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# Quorum sensing signals are produced by *Aeromonas salmonicida* and quorum sensing inhibitors can reduce production of a potential virulence factor

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ABSTRACT: Many pathogens control production of virulence factors by self-produced signals in a process called quorum sensing (QS). We demonstrate that acyl homoserine lactone (AHL) signals, which enable bacteria to express certain phenotypes in relation to cell density, are produced by a wide spectrum of *Aeromonas salmonicida* strains. All 31 typical strains were AHL producers as were 21 of 26 atypical strains, but on a strain population basis, production of virulence factors such as protease, lipase, A-layer or pigment did not correlate with the production and accumulation of AHLs in the growth medium. Pigment production was only observed in broth under highly aerated conditions. Quorum sensing inhibitors (QSIs) are compounds that specifically block QS systems without affecting bacterial growth and 2 such compounds, sulphur-containing AHL-analogues, reduced production of protease in a typical strain of *Aeromonas salmonicida*. The most efficient compound *N*-(heptylsulfanylacetyl)-L-homoserine lactone (HepS-AHL), reduced protease production by a factor of 10. Five extracellular proteases were detected on gelatin-containing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels and 3 of these were completely down regulated by HepS-AHL. Hence, QSIs can curb virulence in some strains and could potentially be pursued as bacterial disease control measures in aquaculture.

KEY WORDS: Acylated homoserine lactones · *Aeromonas salmonicida* · Pigment · Protease · Quorum sensing inhibitors

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## INTRODUCTION

Fish farming is an intensive production and microbial diseases may occur. Traditionally, antibiotics or chemotherapeutics have been used to control microbial diseases, but this may lead to resistance in both pathogenic and non-pathogenic bacteria (Tsoumas et al. 1989, Spanggaard et al. 1993, Depaola et al. 1995, Schmidt et al. 2000). The use of antibiotics has been dramatically reduced by development and use of vaccines (Grave et al. 1996, Markestad & Grave 1997, NORM-VET 2004); however, vaccines cannot be used at all stages and for all aquacultured species. Research has, therefore, also focused on alternative, potential disease control measures such as probiotics (Gram & Ringø 2005) or specific attenuators of bacterial virulence (Hentzer et al. 2003).

Some Gram-negative bacteria regulate their virulence factors as a function of population cell density (quorum sensing, QS), which they sense by means of extracellular signal molecules such as acylated homoserine lactones (AHLs). QS systems and/or QS signals have been found in many Gram-negative fish pathogenic bacteria such as *Aeromonas hydrophila*, *A. salmonicida*, *Vibrio harveyi*, *V. anguillarum*, *V. salmonicida*, *V. vulnificus, Edwardsiella tarda* and *Yersinia ruckeri* (Milton et al. 1997, Swift et al. 1997, Freeman & Bassler 1999, Temprano et al. 2001, Buch et al. 2003, Morohoshi et al. 2004, Bruhn et al.

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2005). QS controls several of the putative virulence factors in aquatic bacteria, such as protease in *V. anguillarum* (Croxatto et al. 2002) and *A. hydrophila* (Swift et al. 1999), siderophores in *V. harveyi* (Lilley & Bassler 2000), and haemolysin in *V. vulnificus* (Kim et al. 2003). However, the role of the QS systems in expression of virulence in fish pathogenic bacteria is not at present fully understood.

Quorum sensing inhibitors (QSIs) are compounds that antagonise bacterial QS systems without affecting growth of the bacteria (Smith et al. 2003, Castang et al. 2004, Persson et al. 2005, Rasmussen et al. 2005a,b). QSIs reduce production of toxin from *Vibrio harveyi* (Manefield et al. 2000) and treatment with a QSI, the halogenated furanone C-30, reduced accumulated mortality in rainbow trout infected with *V. anguillarum* (Rasch et al. 2004). However higher concentrations of furanone C-30 resulted in rapid fish death (Rasch et al. 2004), which stressed the need to explore less toxic QSI compounds for treatment. Sulfonated analogues of AHL compounds are such less toxic compounds (Persson et al. 2005) and can inhibit several QS systems (Koch et al. 2005). In the present study, we address the possible involvement of QS systems in expression of virulence factors of *Aeromonas salmonicida* and determine to what extent QSI compounds affect production of virulence factors.

*Aeromonas salmonicida* is the bacterial agent associated with furunculosis in salmonid fish (Austin & Austin 1999). The QS signals, *N*-butanoyl-L-homoserine lactone ($C_4$-HSL) and *N*-hexanoyl-L-homoserine lactone ($C_6$-HSL) (Swift et al. 1997, Bruhn et al. 2005), and the genes involved in production of the AHL synthase (LuxI) and AHL receptor (LuxR) have been detected in *A. salmonicida* (Swift et al. 1997). Several of the virulence factors expressed by *A. salmonicida*, such as α-haemolysin, glycero-phospholipid-cholesterol acyltransferase, lipase and serine protease, are associated with high cell densities and may therefore potentially be controlled by QS (Swift et al. 1997). Exogenous addition of $C_4$-HSL resulted in a small induction of protease production in *A. salmonicida*, whereas addition of *N*-decanoyl-L-homoserine lactone ($C_{10}$-HSL) and *N*-(3-oxodecanoyl)-L-homoserine lactone (oxo-$C_{10}$-HSL) inhibited protease production (Swift et al. 1997). Both the induction by the natural AHL compound and inhibition by longer chained compounds indicate that the protease is under QS control.

Bruhn et al. (2005) reported that AHLs were produced by all of 7 typical *Aeromonas salmonicida* strains but only in 2 of 10 atypical strains (*A. salmonicida* ssp. *pectinolytica* DSM 12609 and *A. salmonicida* ssp. *achromogenes* NCIMB 1110). Typical strains (ssp. *salmonicida*) and ssp. *pectinolytica* produce a brown water-soluble pigment whereas atypical strains (ssp. *achromogenes*, ssp. *masoucida*, and ssp. *smithia*) produce only very low amounts of the brown pigment (Wiklund & Dalsgaard 1998, Pavan et al. 2000). The AHL production was only observed from strains producing the pigment (J. B. Bruhn unpubl.). Hence, we hypothesised that pigment and AHL production could be correlated and investigate this in a larger collection of strains in the present study.

The purpose of the present study was to investigate whether there is any correlation between production of QS signal molecules and virulence factors in *Aeromonas salmonicida* and if there are systematic differences in QS signal production in typical and atypical strains. Furthermore, the possible effect of a series of new QSIs on production of virulence factors in *A. salmonicida* was investigated with the long-term aim of evaluating their potential use in control of fish disease.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** A selection of 31 typical and 26 atypical *Aeromonas salmonicida* was used in this study (see Tables 1 & 2). The strain collection comprised strains isolated over many years from different sources, locations and from both sea and freshwater farms as well as type strains. All but 3 strains had been stored at −80°C immediately after isolation. All strains were grown in Luria-Bertani broth (Bertani 1951) using 5 g $l^{-1}$ of NaCl instead of 10 g $l^{-1}$ ($LB_5$). When appropriate, the broth was supplemented with 1.2% (w/v) agar. AHLs were monitored using *Agrobacterium tumefaciens* strain NT1 with pZLR4 (Cha et al. 1998) and *Chromobacterium violaceum* strain CV026 (Throup et al. 1995, McClean et al. 1997). The growth media for *A. tumefaciens* and *C. violaceum* were supplemented with 20 µg $ml^{-1}$ of gentamicin and kanamycin, respectively. *A. tumefaciens* was grown in AB medium (Clark & Maaløe 1967) supplemented with 2.5 mg $l^{-1}$ thiamine (ABT), 0.5% (w/w) casamino acids and 0.5% (w/w) glucose (ABTG) for estimation of levels of AHLs.

**Production of virulence factors.** Protease production and proteolytic activity was detected on 1.2% agar plates supplemented with 10% (v/v) sterile skimmed milk (105°C for 30 min). The cultures were streaked on the skim milk agar plates and incubated at 25°C. Proteolytic strains caused a clearing zone around the bacterial growth. Quantitative assessment of protease production was assayed in sterile filtered culture supernatants (Windle & Kelleher 1997, Denkin & Nelson 1999). In brief 100 µl of sample was incubated with 100 µl azocasein (sulfanilamide-azocasein, cat. no. A-2765, Sigma) (5 mg $ml^{-1}$ dissolved in 50 mM Tris-HCl, pH 8) at 30°C for 90 min. The reaction was terminated by addition of 400 µl trichloracetic acid (10% w/v) and the precipitated

protein was removed by centrifugation (16 000 × *g*, 3 min). We transferred 500 µl supernatant to 700 µl of 525 mM NaOH and measured optical density (OD) at 442 nm on a Novaspec II visible spectrophotometer (Pharmacia Biotech). Each sample was assayed in triplicate and 2 controls were prepared for each sample. Controls were vials to which the sample was added after trichloracetic acid. Protease profiles were analysed by electrophoresis of sterile filtered culture supernatants on zymogram gels (ready gel zymogram gel 12% with casein, cat. no. 161–1114, and ready gel zymogram gel 10% with gelatin, cat. no. 161–1113, Bio-Rad). Protease size was determined by using a dual colour molecular weight standard (Precision Plus Protein Standard cat. no. 161-0374, Bio-Rad), which was cut off the gel before staining with Coomassie Brilliant Blue (CBB) R-250 (cat. no. 443283M, BDH) (Rajmohan et al. 2002). Lipase activity was assayed on tributyrin agar (cat. no. PM0004C, Oxoid). We spotted 10 µl of an overnight culture on the agar plates, and the plates were incubated at 25°C until visible growth. Lipolytic activity was seen as clear zones around the colonies. *Pseudomonas aeruginosa* ATCC 27853 was used as positive control and *Escherichia coli* MG1655 as negative control for lipolytic activity. Presence of A-layer was evaluated on CBB agar (Bernoth 1990). Cultures grown in $LB_5$ were streaked on CBB-plates and incubated at 20°C and presence of A-layer was seen as blue colonies whereas A-layer negative strains had white colonies. For strains with both blue and white colonies on the plates the proportion of blue vs. white colonies was estimated. For quantitative evaluation of pigment production, sterile filtered bacterial supernatants were measured spectrophotometrically on as Novaspec II visible spectrophotometer (Pharmacia Biotech) at 410 nm with the appropriate growth medium as reference. The cultures were grown in 50 ml $LB_5$ broth in 250 ml bottles covered with oxygen permeable film at 200 rpm on a rotational shaker at 15°C.

**Production of QS signals, acylated homoserine lactones.** The *Aeromonas salmonicida* strains were streaked on a $LB_5$ agar plates (without added antibiotic) in parallel with each of the monitor strains *Agrobac-*

Table 1. *Aeromonas salmonicida.* Production of virulence factors and acylated homoserine lactones (AHLs) in typical strains. Protease activity detected on casein agar; A-layer detected on Coomassie Brilliant Blue (CBB) agar; pigment in Luria-Bertani broth using 5 g $l^{-1}$ NaCl ($LB_5$) at 15°C measured as optical density at 410 nm after 7 d growth; lipase detected on tributyrin agar; AHLs detected with *Chromobacterium violaceum* CV026, and *Agrobacterium tumefaciens* pZLR4. Sea: sea farm; Fresh: freshwater farm. +: positive; (+): slightly positive; –: negative; +/– majority positive

| Strain | Source | Location | Protease | A-layer | Pigment | Lipase | AHL production | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | CV026 | pZLR4 |
| NCIMB 1102 | Atlantic salmon | Scotland | + | + | 6.56 | + | + | + |
| 02-9-1 | Rainbow trout | Denmark; Sea | + | +/– | 6.38 | + | + | + |
| 02-9-37 | Rainbow trout | Denmark, Sea | + | + | 7.26 | + | + | + |
| 93-8-294 | Rainbow trout | Denmark, Sea | + | + | 6.12 | + | + | + |
| 94-6-87 | Rainbow trout | Denmark, Sea | + | + | 6.12 | + | + | + |
| ATCC 14174 | Brook trout | USA | – | – | 6.48 | + | + | + |
| 820615-1/2 | Rainbow trout | Denmark, Sea | – | + | 4.70 | – | + | + |
| 820618-2/1 | Rainbow trout | Denmark, Fresh | – | + | 5.78 | + | + | + |
| 830608-1/2 | Rainbow trout | Denmark, Fresh | + | – | 7.28 | + | + | + |
| 830719-2/2 | Rainbow trout | Denmark, Sea | + | + | 6.76 | + | + | + |
| 830803-1/2 | Rainbow trout | Denmark, Sea | + | + | 6.96 | + | + | + |
| 850612-1/1 | Rainbow trout | Denmark, Sea | – | + | 5.48 | + | + | + |
| 850613-1/1 | Rainbow trout | Denmark, Sea | + | – | 7.10 | + | + | + |
| 860619-3/3 | Rainbow trout | Denmark, Sea | + | + | 6.80 | + | + | + |
| 860815-1/2 | Rainbow trout | Denmark, Sea | + | – | 6.96 | + | + | + |
| 870708-1/4 | Rainbow trout | Denmark, Fresh | + | + | 6.18 | (+) | + | + |
| 880819-2/1 | Rainbow trout | Denmark, Fresh | + | + | 6.14 | + | + | + |
| 880827-1/2 | Rainbow trout | Denmark, Sea | + | + | 7.14 | + | + | + |
| 940818-1/1 | Rainbow trout | Denmark, Sea | + | + | 5.88 | + | + | + |
| 950901-3/1 | Rainbow trout | Denmark, Fresh | – | + | 5.10 | + | + | + |
| 960828-1/1 | Rainbow trout | Denmark, Sea | + | + | 7.18 | + | + | + |
| 980623-1/1 | Sea trout | Denmark, Fresh | + | + | 5.46 | – | + | + |
| 990622-1/9 | Sea trout | Denmark, Sea | + | + | 4.66 | + | + | + |
| 020625-6/2 | Common whitefish | Denmark, Fresh | + | + | 5.30 | + | + | + |
| 030715-1/8 | Common whitefish | Denmark, Fresh | + | + | 6.12 | + | + | + |
| 030729-1/1 | Rainbow trout | Denmark, Sea | + | + | 5.20 | + | + | + |
| 040115-2/2 | Atlantic salmon | Denmark, Fresh | + | + | 5.88 | + | + | + |
| 040811-1/2 | Charr | Denmark, Fresh | + | + | 4.94 | + | + | + |
| 040915-1/2 | Rainbow trout | Denmark, Sea | + | + | 5.60 | + | + | + |
| 050225-1/1A | Atlantic salmon | Denmark, Fresh | + | + | 5.80 | + | + | + |
| Jno 3175/88 | Atlantic salmon | Norway | + | + | 6.80 | + | + | + |

*terium tumefaciens* and *Chromobacterium violaceum* as described by Ravn et al. (2001). *A. tumefaciens* plates were supplemented with X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, cat. no. A4978, AppliChem) to a final concentration of 50 µg $ml^{-1}$. The plates were incubated at 25°C until both strains showed good growth. *Aeromonas* strains causing blue colour in *A. tumefaciens* or violet pigmentation in *C. violaceum* were positive for AHL production. Semiquantitative determination of AHL concentrations was determined by testing extracts of liquid cultures in a well diffusion assay (Ravn et al. 2001) with $C_4$-HSL (cat. no. 09945, Sigma-Aldrich) as standard. We lowered 25 ml of bacterial cultures to pH 2 with 2 M HCl to ensure that the lactone ring of the AHL molecule was closed. The culture was extracted with an equal volume of acidified ethyl acetate (ethyl acetate with 0.5% formic acid). The mixture was shaken vigorously and after phase separation, the ethyl acetate phase was transferred, evaporated under nitrogen flow and redissolved in 1 ml of acidified ethyl acetate. The samples were stored at –20°C until further analyses.

**Growth experiments.** *Aeromonas salmonicida* strain 02-9-1 was isolated from a furunculosis outbreak in salmon. It produces extracellular virulence factors including a high level of pigment. The strain produces several AHLs with $C_4$-HSL as the dominant molecule (called 'BHL' in Bruhn et al. 2005). Strain 02-9-1 was cultured on $LB_5$ agar and inoculated in 50 ml $LB_5$ broth at 15°C and 200 rpm for 2 d. We inoculated 2000 ml baffled flasks with 1500 ml of $LB_5$ with *A. salmonicida* 02-9-1 to a final concentration of approx. $10^3$ CFU $ml^{-1}$. The flasks were incubated at 15°C and 200 rpm. At appropriate time intervals samples were taken for measurement of cell density ($OD_{450nm}$), CFU $ml^{-1}$ on $LB_5$ agar, and pigment production as described above. AHLs were extracted as described above, and sterile filtered culture supernatants were stored at –20°C for further analysis of protease activity and protease profiles.

**Effect of QSI compounds on growth and protease production.** The QSI compounds *N*-(propylsulfanylacetyl)-L-homoserine lactone (ProS-AHL), *N*-(pentylsulfanylacetyl)-L-homoserine lactone (PenS-AHL) and

Table 2. *Aeromonas salmonicida*. Production of virulence factors and acylated homoserine lactones (AHLs) in atypical strains. Protease activity detected on casein agar; A-layer detected on Coomasie Brilliant Blue (CBB) agar; pigment in Luria-Bertani broth using 5 g $l^{-1}$ NaCl ($LB_5$) at 15°C measured as optical density at 410 nm after 7 d growth; lipase detected on tributyrin agar; AHLs detected with *Chromobacterium violaceum* CV026, and *Agrobacterium tumefaciens* pZLR4. +: positive; (+): slightly positive; –: negative; +/–: majority positive; –/+: majority negative

| Strain | Source | Location | Protease | A-layer | Pigment | Lipase | AHL production CV026 | AHL production pZLR4 |
|---|---|---|---|---|---|---|---|---|
| ssp. *smithia* NCIMB 13210 | Roach | UK | + | + | 0.12 | – | – | – |
| ssp. *pectinolytica* DSM 12609 | River water | Argentina | + | – | 6.52 | + | + | + |
| NCIMB 1110 | Sea trout | Scotland | + | – | 0.59 | + | + | + |
| V179 | Wrasse | Scotland | – | – | 0.15 | – | + | – |
| 909/81 | Atlantic salmon | Norway | + | + | 1.46 | (+) | + | + |
| 2013/81 | Atlantic salmon | Norway | – | – | 3.04 | + | + | + |
| 1777/92 | Wolf fish | Norway | + | + | 6.80 | + | + | + |
| 2656/92 | Halibut | Norway | + | + | 1.96 | + | + | + |
| M45/89 | Arctic char | Iceland | + | + | 2.74 | – | + | – |
| S226/90 | Brown trout | Iceland | + | + | 3.10 | (+) | + | + |
| M283/89 | Atlantic salmon | Iceland | + | + | 3.08 | (+) | + | + |
| T233/91 | Atlantic cod | Iceland | + | + | 3.28 | (+) | + | + |
| T3-A1 | Haddock | Iceland | + | + | 2.64 | (+) | + | + |
| No. 1 | Sea trout | Finland | – | + | 1.73 | + | + | + |
| No. 2 | Brown trout | Finland | – | + | 0.09 | – | – | – |
| No. 3 | Grayling | Finland | – | + | 0.13 | – | – | – |
| 850319-1/4 | Sandeel | Denmark | – | – | 4.56 | (+) | + | + |
| 920225-1/2 | Eel | Denmark | – | + | 0.08 | + | – | – |
| 860613-1/1 | Atlantic salmon | Faroe Islands | – | –/+ | 3.02 | – | + | + |
| 329/89 | Atlantic salmon | Sweden | – | + | 0.24 | + | + | – |
| 298/89 | Arctic char | Sweden | – | + | 0.14 | (+) | + | + |
| 420/88 | Brown trout | Sweden | + | + | 1.83 | + | + | + |
| 261/89 | Brown trout | Sweden | – | + | 1.83 | (+) | + | + |
| 870626-1/1 | Blenny | Denmark | + | +/– | 5.08 | (+) | + | + |
| 911025-1/2 | Goldfish | Denmark | – | – | 1.13 | + | – | – |
| 911030-1/1 | Fourbed rockling | Denmark | + | – | 5.16 | – | + | + |

*N*-(heptylsulfanylacetyl)-L-homoserine lactone (HepS-AHL) (Persson et al. 2005) were dissolved in dimethylsulfoxide (DMSO) and stored at room temperature. *Aeromonas salmonicida* was grown with or without addition of 10 µM of ProS-AHL, PenS-AHL and HepS-AHL and 0.1% DMSO (solvent used as control) as described above. Samples were taken at regular intervals for determination of cell density ($OD_{450nm}$ and CFU $ml^{-1}$), pigmentation and protease level.

## RESULTS

### Characterization of typical and atypical *Aeromonas salmonicida*

AHLs were detected in all 31 of the typical strains and in 21 out of 26 (86%) of the atypical strains (Tables 1 & 2) using the monitor strains *Agrobacterium tumefaciens* and *Chromobacterium violaceum*. Three atypical strains were only AHL-positive in the *C. violaceum* monitor. Protease activity was detected in 26 out of 31 typical strains and in 13 out of 26 atypical strains. The formation of an A-layer was variable and some strains appeared on CBB-agar-plates with both A-layer positive and A-layer negative colonies. However, only 4 of 31 typical strains and 7 of 26 atypical strains were completely A-layer negative (Tables 1 & 2). All typical strains produced pigment in liquid cultures resulting in dark brown to nearly black cultures after 3 to 4 d of aerated incubation. The pigmentation was quantified by OD at 410 nm and after 7 d of growth, all of the typical strains showed pigmentation levels corresponding to 4.6 to 7.3 $OD_{410nm}$ (Table 1). The level of pigmentation among most of the atypical strains was much lower between 0.09 and 3.1 $OD_{410nm}$, except for the strains 1777/92, 850319-1/4, 870626-1/1, 911030-1/1 and ssp. *pectinolytica*, which all developed pigmentation to the same extent as the typical strains. Considering the lower growth rate of many of the atypical strains, pigmentation was also measured in these strains after 9 d of incubation in growth medium; however, this did not alter the pigmentation patterns described above. Of the typical strains, 29 were lipase positive, 19 of 26 atypical strains were positive and of these, several formed only a weak zone on tributyrin agar. All typical strains produced AHLs as detected by the 2 monitor strains, and all but 5 atypical strains produced AHLs. No apparent correlation between AHL production, or any of the potential extracellular virulence factors (protease, A-layer, pigment and lipase) could be detected (Tables 1 & 2).

### Influence of growth conditions on production of virulence factors

The aerated cultures of *Aeromonas salmonicida* strain 02-9-1 grew faster and to approximately 10-fold higher level of cell density as compared to those incubated under stagnant conditions (Fig. 1A). The average protease activity of the cultures was 0.45 $\Delta OD_{442nm}$ in the aerated cultures as compared to 0.13 $\Delta OD_{442nm}$ for the static cultures (Fig. 1B). However, considering the lower cell density of the static culture, this corresponds to a higher specific protease activity of the static cultures. No pigment could be detected in the static cultures after 96 h, whereas the aerated cultures were

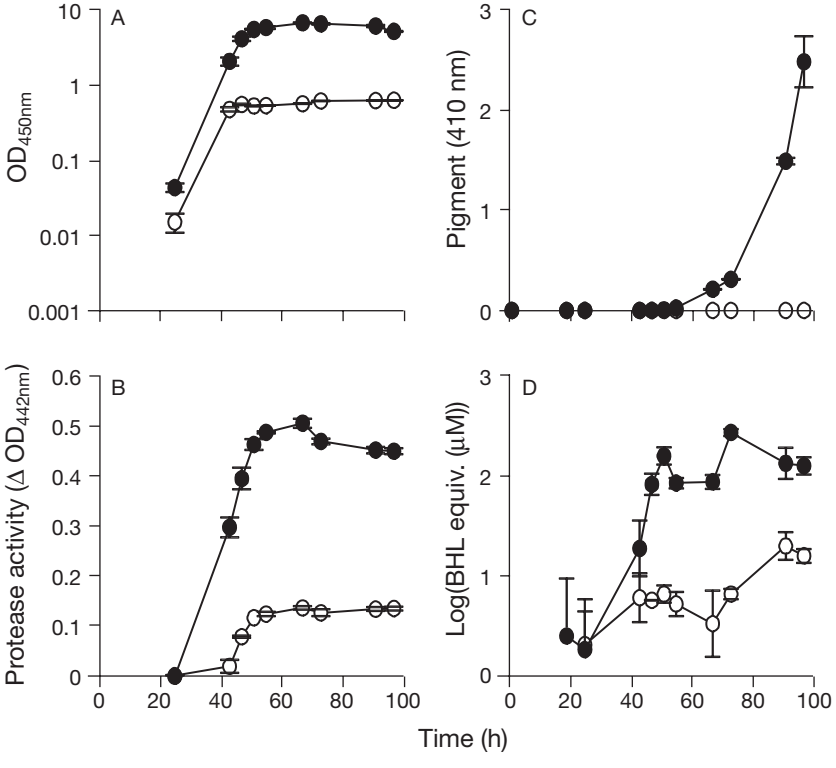


Fig. 1. *Aeromonas salmonicida* 02-9-1. Effect of aeration on (A) optical density (OD), (B) protease activity, (C) pigment production, and (D) acylated homoserine lactone (AHL) production when grown in Luria-Bertani broth (Bertani 1951) using 5 g $l^{-1}$ of NaCl ($LB_5$) at 15°C, either in static conditions (○) or aerated on a rotational shaker at 200 rpm (●). Values are means ± SD of double determinations. $\Delta OD_{442nm}$: change in OD at 442 nm; BHL: *N*-butanoyl homoserine lactone

very darkly pigmented with average pigmentation of 2.48 $OD_{410nm}$ measured on a sterile filtered culture supernatant (Fig. 1C). Even after prolonged incubation for up to 2 wk, pigmentation did not develop in the static cultures. According to the semiquantitative well diffusion assay, the concentration of AHLs reached a final level of approximately 5 µM $C_4$-HSL equivalents in the aerated cultures, whereas the final level of $C_4$-HSL equivalents in the static cultures was approximately 0.4 µM (Fig. 1D). Considering the difference in cell density of a factor 10, this indicates that the production of AHL per cell unit is similar under both growth conditions. pH remained at pH 7 in the static cultures but increased to approximately 8.1 after 96 h of growth under aerated conditions. After prolonged incubation (6 to 7 d) cell death and lysis was observed in the aerated cultures probably as a consequence of high pH (data not shown).

### Effect of QSI compounds on growth and protease activity

The effect of the QSI compounds ProS-AHL, PenS-AHL and HepS-AHL on exoprotease production was assessed in $LB_5$ broth at 15°C. None of the 3 compounds influenced growth of *Aeromonas salmonicida* 02-9-1 at concentrations of 10 µM (Fig. 2A). The protease activity in supernatants from *A. salmonicida* 02-9-1 grown without QSI compounds was 0.3 $\Delta OD_{442nm}$ after 40 h whereas the presence of QSI compounds resulted in a significant decrease in protease activity being 0.2, 0.09 and 0.04 $\Delta OD_{442nm}$ for *A. salmonicida* grown with ProS-AHL, PenS-AHL, and HepS-AHL, respectively (Fig. 2B). The magnitude of repression varied depending on the QSI compound; ProS-HSL caused a 1.5-fold decrease, PenS-HSL a 3.3-fold decrease, and HepS-AHL a 7.5-fold decrease. By the end of the experiment (92 h of incubation) the protease activity of the *A. salmonicida* culture treated with ProS-AHL had reached the level of the untreated controls (0.42 to 0.44 $\Delta OD_{442nm}$), whereas the protease activity of the cultures treated with PenS-AHL and HepS-AHL was lower (0.37 $\Delta OD_{442nm}$) (Fig. 2B). Zymograms of sterile filtered supernatants of *A. salmonicida* 02-9-1 growth revealed 5 bands with gelatinolytic activity of approximately 35, 40, 58, 91, and 104 kDa. When *A. salmonicida* 02-9-1 was grown in the presence of 10 µM HepS-AHL, 3 of these bands (35, 40, and 58 kDa) completely disappeared. The 40 kDa band was weak when *A. salmonicida* 02-9-1 was grown with 10 µM PenS-AHL as compared to the untreated control, and the bands of 35 and 58 kDa completely disappeared. After 50 h of growth there was no difference between the gelatinolytic bands for *A. salmonicida* 02-9-1 grown in the presence or absence of the QSI compounds (data not shown). Casein-containing zymogram gels revealed the presence of only one caseinolytic band in sterile filtered supernatants taken after 39 and 50 h of growth, with an approximately size of 52 kDa. This band was present both when *A. salmonicida* 02-9-1 was grown in the presence or absence of the QSI compounds. The 3 QSI compounds had no effect on the pigment production.

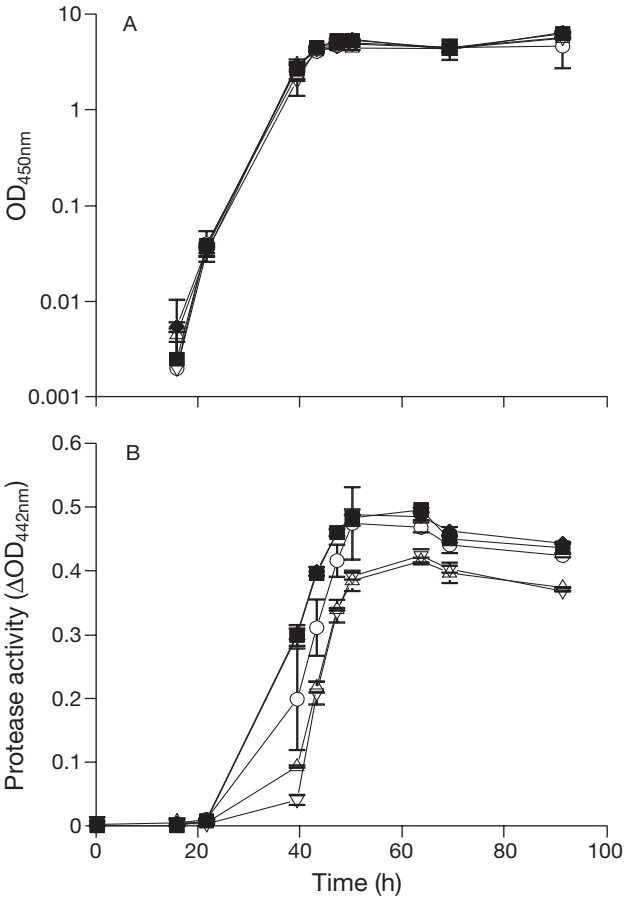


Fig. 2. *Aeromonas salmonicida* 02-9-1. Effect of the quorum sensing inhibitor compounds *N*-(propylsulfanylacetyl)-L-homoserine lactone (ProS-AHL), *N*-(pentylsulfanylacetyl)-L-homoserine lactone (PenS-AHL) and *N*-(heptylsulfanylacetyl)-L-homoserine lactone (HepS-AHL) on (A) optical density (OD) and (B) protease activity when grown in Luria-Bertani broth (Bertani 1951) using 5 g $l^{-1}$ of NaCl ($LB_5$) broth at 15°C. ■: *A. salmonicida* 02-9-1; ◆: 02-9-1 + 0.1% DMSO; ○: 02-9-1 + 10 µM ProS-AHL; △: 02-9-1 + 10 µM PenS-AHL; ▽: 02-9-1 + 10 µM HepS-AHL. Values are means ± SD of double determinations. $\Delta OD_{442nm}$: change in OD at 442 nm

## DISCUSSION

Quorum sensing signal molecules were detected in the vast majority of strains in a collection of both typi-

cal and atypical *Aeromonas salmonicida*. It has been known for some time that QS systems exist in *A. salmonicida* (Swift et al. 1997), but it has not yet been recognized as a widespread phenomenon which occurrs in most independent isolates. Also, AHL production was widespread in *Vibrio anguillarum* isolates, where 148 of 150 produced AHLs but no correlation between serotype, virulence or origin could be found (Buch et al. 2003). Recently, Bruhn et al. (2005) found AHL production in all 7 typical *A. salmonocida* strains investigated, but only in 2 of 10 atypical strains. Furthermore, the 2 atypical strains that produced AHLs were the only 2 atypical strains developing pigmentation and this leads us to hypothesize that AHL production could be involved in regulation of pigment production. However, all of 31 typical and 21 of 26 atypical strains produced AHLs, demonstrating that AHL production in *A. salmonicida* is widespread, and we did not, on a population basis, find any correlation between AHL production and pigmentation.

Pigmentation has previously been described as being an aerobic phenomenon (Aurstad & Dahle 1972, Donlon et al. 1983) and we did only detect pigment in the aerated *Aeromonas salmonicida* cultures. The brownish melanin-like pigments formed by several microorganisms are believed to be a protective mechanism against oxidative radicals of the oxidative burst (de Cassia & Pombeiro-Sponchiado 2005); hence, it is likely that aeration induces their production. D-glucose may repress pigment formation (Altmann et al. 1992, Koppang et al. 2000), but as all the experiments were performed in $LB_5$ broth, which does not contain glucose, this has not influenced the results. The majority of the atypical strains in this study have been examined for caseinolytic activity by Gudmundsdóttir (1996); except for 3 strains, our reporting of positive and negative strains corresponds to the earlier finding. We did not detect clearing zone on casein agar around 2013/18, 298/89 and 261/89, whereas Gudmundsdóttir (1996) reported low caseinolytic activity for these strains. However, different assays have been used and our casein agar may be slightly less sensitive than the azocasein assay used by Gudmundsdóttir (1996).

The extracellular protease activity was significantly lowered when *Aeromonas salmonicida* strain 02-9-1 was grown in the presence of the QSI compounds ProS-AHL, PenS-AHL and HepS-AHL. As none of these compounds altered growth rate or maximum cell density, the effect is not due to growth repression, and we have previously reported that the compounds do not per se act as protease inhibitors (Rasch et al. 2007). Therefore, the effect of the QSI compounds is believed to be an effect directly on the QS system. PenS-AHL and HepS-AHL also inhibited QS-regulated protease production in a *Pectobacterium* (Rasch et al. 2006). Although no apparent connection could be made between the ability to produce AHLs and virulence factors, the fact that known QSI compounds were able to inhibit the production of protease indicate that protease production is under QS regulation in *A. salmonicida*. Protease production has previously been described as QS-regulated in *A. salmonicida*, as Swift et al. (1997) observed that addition of exogenous $C_4$-HSL resulted in a small stimulation of protease production, and addition of the longer chained $C_{10}$-HSL and 3-oxo-$C_{10}$-HSL inhibited protease production. Also, protease production is QS-regulated in a number of other Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Serratia liquefaciens* and *Vibrio anguillarum* (Passador et al. 1993, Eberl et al. 1996, Croxatto et al. 2002). In *P. aeruginosa* the QS-regulated gene encoding LasB protease has been repressed by the QSI compounds C-30, patulin, penicillic acid, 4-nitro-pyridine-*N*-oxide and garlic extract (Hentzer et al. 2003, Rasmussen et al. 2005a,b).

Zymograms of supernatants from *Aeromonas salmonicida* 02-9-1 revealed 5 bands with gelatinolytic activity with molecular sizes of approximately 35, 40, 58, 91, and 104 kDa. Arnesen et al. (1995) detected at least 9 bands with molecular weights ranging from 37 to 100 kDa, and characterized a gelatinolytic metalloprotease from *A. salmonicida* subsp. *salmonicida* with a molecular weight of about 37 kDa. The major protease activity was found in the bands of 37, 89 and 100 kDa (Arnesen et al. 1995). Considering the accuracy of zymograms for determination of molecular weight it seems likely that these 3 bands correspond to the bands from *A. salmonicida* 02-9-1 of 35, 91 and 104 kDa, respectively. The 35 kDa protease disappeared when *A. salmonicida* was grown in the presence of PenS-AHL and HepS-AHL suggesting that this particular protease is QS-regulated. The protease from *A. salmonicida* previously described as QS-regulated was a serine protease (Swift et al. 1997) but the molecular weight of the molecule was not determined.

The strategy of blocking quorum sensing for control of fish disease has been explored in recent years. Rasch et al. (2004) showed that the QSI compound furanone C-30 reduced mortality of rainbow trout infected with *Vibrio anguillarum*, but were not able to explain the effect of C-30 on the identified QS system on the bacteria. *V. anguillarum* strains mutated in the AHL synthase genes were as virulent as the wild type strain when infecting trout (Milton et al. 1997) and the brine shrimp *Artemia franciscana* (Defoirdt et al. 2005), but the QS system of *V. anguillarum* is extremely complex (Milton 2006), and ensuring a complete inhibition of the QS system is difficult. A mutation in the AHL synthase gene of *Aeromonas hydrophila* did not have any effect on virulence towards *Artemia franciscana* (Defoirdt et al. 2005).

On a population level, AHLs do not appear to be important for regulation of virulence factors in *Aeromonas salmonicida* and this would indicate that QSI compounds are of little interest in furunculosis control. In contrast, our finding that QSIs can inhibit protease production in a strain of *A. salmonicida* indicates that further studies on the QS regulatory network, its potential involvement in virulence of *A. salmonicida* and the effects of QSI compounds should be pursued.

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