Quorum Sensing-Dependent Regulation and Blockade of Exoprotease Production in Aeromonas hydrophila

SIMON SWIFT,1,2* MARTIN J. LYNCH,1,2 LEIGH FISH,2† DAVID F. KIRKE,1,2 JUAN M. TOMÁS,3 GORDON S. A. B. STEWART,2 AND PAUL WILLIAMS1,2,4

Institute of Infections and Immunity1 and School of Clinical Laboratory Sciences,4 Queen’s Medical Centre, University Park, University of Nottingham, Nottingham, NG7 2UD, and School of Pharmaceutical Sciences, University Park, University of Nottingham, Nottingham, NG7 2RD,2 United Kingdom, and Departamento de Microbiología, Facultad de Biología, Universidad de Barcelona, 08071 Barcelona, Spain3

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In Aeromonas hydrophila, the ahyl gene encodes a protein responsible for the synthesis of the quorum sensing signal N-butanoyl-L-homoserine lactone (C4-HSL). Inactivation of the ahyl gene on the A. hydrophila chromosome abolishes C4-HSL production. The exoprotease activity of A. hydrophila consists of both serine protease and metalloprotease activities; in the ahyl-negative strain, both are substantially reduced but can be restored by the addition of exogenous C4-HSL. In contrast, mutation of the LuxR homolog AhyR results in the loss of both exoprotease activities, which cannot be restored by exogenous C4-HSL. Furthermore, a substantial reduction in the production of exoprotease by the ahyl− parent strain is obtained by the addition of N-aclyhomoserine lactone analogs that have acyl side chains of 10, 12, or 14 carbons. The inclusion of N-(3-oxododecanoyl)-L-homoserine lactone or N-(3-oxotetradecanoyl)-L-homoserine lactone at 10 μM in overnight cultures of A. hydrophila abolishes exoprotease production in azocasein assays and reduces the activity of all the exoprotease species seen in zymograms.

Aeromonas species are pathogens of humans and fish. Aeromonas salmonicida is the causative agent of furunculosis in salmonid fish, whereas Aeromonas hydrophila is responsible for motile aeromonad septicemia; both are significant problems in aquaculture (11). Importantly, interest in the pathogenesis of Aeromonas now extends beyond the economic consequences to the fish farming industry, as members of this genus are increasingly implicated in intestinal and extraintestinal infections in humans (54).

The virulence of Aeromonas spp. is multifactorial. Surface-associated factors include adhesins (e.g., pili), the S-layer, and lipopolysaccharide. Extracellular factors include siderophores for iron acquisition and an array of exoenzymes and exotoxins, i.e., enterotoxins, glycerophospholipid-cholesterol acetyltransferase (GCAT), hemolysins, lipases, and proteases (30, 33, 38, 54). Many of the proteins involved in pathogenicity are reliant on the general secretory pathway for export (16, 38).

The regulation of virulence determinants by pathogenic bacteria, such as Aeromonas, throughout the infection and transmission cycle is an important consideration for the etiology of disease. A major objective of an infecting bacterium is the evasion of host defenses. Hence the premature elaboration of an aggressive phenotype, which could be recognized by the host as the signal to elicit the induction of immune defenses, would constitute a poor strategy for a pathogen. Where the bacterium is able to evade host defenses and find a suitable niche, it can then proliferate to a level where the combined aggressive phenotype of the population is capable of overwhelming host defenses. In this respect, the regulation of gene expression by the process termed quorum sensing (12) can be used for a concerted activation of a modulon of genes coding for the components of an aggressive phenotype only when a bacterial population sufficient to make the phenotype effective is present. Quorum sensing relies on the release of a low-molecular-mass signalling molecule into the extracellular milieu (for reviews, see references 12, 13, 46, and 52). Accumulation of the signal (often an N-acylhomoserine lactone [AHL]) above a threshold concentration, indicative of a critical cell population density, activates the relevant gene expression. The system has been shown to regulate virulence and secondary metabolism in a number of gram-negative bacteria (for reviews, see references 12, 13, 46, and 52), where the AHL is produced by members of the LuxI family of synthases and recognized by the LuxR family of response regulators.

The discovery of AHL-based quorum sensing in Aeromonas (51) has placed our focus on this genus for the elucidation of its role in pathogenesis. A number of investigations of different strains of Aeromonas have demonstrated that exoprotease activity correlates with the establishment of infection (8, 9, 25, 44). There is, however, good evidence for A. salmonicida that protease is not an absolute requirement for pathogenicity. Vipond et al. (58) demonstrated that a defined protease mutant of a highly pathogenic strain of A. salmonicida exhibited no significant change in virulence. This study was of further interest because the protease-dependent activation of GCAT was also abolished (58).

The regulation of exoprotease activity by Aeromonas may be important because if it is elaborated too early, host defenses will be alerted and the bacterial infection may well be contained. Exoprotease production is therefore a likely candidate for quorum sensing-dependent regulation. A number of factors contribute data to this concept. Proteolytic activity is observed in the culture supernatant when cells are at high population density in the stationary phase of growth (6, 45), a phenome-
non closely associated with quorum sensing control. In *Pseudomonas aeruginosa* (15, 22) and *Erwinia carotovora* (22, 39), protease expression is positively regulated by quorum sensing.

To explore the role of quorum sensing in regulating exoproteases in *A. hydrophila*, we mutagenized *ahyI*, encoding the N-butanol-1-homoserine lactone (C4-HSL)-synthase of *A. hydrophila*, and *ahyR*, encoding a LuxR-type response regulator.

In this study, we show that quorum-sensing regulates both secretion and metalloprotease activities and demonstrate that exoprotease production can be blocked by C4-HSL analogs.

**MATERIALS AND METHODS**

**Strains.** *A. hydrophila* AH-1 is a spontaneous mutant of *A. hydrophila* AH-1 that is *S*-Slayer and the O-1 antigen while retaining other surface characteristics (57). *Escherichia coli* JM109 [recA1 endA1 gyrA96 thiR17 relA1] supE44 recA1 (Δlac–proAB–lacI) F′ proA34 proB4 lacZ ΔM15 (60) was used as the host for plasmids not requiring the xprI protein for replication. E. coli CC118 xprI (ΔxprI gyrA96 thiR17 recA1 ΔlacsX47 lacIΔM15 galK galU proA34 thiO rpsE rpoB argE DlacI159) was used as a permissive host for transcription. E. coli CC118 xprI (ΔxprI gyrA96 thiR17 recA1 ΔlacsX47 lacIΔM15 galK galU proA34 thiO rpsE rpoB argE DlacI159) was used as a permissive host for transcription.

**Construction of an A. hydrophila ahyR mutant strain.** Overlap extension PCR (17a) was used to generate an in-frame deletion of the *ahyR* gene on the *A. hydrophila* AH-1 chromosome. Two PCR fragments were generated from the template pAH17 with the primer pairs AHYR-1 (5′-GAGTACCTGACGATTTCCTGGCAG) and AHYR-3 (5′-CTTGAGTGAGCAACTATCCATCGGCGGCG) plus AHYR-4 (5′-GGAAGGTTTGTCGACCAAGCTTC). The resulting products contained a 315-bp fragment containing the 5′ end of *ahyR* and a 302-bp fragment containing the 3′ end of *ahyR*, respectively. A 17-bp overlap in their sequences (underlined) permitted amplification of a 600-bp product during a second PCR with primers AHYR-1 and AHYR-4. The resultant product contained a deletion from nucleotides 320 to 466 of *ahyR* (GenBank accession no. X98469) corresponding to AhyR amino acid residues 107 to 156 and was T-cloned into pUC57/T. DNA sequence sets were used to confirm that the cloned overlap extension PCR product was correct. The T-cloned PCR product was transferred to pDM4 as a suicide plasmid. In all experiments, the A. hydrophila strains were grown at 37°C. In cases of mixed cultures, e.g., conjugations, incubations were at 30°C.

**Plasmids.** pAH17 contains an active *ahyR* gene, confers ampicillin resistance, and has the pUC origin of replication (51). pAH17 is analogous to pAHH1 (51), pBScam, and an ampicillin- and chloramphenicol-resistant, AHL-negative derivative, pAH17 (5′-GAGTACCTGACGATTTCCTGGCAG) plus AHYR-2 (5′-CTTGAGTGAGCAACTATCCATCGGCGGCG) containing the pUC origin of replication (51). pAHH2 is analogous to pAHH1 (51), and has the pUC origin of replication (51). pAHH2 is analogous to pAHH1 (51).

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FIG. 1. Structures of AHL molecules used in this study. See text for definitions.

and the metalloprotease inhibitor EDTA was included at 10 mM (25). For quantitative hemolysin assay, doubling dilutions of sterile filtered culture supernatant were prepared in a U-bottomed microplate with phosphate-buffered saline, pH 7.2 (70 mM phosphate, 150 mM sodium chloride), to leave a 50-μl volume. Then 100 μl of 3% (vol/vol) sheep blood in phosphate-buffered saline was added to each and incubated at 37°C for 1 to 2 h, and the lowest dilution at which hemolysis occurred was recorded.

AHLs. The AHLs used in this study, C4-HSL, C6-HSL, C8-HSL, 3-oxo-C4-HSL, 3-oxo-C6-HSL, and 3-oxo-C8-HSL, were synthesized as described by Chhabra et al. (3). Figure 1 shows the structures of the major AHLs used in this study.

RESULTS

Exoprotease activity is abolished in A. hydrophila quorum sensing mutants. Agar plate assays were used to qualitatively screen candidate phenotypes regulated by quorum sensing in the ahyl mutant strains AH-1NahyI-1 to -6. All behaved the same, and AH-1NahyI-6 was taken as a representative for use in further assays. In this mutant, exoprotease activity on SMLB was substantially down-regulated, but amylase, nuclease, and lipase activities were unaffected and β-hemolysin activity was increased. Microplate assay of β-hemolysin demonstrated a twofold increase in activity in the mutant strain. Analysis of the API-20E profile of mutants AH-1NahyI-1 to -6 and parent showed that each mutant was unable to liquefy gelatin (a function of protease activity). The inclusion of the major AHL product of AhyI, C4-HSL, at 1 μM in SMLB and in API-20E assays restored the exoprotease activity and consequently the ability to both digest casein and liquefy gelatin.

The analysis of supernatant proteins on denaturing PAGE highlights a number of quorum sensing-dependent proteins (arrowed in Fig. 2A). The largest of these is also observable on nondenaturing PAGE (Fig. 2B) and is possibly multimerized aerolysin, formed in a process reliant upon protease activity (7, 18). The reduction in the number of protein species in the lanes where concentrated supernatants were loaded is likely due simply to proteolytic degradation. The resistance to protease of the large protein present in the stacking gel (Fig. 2B, lane 3) is further evidence for this protein being a multimerized form of aerolysin, as this has been reported to be resistant to proteolytic digestion (7, 18).

Analysis of supernatants on casein and gelatin zymograms (Fig. 2C and D) revealed a reduction in the number of bands exhibiting protease activity in the ahyl mutant. The analysis of protease activity in Aeromonas supernatants is complicated by the presence of the number of protease bands. Nieto and Ellis (32) suggested that these could represent both additional cryptic proteases and different forms of the same protease after casein overlay analysis of extracellular proteins separated by isoelectric focusing (32). After PAGE performed under nondenaturing conditions equivalent to those used in the zymog-
quirorophila AH-1N and the A. hydrophila mutant strain cultured with C4-HSL we see a protein band migrating with the 60-kDa marker that is most probably the A. hydrophila serine protease. Denatured, prestained markers were run with a gap of at least one lane to sample proteins to minimize any effect of reducing agents present in the markers. Protease bands at 60 kDa are also associated with A. hydrophila and the 60-kDa marker that is most probably the mutant strain cultured with C4-HSL we see a protein band (Fig. 2B), in concentrated supernatants from the ahyI mutant strain AH-1N and the A. hydrophila migrating with the 60-kDa marker that is most probably the mutant strain cultured with C4-HSL in both casein and gelatin zymograms (Fig. 2C and D) but are absent in the A. hydrophila AH-1N ahyI mutant and less intense in A. hydrophila AH-1N cultured with 3-oxo-C12-HSL.

For quantitative assay of ahyI-regulated exoprotease activity in A. hydrophila, assays using culture supernatants from AH-1N ahyI-6 and the isogenic parent were performed after overnight growth in LB at 30°C in the presence of 0, 1, and 10 μM C4-HSL. Although little effect is seen on the parent, a dose response-dependent restoration of exoprotease activity is seen in the ahyI mutant (Fig. 3A). As exoprotease production in A. hydrophila is population density dependent, we also assayed exoprotease activity in the supernatant throughout growth to the stationary phase. The exoprotease induction profiles from the parent and from the mutant grown in the presence of 1 μM C4-HSL are similar, but little activity is detected in the ahyI mutant in the absence of C4-HSL (Fig. 4A). Furthermore, this experiment shows that the exogenous provision of the quorum sensing signalling molecule does not immediately induce any detectable increase in exoprotease production.

The exoprotease activity of the A. hydrophila ahyR mutant matches that of the ahyI mutant strain in agar plate assays for exoprotease, lipase, hemolysin, nuclease, and amylase activities. Analysis of exoprotease activity from supernatants of overnight A. hydrophila cultures in azocasein assays (Fig. 5) demonstrates that the A. hydrophila AH-1N ahyR mutant is substantially reduced in its ability to produce exoprotease activity. Complementation of the ahyR mutation with a plasmid encoding the ahyR gene restores approximately 50% of protease production (Fig. 5). We cannot explain why full complementation is not obtained but believe that this may be an effect of the introduction of multiple copies of ahyR uncoupled from its normal regulation into A. hydrophila.

The effect of the ahyR mutation on protease production does not appear to be due to an effect on AHL production. C. violaceum CV026 T-streaks show that the A. hydrophila AH-1N ahyR mutant and A. hydrophila AH-1N produce similar levels of AHL after overnight incubation (data not shown) and that the exogenous addition of 5 μM C4-HSL to the A. hydrophila AH-1N ahyR mutant does not restore any protease production (data not shown).

Metalloprotease and serine protease activities are under quorum sensing control. Previous studies have demonstrated that A. hydrophila possesses both serine protease and metalloprotease activities (25). To investigate the contribution of quorum sensing to the activation of both activities in A. hydrophila, exoprotease activity was assayed in the supernatant of overnight cultures of the ahyI-negative mutant AH-1NahyI-6 induced with 1 μM C4-HSL. In the presence of 10 mM EDTA (metalloprotease inhibitor), 1 mM PMSF (serine protease inhibitor), or 10 mM EDTA plus 1 mM PMSF, it was demonstrated that both activities were activated by C4-HSL (Fig. 3B). Serine protease accounted for approximately 60% of the induced activity, while metalloprotease accounted for approximately 30%. The residual 10% activity is presumably attributable to the azocaseinolytic activity of one or more of the additional exoproteases observed in zymography (Fig. 2C and D). Further evidence for the control of both protease activities

was obtained from SDS-PAGE analysis of the TCA-precipitable exoproducts. A. hydrophila AH-1N ahyI-6 lacks proteins at approximately 35 kDa and approximately 65 kDa that are present in the wild type and in the mutant cultured with 1 μM C4-HSL (Fig. 2A). The molecular masses of these proteins

FIG. 3. Exoprotease production by A. hydrophila AH-1N and AH-1NahyI-6. (A) Response to C4-HSL (added to give 0, 1, or 10 μM) in the culture medium of A. hydrophila AH-1N (solid bars) and AH-1NahyI-6 (open bars); (B) ratio of serine protease and metalloprotease activities. The exoprotease activity induced by 1 μM C4-HSL in A. hydrophila AH-1NahyI-6 supernatant and that remaining after inhibition with 1 mM PMSF (due to metalloprotease), 10 mM EDTA (due to serine protease), or 1 mM PMSF and 10 mM EDTA are shown in panel B. In both panels, n = 3 and error bars represent 1 standard deviation.
correspond to the published sizes of the serine protease (70 kDa [25, 42]) and metalloprotease (35 kDa [25, 41]) of *A. hydrophila*.

Under suitable culture conditions, protease production by *A. hydrophila* at a high population density occurs at 22 and 30°C; however, a growth temperature of 37°C inhibits exoprotease production (27, 36). *A. hydrophila* AH-1N and the *ahyI* mutant AH-1N*ahyI*-6 were incubated in LB overnight at 22, 30, and 37°C. In line with previous studies (27, 36), no protease expression was seen at 37°C, and the addition of 1 μM C4-HSL to either parent or mutant failed to stimulate activity (data not shown). Nevertheless, *C. violaceum* CV026 T-streak experiments showed that *A. hydrophila* AH-1N does produce C4-HSL at 37°C, and the serine protease and metalloprotease are active in assays at 37°C, demonstrating that protease production is inhibited at 37°C in a quorum sensing-independent manner.

**Inhibition of exoprotease activity by quorum sensing blocking.** Empirical studies with AHL analogs of the natural ligands for LuxR, LasR, and CarR have shown certain compounds to be antagonistic (3, 37, 48, 61). The application of this antagonism to the induction of pigment by *C. violaceum* CV026 by 3-oxo-C6-HSL has been used as an assay for long-chain (C_8-10) AHLS (28) and in the characterization of 3-oxo-C10-HSL, the AHL produced via VanI in *Vibrio anguillarum* (31). In *A. salmonicida*, 3-oxo-C10-HSL has been shown to antagonize both the time of induction and final level of exoprotease (51). In this study, we demonstrate that 3-oxo-C10-HSL has the same inhibitory effect on exoprotease production by *A. hydrophila* (Fig. 4B). To further investigate this phenomenon, we analyzed the antagonistic effects of a range of C4-HSL analogs toward exoprotease production by *A. hydrophila* (Fig. 6A) and the *ahyI* mutant AH-1N*ahyI*-6 (Fig. 6B). Exoprotease activity in the supernatant was assayed against azocasein after overnight incubation at 30°C in the presence of AHLS. Results consistently showed that AHLS with an acyl chain of C_{10}, C_{12}, or C_{14} at 10 μM antagonized protease production and that for the parent strain, 3-oxo-C12-HSL and 3-oxo-C14-HSL almost totally inhibited protease production (Fig. 6A). AHLS with acyl chains of C_6 and C_8 had little or no antagonistic activity in equivalent experiments (data not shown). Denaturing and nondenaturing PAGE and zymography also demonstrate the activity of 3-oxo-C12-HSL upon the expression of *Aeromonas* exoproteases (Fig. 2).

**DISCUSSION**

Mutagenesis of the *ahyI* and the *ahyR* genes abolishes the ability of *A. hydrophila* to produce C4-HSL, which in turn substantially reduces exoprotease production. Addition of exogenous C4-HSL restored exoprotease production to the *ahyI* mutant but not to the *ahyR* mutant. The serine protease(s) *A. hydrophila* and *A. salmonicida* secrete is via the general secretory pathway (16, 38). We have previously shown that unlike the case of *P. aeruginosa* (2), the general secretory pathway of *A. salmonicida* (*exe*) is not under the control of quorum sensing (51). In this study we show that apart from exoproteases, the
secretion of proteins using the Exe system, e.g., aerolysin, amylase, and lipase (16), is unaffected by the ahyI mutation, implying that the regulation of protease production by quorum sensing is direct. Further work in A. hydrophila is required to prove this assumption, as the ahyRI system may control expression of a regulatory protein that directly activates transcription of the genes encoding the exoprotease activities.

This work shows that both serine protease and metalloprotease activities are under quorum sensing control and that the inhibition of protease production at low cell population densities and at 37°C (in both ahyI mutant and parent strain) cannot be recovered simply by the addition of C4-HSL. PAGE analysis of A. hydrophila and A. hydrophila ahyI mutant culture supernatant highlights a number of quorum sensing-dependent proteins (Fig. 2A). Identification of some of these requires further work; however, the high-molecular-mass band that barely migrates into the gel is likely to be a multimerized form of the pore-forming toxin aerolysin, absent from the ahyI mutant because of the requirement for proteolytic processing during multimerization (7, 18). No other regulated traits were observed, although we detected an increase in the activity of β-hemolysin, as has been noted previously for protease mutants of A. hydrophila (1). The molecular basis of this phenomenon is still to be elucidated (1).

There is considerable evidence implicating exoprotease activity as a factor in the virulence of Aeromonas. Histopathology studies have revealed tissue damage associated with proteolytic activity (8, 44), and the injection of exoprotease can re-create certain aspects of the pathology of an Aeromonas infection (44). In experimental animal models, protease-null mutants of both A. hydrophila and A. salmonicida exhibit reduced virulence (25, 44), although recent work with defined mutants of A. salmonicida contradicts this finding (58). Nevertheless, the evidence suggests a role for exoprotease in the establishment of infection. This role is consistent with the correlation between the increasing levels of exoprotease inhibitors in fish serum and the decreasing susceptibility to furunculosis seen in comparisons of rainbow trout (Oncorhynchus mykiss), atlantic salmon (Salmo salar), and brown trout (Salmo trutta) (9). Furthermore, a comparison of the furunculosis-resistant rainbow trout with the furunculosis-sensitive brook trout (Salvelinus fontinalis) demonstrated a 10-fold reduction in the levels of α2-macroglobulin (10). The exoprotease of A. salmonicida has been proposed as a candidate vaccine target, and its effective use in fish vaccination trials provides practical evidence for a key role in pathogenicity (4).

The regulation of exoprotease activity is therefore important, for if it is expressed too early, effective host defenses will be induced and the infection will most probably be contained. Indeed, null mutations in the S-layer (33), protease (58), hemolysin (59), and GCAT (58) suggest that while Aeromonas spp. can be effective pathogens when lacking one or more secreted virulence factors, a significant reduction in pathogenicity occurs when the ability of the bacterium to evade host defenses is compromised. The tight regulation of virulence gene activation by quorum sensing is seen, for example, in Agrobacterium (20, 34, 62) and Erwinia (5), where it can be proposed to prevent host alert through prevention of exoenzyme production at low cell numbers. A similar situation may exist in Aeromonas (Fig. 4A), and the role of quorum sensing may simply be to rapidly induce the expression of certain virulence factors when a significant population density has been achieved.

Mechanisms have been described whereby information about, for example, ambient temperature (19, 29) and oxygen tension (21, 50) can be transduced and effect changes in gene expression. Prevailing growth environment with both nutrient deprivation and growth rate influencing expression (36) regulates the synthesis of protease. Exoprotease production is therefore dependent on the particular environmental stimuli such as iron, nitrogen availability, temperature, pH, oxygen concentration but in general does not occur until a high cell population density is achieved (1, 6, 14, 26, 27, 36, 40). The integration of these regulatory networks at the corresponding protease promoters with quorum sensing signals is, therefore, an important question for the future.

The potential of blocking quorum sensing to control viru-
ulence and hence prevent infection by *A. hydrophila* was examined in empirical studies where C4-HSL analogues were added to cultures of *A. hydrophila*. The inhibition of protease activity by the AHLs 3-oxo-C12-HSL and 3-oxo-C14-HSL is an indication of the potential value of the blockade of quorum sensing. Interestingly, a second fish pathogen, *V. anguillarum*, produces 3-oxo-C10-HSL, which we have shown has antagonistic activity toward the protease activities of both *A. hydrophila* (Fig. 4B and 6) and *A. salmonicida* (51). Therefore, a role for this molecule in nature might be to antagonize quorum sensing-dependent virulence in *Aeromonas* and perhaps provide *V. anguillarum* with a competitive edge.

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REFERENCES


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