was motile. AH11NOVO had significantly ($P < 0{\cdot}05$) lower *in vitro* chemotactic response to catfish mucus than that of AH11P. Although the ability of AH11NOVO to attach catfish gill cells was similar to that of AH11P, the ability of AH11NOVO to invade catfish gill cells was significantly ($P < 0{\cdot}05$) lower than that of AH11P.
Conclusions: The novobiocin-resistant AH11NOVO is attenuated and different from its parent AH11P in pathogenicity.
Significance and Impact of the Study: The significantly lower chemotactic response and invasion ability of AH11NOVO compared with that of its virulent parent strain AH11P might shed light on the pathogenesis of *Aer. hydrophila*.

Introduction

Aeromonas hydrophila, a Gram-negative, motile, rod-shaped bacterium commonly found in aquatic environments throughout the world, is the causative agent of motile aeromonad septicaemia (MAS; Harikrishnan *et al.* 2003), which is also known as epizootic ulcerative syndrome (Mastan and Qureshi 2001). The symptoms of MAS include swelling of tissues, dropsy, red sores, necrosis, ulceration and haemorrhagic septicaemia (Karunasagar *et al.* 1989). Fish species affected by MAS include tilapia (Abd-El-Rhman 2009; Tellez-Bañuelos *et al.* 2010), catfish (Majumdar *et al.* 2007; Ullal *et al.* 2008), goldfish (Irianto *et al.* 2003; Harikrishnan *et al.* 2009), common carp (Jeney *et al.* 2009; Yin *et al.* 2009) and eel (Esteve *et al.* 1994). Although usually considered as a secondary pathogen associated with disease outbreaks, *Aer. hydrophila* could also emerge as a primary pathogen (Pridgeon and Klesius 2011a), causing outbreaks in fish farms with high mortality rates and severe economic losses to the aquaculture industry worldwide.

To control MAS, feeding infected fish with antibiotic-medicated feed is a general practice (DePaola *et al.* 1995). However, this practice is expensive and usually ineffective as sick fish tend to remain off feed. In addition, MAS caused by *Aer. hydrophila* can be very acute, causing

mortality within 24 h (Pridgeon and Klesius 2011a). Furthermore, there are only three antibiotics currently approved by FDA for use in aquaculture: oxytetracycline (Terramycin), sulfadimethoxine (Romet-30) and florfenicol (Aquaflor). The widespread use of the limited number of antibiotics for treating bacterial diseases in aquaculture has led to the development of antibiotic resistance in many fish pathogens worldwide (Pridgeon *et al.* 2011). Therefore, alternative control methods are urgently needed for the aquaculture industry.

Use of vaccine is an alternative control method to prevent MAS. The most extensively studied *Aer. hydrophila* vaccines are bacterins consisting of formalin or heat-killed bacteria of pathogenic *Aer. hydrophila* strains (Ruangpan *et al.* 1986; Chandran *et al.* 2002; John *et al.* 2002). In addition, recombinant protein vaccines such as *Aer. hydrophila* outer membrane proteins and bacterial lysates have been demonstrated to elicit protection against *Aer. hydrophila* challenge (Khushiramani *et al.* 2007; LaPatra *et al.* 2010; Poobalane *et al.* 2010; Guan *et al.* 2011). Furthermore, live attenuated vaccines such as *aroA* mutant and transposon Tn916-generated mutants have been reported to confer significant protection against homologous *Aer. hydrophila* challenge (Hernanz Moral *et al.* 1998; Liu and Bi 2007).

To develop effective live bacterial vaccines, novobiocin-resistant strategy has been successfully used to attenuate *Edwardsiella ictaluri* (Pridgeon and Klesius 2011b) and *Streptococcus iniae* (Pridgeon and Klesius 2011c). In addition, attenuated *Aer. hydrophila* have been obtained through selection for resistance to both novobiocin and rifampicin (Pridgeon and Klesius 2011d). However, it is currently unclear whether *Aer. hydrophila* strains could be attenuated by novobiocin alone for the purpose of vaccine development. In addition, it is currently unknown whether the trait of novobiocin resistance will affect the growth and pathogenic characteristics of *Aer. hydrophila*, such as chemotaxis, motility, attachment and invasion. Therefore, the objectives of this study were as follows: (i) To determine whether novobiocin-resistant strategy could be used to attenuate a virulent *Aer. hydrophila* AH11P strain; and (ii) To characterize the growth and pathogenicity differences between the novobiocin-resistant *Aer. hydrophila* AH11NOVO strain and its virulent parent strain AH11P.

Materials and methods

Induction of novobiocin resistance in *Aeromonas hydrophila*

Aeromonas hydrophila AH11P strain was obtained from diseased channel catfish from Alabama in 2005. The bacteria strain was identified by gas chromatography analysis of fatty acid methyl ester (FAME) using the MIDI microbial identification system (MIDI, Newwark, DE, USA) according to established procedures (Shoemaker *et al.* 2005). The archived strain was recovered from frozen stocks (2 ml aliquots stored at −80°C) and grown in tryptic soy broth (TSB; Fisher Scientific, Pittsburgh, PA, USA) for 24 h at 28°C. The recovered AH11P strain was used to obtain the novobiocin-resistant strain AH11NOVO. Novobiocin sodium salt was purchased from Promega (Madison, WI, USA). The initial concentration of novobiocin that allowed growth of *Aer. hydrophila* was 10 μg ml^{-1}. After 20 passages of *Aer. hydrophila* AH11P in TSB containing the same or higher concentrations of novobiocin, the antibiotic-resistant strain AH11NOVO was able to grow in TSB containing 9600 μg ml^{-1} of novobiocin. The parent strain AH11P and the novobiocin-resistant strain AH11NOVO were then cultured on 5% sheep blood agar plates (Thermo Fisher Scientific Remel Products, Lenexa, KS, USA) for bacterial identification. Bacteria strains were identified by gas chromatography analysis of FAME using the MIDI microbial identification system (MIDI) according to established procedures (Shoemaker *et al.* 2005). In addition, DNA gyrase B gene specific primers (forward 5′-AGTGTGCGTCCCAGGTA TTC-3′ and reverse 5′-CTTCCTGATAGGCGTCGTTC-3′) were used to amplify partial DNA gyrase B gene from both strains by polymerase chain reaction (PCR) and the purified PCR products were subjected to DNA sequencing.

Virulence of the novobiocin-resistant *Aeromonas hydrophila* AH11NOVO to channel catfish

All fish treatment protocols were approved by Institutional Animal Care and Use Committee at the Aquatic Animal Health Research Laboratory following related guidelines. The health status of fish used in this study was randomly checked by culturing kidney samples on tryptic soy agar (TSA) plates. Only culture negative catfish were considered healthy and therefore used in this study. To study the virulence of the novobiocin-resistant *Aer. hydrophila* strain AH11NOVO to healthy channel catfish compared with its parent strain AH11P, all bacteria were cultured overnight in TSB at 28°C. Optical density (OD) of the bacterial culture was measured at 540 nm and adjusted to OD = 1·0 using Thermospectronic spectrophotomer (Fisher Scientific). Serial dilutions of each strain (in triplicates) were then immediately prepared in TSB, and 100 μl of serially diluted *Aer. hydrophila* was plated on TSA plates immediately. After the TSA, plates were incubated at 28°C for 24 h, the average number of colony forming unit (CFU) per millilitre was then calculated for both strains. Three

different doses ($1{\cdot}6 \times 10^8$, $8{\cdot}0 \times 10^7$, and $1{\cdot}6 \times 10^7$ colony forming unit per fish) were used to determine whether AH11NOVO was attenuated. Channel catfish (mean weight of 12 ± 2·6 g) were exposed to AH11P or AH11NOVO through intraperitoneal (IP) injection. All channel catfish (Industry pool strain, USDA, ARS; Catfish Genetics Research Unit, Stoneville, MS, USA) used in this study were raised at the USDA-ARS Aquatic Animal Health Research facility at Auburn, Alabama. A total of 60 fish were used in each treatment group (20 fish per tank, three replicates). Published fish maintaining conditions (Pridgeon and Klesius 2011b) were used in this study. Mortalities were recorded daily for 14 days post challenge and the presence or absence of *Aer. hydrophila* in dead fish were determined from bacterial cultures derived from the brain and kidney samples streaked on blood agar plates followed by FAME analysis using MIDI microbial identification system (MIDI).

Vaccination of channel catfish with AH11NOVO followed by challenge with AH11P

The attenuated *Aer. hydrophila* AH11NOVO was cultured in TSB broth at 28°C with shaking at 125 rev min^{-1} overnight before vaccination. Channel catfish were vaccinated with $1{\cdot}6 \times 10^8$ CFU per fish of the novobiocin-resistant *Aer. hydrophila* AH11NOVO in a total volume of 100 μl by IP injection. The vaccination dose of $1{\cdot}6 \times 10^8$ CFU per fish was chosen because this was the highest safe dose of AH11NOVO to catfish used in this study. As sham-vaccination controls, 100 μl of sterile TSB was injected into each fish. A total of 60 fish were used in each treatment group (20 fish per tank, three replicates). At 14 and 28 days post vaccination (dpv), fish were challenged with the virulent parent strain AH11P through IP injection. Mortalities were recorded for 14 days postchallenge and the presence or absence of *Aer. hydrophila* in dead fish was determined as described earlier. Results of challenge were presented as relative per cent of survival (RPS) as described previously (Amend 1981). RPS was calculated according to the following formula: RPS = [1 − (vaccinated mortality ÷ control mortality)] × 100.

Colony size and cell size of AH11NOVO compared with AH11P

To determine the colony size of AH11NOVO compared with AH11P, both bacteria were cultured overnight in TSB at 28°C. Optical density (OD) of the bacterial culture was measured at 540 nm and adjusted to OD = 1·0. Serial dilutions of each strain (in triplicates) were then prepared in TSB and 100 μl of serially diluted *Aer. hydrophila* was immediately plated onto TSA plates. After overnight growth, the colonies of AH11NOVO or AH11P grown on TSA plates were photographed using Gel Logic 440 Imaging system (Kodak, New Haven, CT, USA). To determine the cell size of AH11NOVO compared with AH11P, equal volume of bacterial suspension in phosphate-buffered saline and 5% skim milk were mixed together on clean microscope slides and then streaked across the slides using a second slide in a swift motion. After air drying, the slides were stained with Gram's crystal violet solution for 1–2 min. The excess stain was washed off with 20% copper sulfate ($CuSO_4$) solution and the slides were air dried in a vertical position. The cells were observed using an 100× oil immersion lens with a BX41 microscope (Olympus, Tokyo, Japan).

Cell growth and proliferation profile of AH11NOVO compared with AH11P

Cell growth and proliferation assays were performed using published procedures (Pridgeon *et al.* 2011). Briefly, both AH11NOVO and AH11P were cultured overnight in TSB at 28°C. Optical density (OD) of the bacterial culture was measured at 540 nm and adjusted to OD = 1·0. Serial dilutions of both AH11NOVO and AH11P were prepared in TSB and six dilutions (1 : 20, 1 : 40, 1 : 80, 1 : 160, 1 : 320 and 1 : 640) were made in triplicate in sterile 96-well microtitre plates. Plates were incubated at 28°C with constant shaking and the optical density at 540 nm was measured at different time points (0-, 1-, 2-,3-, 4-, 5-, 6-, 7-, 23-, 24- and 25-h postincubation) using a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, CA, USA). The number of viable bacteria in each well was then determined by CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS; Promega) using published procedures (Pridgeon *et al.* 2011). The optical density of the 96-well plate measured at 490 nm soon after MTS incubation was considered as the 0-h of the MTS incubation. Relative increased OD value was calculated using the following formula: Increased OD value = OD value after incubation − OD value at 0 h of the incubation. The increased OD values after incubation were then plotted against its respective incubation time.

In vitro motility of AH11NOVO and AH11P in semi-solid agar medium

To determine the *in vitro* motility of AH11NOVO in semi-solid agar medium, tryptic soy broth containing agar at concentration of 2·5 g ml^{-1} was prepared in 14-ml sterile culture tubes. Both AH11NOVO and AH11P were cultured overnight in TSB at 28°C. Optical density of the bacterial culture was measured at 540 nm and adjusted to OD = 1·0. A 10-μl sterile loop was used

to inoculate AH11NOVO or AH11P to the semi-solid agar medium by stabbing through the centre of the medium with inoculating needle reaching approximately half of the depth of the medium. Each sample was inoculated into four tubes. Inoculated test tubes containing the semi-solid agar were then incubated at 28°C for 48 h. After the 48 h incubation, *in vitro* motility results were recorded using Logic 440 Imaging system (Kodak).

In vitro attachment and invasion of *Aeromonas hydrophila* to G1B catfish gill cells

In vitro competitive attachment and invasion assays were performed according to published procedures (Pridgeon *et al.* 2011) with slight modifications. Briefly, catfish G1B gill cells (American Type Culture Collection, Manassas, VA, USA) in F-12K media were split into 48-well tissue culture plates with final concentration of 1×10^5 cells per well and grown at 25°C for 24 h. Overnight culture of AH11NOVO and AH11P (OD = 0·8) were diluted to 1 : 10 and mixed at a ratio of 1 : 1. Mixed bacterial samples were added to 48-well plates that containing G1B gill cells. Gill cells in the absence of any bacteria were used as negative control. Plates were incubated at 25°C for 1 h. For the attachment assay, the culture medium and unattached bacteria were removed by washing each well three times gently with 2 ml of 1× Hank's balanced salt solution. Trypsin-EDTA was added to each well to detach G1B gill cells from the culture plates. The solution in each well containing G1B cells and any attached bacteria were then serially diluted and plated on triplicate TSA plates. For the invasion assay, culture medium containing 5 mg ml^{-1} gentamicin (Sigma-Aldrich, St Louis, MO, USA) was added to each well to kill any extracellular bacteria. Plates were incubated at 25°C for 1 h. The culture medium containing gentamicin and any extracellular bacteria were then gently removed. TSB containing 0·1% Triton X-100 (Sigma-Aldrich) was then added to each well to release any intracellular bacteria inside G1B cells. The solution was then serially diluted and plated onto triplicate TSA plates. To determine the input number of AH11NOVO and AH11P added to each well, serial dilutions of each strain (in triplicates) were also prepared in TSB and 100 μl of serially diluted *Aer. hydrophila* was plated onto TSA plates. All TSA plates were incubated at 28°C for 24 h. To determine whether the attached or invaded bacteria were AH11NOVO or AH11P, all individual colonies grown on TSA were subsequently picked to grow overnight in TSB in 96-well plate in the presence and absence of novobiocin (9·6 mg ml^{-1}). The 96-well plate was then incubated at 28°C. The number of colonies that grew in the presence of 9·6 mg ml^{-1} novobiocin was counted as that of AH11NOVO. The colony number of AH11P was calculated by using the following formula: colony number of AH11P = total colony number − colony number of AH11NOVO. The experiment was repeated three times. The attachment and invasion rate of AH11NOVO or AH11P was calculated using the following formula: attachment or invasion rate = [(number of attached or invaded bacteria) ÷ total number of bacteria added] × 100%.

In vitro chemotactic response of AH11NOVO and AH11P to catfish mucus

In vitro chemotaxis assays were performed according to published procedures (Klesius *et al.* 2010). Briefly, healthy channel catfish mucus was collected from the skin of the catfish and the mucus proteins were prepared according to published procedures (Klesius *et al.* 2010). The mucus protein concentration was then determined using BCA Protein assay (Pierce, Rockford, IL, USA) and adjusted to 0·2 mg ml^{-1} with 1× PBS. Chemotaxis assay was performed using blind-well chemotaxis chambers (Corning CoStar, Cambridge, MA, USA) as described by Klesius *et al.* (2010). Briefly, the bottom chambers were filled with 200 μl of either *Aer. hydrophila* (1×10^9 CFU ml^{-1}) parent or vaccine strain. The bottom chamber was separated from the upper chamber by an 8-μm pore diameter polycarbonate membrane filter (Nucleopore, Pleasontan, CA, USA). Triplicate mucus samples (0·2 mg protein ml^{-1}) were added to the upper compartment of the chamber of each parent and vaccine strain. As negative controls, lower chamber with tryptic soy broth and upper chamber with mucus samples was also included in the assay. The chambers were incubated for 90 min at 28°C on a horizontal platform shaker. Following incubation, 100 μl was transferred to a flat-bottom 96-well microtitre plate (Thermo Scientific, Milford, MA, USA). The number of viable bacterial cells in each well was then determined by CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay (MTS) using published procedures (Pridgeon *et al.* 2011). Relative increased OD value was calculated using the following formula: ΔOD 490 nm (sample) = OD 490 nm value (after incubation) − OD 490 nm value (0 h of the incubation). The relative chemotaxis index of AH11NOVO or AH11P to fish mucus was calculated using the following formula: ΔΔ chemotaxis index = ΔOD 490 nm of test sample (AH11NOVO or AH11P) – ΔOD 490 nm of mucus sample alone (without bacteria in the lower chamber). The experiments were repeated four times.

Statistical analysis

All statistical analyses were performed using SigmaStat 3·5 software (Systat Software, Inc, Point Richmond, CA,

USA). Differences in cell growth, cell proliferation, chemotaxis, attachment and invasion were analysed using Student *t*-test and the significance level was defined as $P < 0{\cdot}05$.

Results

FAME profiles and partial DNA gyrase B sequencing of AH11NOVO and AH11P

MIDI microbial identification system revealed that both AH11NOVO and AH11P shared high similarity indices (0·488 and 0·481, respectively) with *Aer. hydrophila* deposited at the RCLN50 database. The FAME profile of AH11NOVO and AH11P are shown in Fig. 1. Of all the fatty acids, the percentage/peak of 16 : 1 w7c/16 : 1 w6c fatty acid in AH11P was the highest (37·18%), followed by the peak of 16 : 0 fatty acid (20·97%) and 18 : 1 w7c fatty acid (19·49%). Similarly, the major fatty acids in AH11NOVO were 16 : 1 w7c/16 : 1 w6c fatty acid (37·80%), 16 : 0 fatty acid (22·16%) and 18 : 1 w7c fatty acid (19·16%). DNA sequencing results revealed that both AH11NOVO and AH11P shared 99% similarities with the DNA gyrase B gene of *Aer. hydrophila* subsp. *hydrophila* ATCC 7966 (GenBank accession no. CP000462), with *e*-value of 0.

Virulence of AH11NOVO compared with AH11P

Virulence of AH11NOVO and AH11P to channel catfish was summarized in Table 1. All dead fish after exposure to AH11P or AH11NOVO throughout this study were culture positive for *Aer. hydrophila*. At injection doses of $1{\cdot}0 \times 10^8$ CFU per fish, the parent strain AH11P killed 100% fish (Table 1). However, when higher amounts $1{\cdot}6 \times 10^8$ CFU per fish of novobiocin-resistant AH11NOVO were injected to fish, no fish died (Table 1).

Vaccination with AH11NOVO followed by challenge with AH11P

When novobiocin-resistant AH11NOVO vaccinated channel fish were challenged with its virulent parent AH11P at 14 dpv, RPS of vaccinated fish was 100% (Table 2). When AH11NOVO vaccinated channel fish were challenged with AH11P at 28 dpv, RPS of vaccinated fish ranged from 50 to 87·5% (Table 2). When AH11NOVO vaccinated fish or TSB sham-vaccinated fish were challenged with AH11P at 14 dpv, the cumulative mortalities of AH11NOVO vaccinated fish at different time points after challenge were significantly ($P < 0{\cdot}05$) lower than that of TSB sham-vaccinated fish (Fig. 1a). Similarly, at 28 dpv, the cumulative mortalities of AH11NOVO vaccinated fish at different time points after challenge with AH11P were significantly ($P < 0{\cdot}05$) lower than that of TSB sham-vaccinated fish (Fig. 1b).

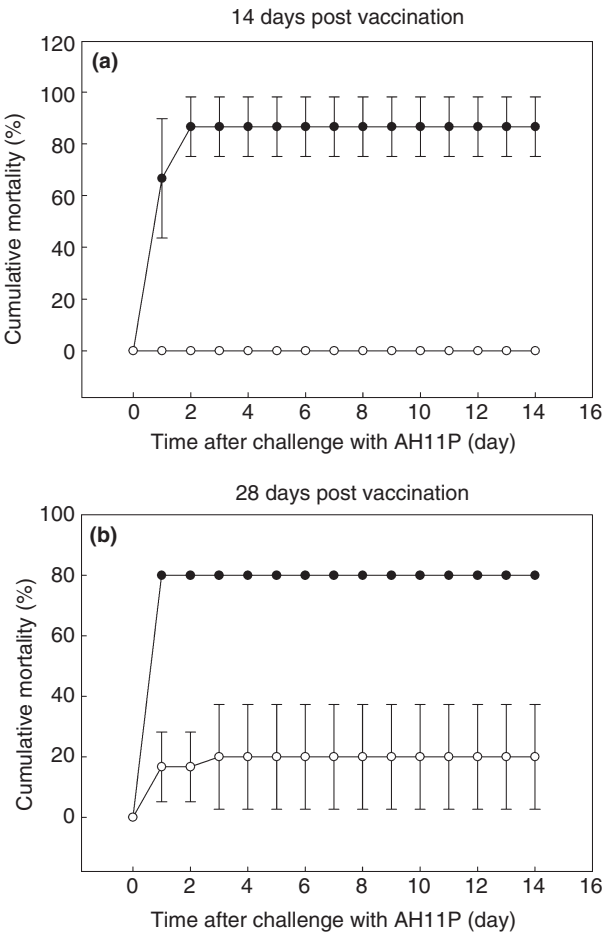

Figure 1 Daily mean per cent cumulative mortality of channel catfish intraperitoneally vaccinated with or without *Aeromonas hydrophila* AH11NOVO and challenged with *Aer. hydrophila* AH11P through intraperitoneal injection. (a) Fourteen days post vaccination; (b) 28 days post vaccination. Daily mean per cent cumulative mortalities were calculated from three vaccination trials. (●) TSB sham vaccinated fish and (○) AH11NOVO vaccinated fish.

Table 1 Virulence of novobiocin-resistant *Aeromonas hydrophila* AH11NOVO and its parent isolates AH11P to channel catfish by intraperitoneal injection

Isolate name	Injection dose (CFU per fish)	Mortality ± SD (%)
AH11NOVO	$1{\cdot}6 \times 10^8$	0 ± 0
AH11P	$1{\cdot}0 \times 10^8$	100 ± 0
AH11NOVO	$8{\cdot}0 \times 10^7$	0 ± 0
AH11P	$5{\cdot}0 \times 10^7$	87 ± 12
AH11NOVO	$1{\cdot}6 \times 10^7$	0 ± 0
AH11P	$1{\cdot}0 \times 10^7$	0 ± 0
TSB	0	0 ± 0
Untreated	0	0 ± 0

Table 2 Cumulative mortality and relative per cent survival of AH11NOVO vaccinated catfish challenged with AH11P

Trial No.	Vaccine group	Vaccine dose (CFU per fish)	Isolate used for challenge	Challenge dose (CFU per fish)	dpv	Mortality (%)	RPS (%)
I	Sham TSB	–	AH11P	$5{\cdot}0 \times 10^7$	14	100	–
I	AH11NOVO	$1{\cdot}6 \times 10^8$	AH11P	$5{\cdot}0 \times 10^7$	14	0	100
II	Sham TSB	–	AH11P	$5{\cdot}0 \times 10^7$	14	80	–
II	AH11NOVO	$1{\cdot}6 \times 10^8$	AH11P	$5{\cdot}0 \times 10^7$	14	0	100
III	Sham TSB	–	AH11P	$5{\cdot}0 \times 10^7$	14	80	–
III	AH11NOVO	$1{\cdot}6 \times 10^8$	AH11P	$5{\cdot}0 \times 10^7$	14	0	100
I	Sham TSB	–	AH11P	$5{\cdot}0 \times 10^7$	28	80	–
I	AH11NOVO	$1{\cdot}6 \times 10^8$	AH11P	$5{\cdot}0 \times 10^7$	28	10	87·5
II	Sham TSB	–	AH11P	$5{\cdot}0 \times 10^7$	28	80	–
II	AH11NOVO	$1{\cdot}6 \times 10^8$	AH11P	$5{\cdot}0 \times 10^7$	28	40	50
III	Sham TSB	–	AH11P	$5{\cdot}0 \times 10^7$	28	80	–
III	AH11NOVO	$1{\cdot}6 \times 10^8$	AH11P	$5{\cdot}0 \times 10^7$	28	10	87·5

dpv, days post vaccination; RPS, relative per cent of survival; TSB, tryptic soy broth.

Colony size and cell size of AH11NOVO compared with AH11P

When similar amount of AH11NOVO and AH11P were distributed onto TSA plates and incubated at 28°C for 24 h, AH11P appeared to be larger in colony size (Fig. 2a). However, morphologically, both colonies appeared to have a smooth surface (Fig. 2a). Under the microscope, the cell size of AH11NOVO appeared to be smaller than that of AH11P (Fig. 2b), but the thickness of the cell walls of AH11NOVO appeared to be similar to that of AH11P (Fig. 2c).

Cell proliferation profile of AH11NOVO compared with AH11P

At OD 540 nm = 1·0, the average amount of AH11P was $1{\cdot}01 \pm 0{\cdot}11 \times 10^9$ CFU ml^{-1}, whereas that of AH11NOVO was $1{\cdot}70 \pm 0{\cdot}20 \times 10^9$ CFU ml^{-1}. When the starting amount of bacteria of both AH11NOVO and AH11P was at 1 : 160 dilution from OD 540 nm = 1·0, the relative increase in OD 540 nm values of AH11P at 1- and 2-h postincubation appeared to be lower than that of AH11NOVO (Fig. 3a). However, at 3-h postincubation and later time points, the relative increase in OD 540 nm value of AH11P was significantly ($P < 0{\cdot}05$) higher than that of AH11NOVO (Fig. 3a). Similar pattern was also observed when the starting amount of both bacteria in each well was at other dilutions (data not shown). Cell proliferation assays revealed that the relative increase in OD 490 nm value of AH11P was significantly ($P < 0{\cdot}05$) higher than that of AH11NOVO at 5-, 6-, 7- and 23-h post-MTS incubation (Fig. 3b). However, at both 24- and 25-h post-MTS incubation, the relative increase in OD 490 nm value of AH11P was not significantly different from that of AH11NOVO (Fig. 3b).

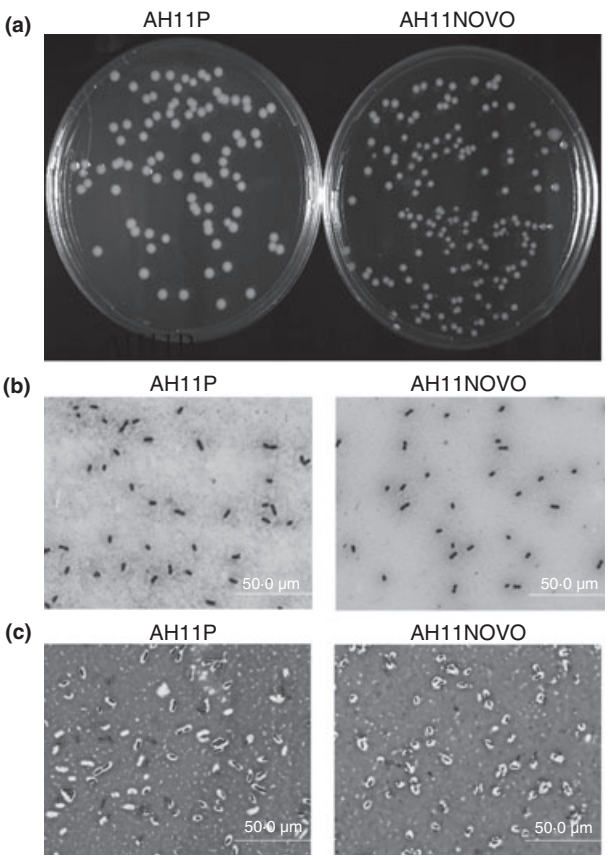

Figure 2 Colony size and cell size of AH11NOVO compared to AH11P. (a) Overnight growth on blood agar plates; (b) Intact cells under microscope; (c) Cell wall stripped cells under microscope. Pictures shown were representatives of four replicates. Scale bar: 50 μm.

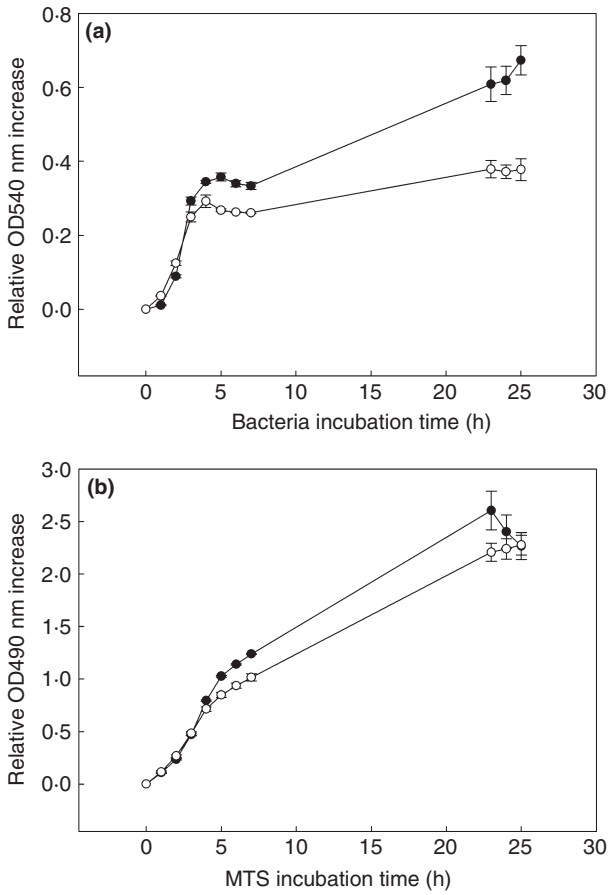

Figure 3 Cell growth and proliferation of AH11NOVO and AH11P. (a) Relative cell growth determined by the turbidity measured at 540 nm; (b) Relative cell proliferation determined by MTS assay measured at 490 nm. Data were presented as mean ± standard deviation (SD) from three replicates. (●) AH11P and (○) AH11NOVO.

In vitro motility of AH11NOVO and AH11P in semi-solid agar medium

When grown in semi-solid agar medium, the virulent parent strain of *Aer. hydrophila* AH11P showed diffused growth throughout the entire medium, whereas the novobiocin-resistant AH11NOVO only showed growth confined to the line of inoculation.

In vitro attachment and invasion of AH11NOVO and AH11P to G1B gill cells

The *in vitro* adhesion and invasion abilities of AH11NOVO and AH11P to catfish G1B gill cells are summarized in Fig. 4. Of the $1{\cdot}3 \times 10^7$ CFU of AH11P added to each well of G1B cells, $8{\cdot}0 \pm 2{\cdot}0 \times 10^4$ CFU attached to G1B cells, with the per cent of AH11P attached to G1B cells at $0{\cdot}62 \pm 0{\cdot}15\%$. Of the $2{\cdot}4 \times 10^7$ CFU of AH11NOVO added to each well of G1B cells, $2{\cdot}0 \pm 0{\cdot}8 \times 10^5$ CFU attached to G1B cells, with the per cent of AH11NOVO attached to G1B cells at $0{\cdot}83 \pm 0{\cdot}33\%$. Therefore, on average, the attachment rate of AH11NOVO was higher than that of AH11P. However, there was no significant difference between the attachment rate of AH11NOVO and that of AH11P (Fig. 4a). The invasion rate of AH11P to G1B gill cells was $0{\cdot}036 \pm 0{\cdot}025\%$, which was significantly ($P < 0{\cdot}05$) higher than that of AH11NOVO ($0{\cdot}0014 \pm 0{\cdot}0016\%$; Fig. 4b).

In vitro chemotactic response of AH11NOVO and AH11P to catfish mucus

The results of the *in vitro* chemotactic response of AH11NOVO and AH11P to catfish mucus are summarized in Fig. 5. The average chemotactic index of AH11P to catfish mucus was $2{\cdot}30 \pm 0{\cdot}36$, which was significantly ($P < 0{\cdot}05$) higher than that of AH11NOVO ($1{\cdot}36 \pm 0{\cdot}38$; Fig. 5).

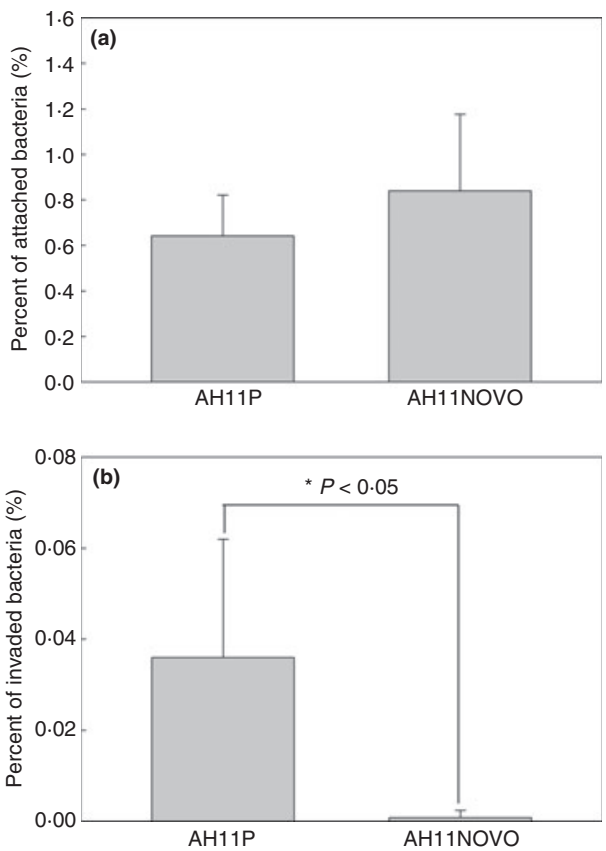

Figure 4 Per cent of AH11NOVO and AH11P attached or invaded to catfish G1B gill cells. (a) Percent of attachment; (b) Percent of invasion. The per cent of the amount of bacteria (colony forming unit, CFU) attached or invaded to G1B cell was determined by surface plating of bacteria onto tryptic soy agar (TSA) plates. Data are presented as mean ± standard deviation (SD) from three replicates. Significant difference ($P < 0{\cdot}05$) was marked by asterisk.

Discussion

Virulence study revealed that AH11P killed 100% channel catfish at dose of $1{\cdot}0 \times 10^8$ CFU per fish. However, at dose of $1{\cdot}0 \times 10^7$ CFU per fish, AH11P failed to kill any catfish used in this study. The LD_{95} dose of another pathogenic strain of *Aer. hydrophila* AL98-C1B was reported to be $2{\cdot}0 \times 10^8$ CFU per fish (Pridgeon and Klesius 2011a). However, the LD_{95} doses of the highly virulent 2009 west Alabama isolates of *Aer. hydrophila* AL98-C1B were $4{\cdot}8 \times 10^5$–$7{\cdot}0 \times 10^5$ CFU per fish (Pridgeon and Klesius 2011a). Taken together, these results suggest that AH11P was not a highly virulent strain of *Aer. hydrophila*.

Using novobiocin-resistant strategy, a novel attenuated *Aer. hydrophila* strain AH11NOVO was obtained from a virulent strain AH11P after 20 passages in novobiocin-containing media. The virulent parent strain AH11P was only able to survive in TSB containing 10 $\mu g\ \mu l^{-1}$ of novobiocin, but the novobiocin-resistant *Aer. hydrophila* AH11NOVO was able to grow in TSB containing 9600 $\mu g\ \mu l^{-1}$ of novobiocin, suggesting that the novel strain of *Aer. hydrophila* AH11NOVO was at least 960 times more resistant to novobiocin than its parent strain AH11P. AH11NOVO was found to be avirulent to channel catfish when injected at $2{\cdot}4 \times 10^8$ CFU per fish, whereas AH11P at similar and lower dose killed 100% fish, suggesting that the trait of novobiocin resistance in AH11NOVO might have resulted in the attenuation of virulence as one of its fitness costs. This is not surprising as decreased virulence as a fitness cost has been reported in novobiocin-resistant *Edw. ictaluri* (Pridgeon and Klesius 2011b) and *Strep. iniae* (Pridgeon and Klesius 2011c). In addition, decreased virulence of *Staphylococcus aureus* has been reported to be associated with antibiotic resistance (McCallum *et al.* 2006). Differential transcriptome analysis on teicoplanin-resistant *Staph. aureus* has revealed that as resistance to antibiotic teicoplanin increased, some virulence-associated genes were down-regulated (McCallum *et al.* 2006). These results suggest that the gain of antibiotic resistance might result in the attenuation of virulence in bacteria. However, the gain of antibiotic resistance in bacteria does not necessarily lead to the attenuation of virulence, because it has been reported that gain of novobiocin resistance trait in *Strep. iniae* isolates such as Kent 02, Uruguay 1, Uruguay A and 15Br failed to lower their virulence (Pridgeon and Klesius 2011c). Therefore, the gain of antibiotic resistance trait in bacteria does not necessarily lead to the attenuation of its virulence.

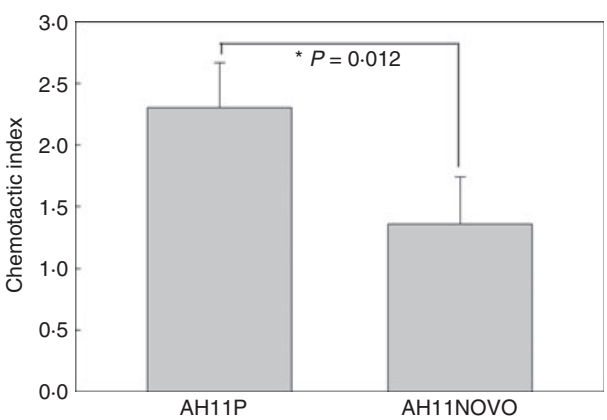

Figure 5 Chemotactic response of *Aeromonas hydrophila* AH11NOVO and AH11P to channel catfish mucus. Data were presented as mean ± standard deviation (SD) from four replicates. Significant difference ($P < 0{\cdot}05$) was marked by asterisk.

When novobiocin-resistant AH11NOVO vaccinated channel fish were challenged with its virulent parent AH11P at 14 dpv, RPS of vaccinated fish was 100%. However, when AH11NOVO vaccinated channel fish were challenged with AH11P at 28 dpv, RPS of vaccinated fish decreased from 100 to 87·5% or 50%. Decreased protection to channel catfish offered by attenuated *Aer. hydrophila* vaccine at 28 dpv compared with that at 14 dpv has been previously reported (Pridgeon and Klesius 2011d), suggesting that booster immunization of AH11NOVO such as oral vaccination after 14 dpv might be necessary to increase its duration of protection.

Cell growth studies revealed that AH11NOVO strain had a much lower growth rate as indicated by its smaller colony size on agar plates and slower cell proliferation profile. Slower growth has been reported as a fitness cost in antibiotic-resistant bacteria (Han *et al.* 2009; Pridgeon and Klesius 2011b,c,d). For example, the macrolide-resistant *Campylobacter jejuni* has a slower growth rate than that of its parent strain, with an average doubling time of 136 *vs* 112 min for the parent strain (Han *et al.* 2009). Taken together, the smaller colony size of AH11NOVO compared with its parent strain AH11P suggests that its slower growth rate might be a fitness cost associated with its resistance to novobiocin.

When grown in semi-solid agar medium, the virulent parent strain of *Aer. hydrophila* AH11P showed diffused growth throughout the entire medium, whereas the novobiocin-resistant AH11NOVO only showed growth confined to the line of inoculation, suggesting that AH11P was motile, whereas AH11NOVO was not. *In vitro* invasion studies revealed that the invasion rate of AH11P to G1B gill cells was significantly higher than that of AH11NOVO. *In vitro* chemotaxis assays revealed that AH11P had significantly higher chemotactic response to catfish mucus than AH11NOVO. As AH11P was virulent to catfish whereas AH11NOVO was avirulent to catfish, these results taken together suggest that

cell motility, cell invasion ability and chemotactic response to host are all important virulence factors of *Aer. hydrophila*. Although cell motility and invasion ability are well known virulence factors (Josenhans and Suerbaum 2002; Zakikhany *et al.* 2008), the importance of chemotaxis affecting the virulence of pathogens are not extensively studied. It has been suggested that chemotaxis is not necessary for *Aer. hydrophila* to become pathogenic to common carp, but may be a necessary parameter for *Aer. hydrophila* to become an obligate pathogen (van der Marel *et al.* 2008). *In vitro* attachment assay revealed that the attaching ability of AH11NOVO to G1B gill cells was not significantly different from that of AH11P, suggesting that attachment ability is not directly linked to virulence. Electron microscopy studies have demonstrated that all motile aeromonads produce fimbriae (pili) that facilitate adhesion, regardless of their virulence (del Corral *et al.* 1990). Taken together, these results suggest that chemotaxis, motility, and attachment are all important factors for pathogens to locate the host and attach to the host, but the ability to invade the host and factors such as extracellular products might be directly linked to virulence.

In summary, a novobiocin-resistant strain AH11NOVO was obtained from a virulent *Aer. hydrophila* strain AH11P through selection of resistance to novobiocin. AH11NOVO provide significant protection to channel fish against AH11P challenge. The cell proliferation rate and chemotactic response of AH11NOVO was found to be significantly lower than that of AH11P. Although the ability of AH11NOVO to attach catfish gill cells was similar to that of AH11P, the ability of AH11NOVO to invade catfish gill cells was significantly lower than that of AH11P. Taken together, these results suggest that chemotaxis, motility and attachment are all important factors for *Aer. hydrophila* to locate the host and eventually attach to the host, but the ability to invade the host might be directly linked to virulence.

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