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Quorum Sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: Identification of the LuxRI Homologs AhyRI and AsaRI and Their Cognate *N*-Acylhomoserine Lactone Signal Molecules

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Spent culture supernatants from both *Aeromonas hydrophila* and *Aeromonas salmonicida* activate a range of biosensors responsive to *N*-acylhomoserine lactones (AHLs). The genes for a quorum sensing signal generator and a response regulator were cloned from each *Aeromonas* species and termed *ahyRI* and *asaRI*, respectively. Protein sequence homology analysis places the gene products within the growing family of LuxRI homologs. *ahyR* and *asaR* are transcribed divergently from *ahyI* and *asaI*, respectively, and in both *Aeromonas* species, the genes downstream have been identified by DNA sequence and PCR analysis. Downstream of both *ahyI* and *asaI* is a gene with close homology to *iciA*, an inhibitor of chromosome replication in *Escherichia coli*, a finding which implies that in *Aeromonas*, cell division may be linked to quorum sensing. The major signal molecule synthesized via both AhyI and AsaI was purified from spent culture supernatants and identified as *N*-(butanoyl)-L-homoserine lactone (BHL) by thin-layer chromatography, high-pressure liquid chromatography analysis, and mass spectrometry. In addition, a second, minor AHL, *N*-hexanoyl-L-homoserine lactone, was identified. Transcriptional reporter studies with *ahyI*::*luxCDABE* fusions indicate that AhyR and BHL are both required for *ahyI* transcription. For *A. salmonicida*, although the addition of exogenous BHL gives only a small stimulation of the production of serine protease with comparison to the control culture, the incorporation of a longer-chain AHL, *N*-(3-oxodecanoyl)-L-homoserine lactone, reduced the final level (by approximately 50%) and delayed the appearance (from an A_{650} of 0.9 in the control to an A_{650} of 1.2 in the test) of protease in the culture supernatant. These data add *A. hydrophila* and *A. salmonicida* to the growing family of gram-negative bacteria now known to control gene expression through quorum sensing.

Quorum sensing (19) is a mechanism for controlling gene expression in response to an expanding bacterial population. In many gram-negative bacteria, the diffusible quorum sensing signal molecule is a member of the *N*-acylhomoserine lactone (AHL) family (18, 45, 51). Accumulation of this molecule above a threshold concentration, through the activity of a signal generator protein, indicates that the population has reached a minimum population size, and the appropriate target gene(s) is activated via the action of a member of the LuxR family of transcriptional activators (18, 45, 51). The LuxR protein appears to consist of two domains containing an AHL binding site within the N-terminal domain and a helix-turn-helix DNA binding motif located within the C-terminal domain (10, 22).

In general, the signal generator proteins responsible for the synthesis of AHLs belong to the LuxI family, the archetypal member of which was originally identified within the *Vibrio* (*Photobacterium*) *fischeri lux* operon as the gene product responsible for the synthesis of *N*-(3-oxohexanoyl)homoserine lactone (13, 14, 16). However, AHL synthesis is not always directed by a LuxI homolog. In both *V. fischeri* and *Vibrio harveyi*, synthesis of the AHLs *N*-octanoylhomoserine lactone and *N*-(3-hydroxybutanoyl)homoserine lactone is driven by the putative AHL synthases AinS (21) and the homologous LuxMN (5), respectively, which do not have homology with the LuxI family. A feature of many quorum sensing systems is the inclusion of the signal generator gene within the regulon that is subject to quorum sensing control (18, 45, 51). The positive feedback which can then occur is pivotal to the rapid amplification of the signal and thus the other gene products of the quorum sensing regulon (18, 45, 51).

AHL-based quorum sensing has been identified in a wide range of gram-negative bacteria (18, 45, 51). The elucidation of this mechanism in the control of bioluminescence in *V. fischeri* (14, 16, 23) and the regulation of virulence and secondary metabolism in *Erwinia carotovora* (3, 27, 42), *Pseudomonas aeruginosa* (29, 30, 40, 41, 61), and *Chromobacterium violaceum* (30, 53, 60) has facilitated the construction of AHL signal molecule biosensors. Since reporter gene expression is activated only in the presence of exogenously supplied AHL, these constructs enable identification of AHL producers through cross-streaking and supernatant assays. Furthermore, they permit the screening of genomic libraries in *Escherichia coli* for AHL signal generator clones (52).

Aeromonas spp. are gram-negative rods belonging to the family *Vibrionaceae*; they are both primary and opportunistic pathogens of mammals and fish (26, 36) and possess a range of virulence determinants, including α-hemolysin (32), glycero-

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TABLE 1. Bacterial strains, plasmids and cosmids used

Strain, plasmid, or cosmids	Relevant genotype and phenotype	Source or reference(s)
Bacterial strains		
A. hydrophila A1	Natural isolate	Juan Tomas
A. salmonicida NCIMB 1102	A-layer-negative strain, laboratory maintained	NCIMB collection
A. salmonicida NCIMB 1102 *exeL*::*lacZ*	Chromosomal insertion of *exeL*::*lacZ* created by conjugation of p4E7 *exeL*::*lacZ* into *A. salmonicida*, Km^r Tc^s	
A. salmonicida MT1326	A-layer-positive, virulent strain	SOAFD collection
C. violaceum CV0blu	Triple mini-Tn*5* mutant derived from *C. violaceum* ATCC 31532. AHL biosensor Hg^r *cviI*::Tn*5xylE* Km^r Cm^r, plus spontaneous Sm^r and another uncharacterized mutation allowing a stronger response to AHLs	35, 53, 60
E. coli JM109	F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15/*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*) *mcrA*	63
Plasmids		
pAHH1	AHL biosensor-activating *A. hydrophila* clone; contains an 11-kb *Hin*dIII fragment in pUCP18, Ap^r	This study
pAHP1	AHL biosensor-activating *A. hydrophila* clone; contains two noncontiguous *Pst*I fragments in pUC18*Not*, Ap^r	This study
pAHP2	AHL biosensor-activating *A. hydrophila* clone containing a 2.8-kb *Pst*I fragment from pAHP1 cloned into pSU8, Cm^r	This study
pAHP3	0.2-kb *Bam*HI deletion of *ahyI* in pAHP2, AHL negative, Cm^r	This study
pAHP8	1.43-kb *Xho*I-*Pst*I fragment of pAHP2 cloned into pSU8 *Sal*I-*Pst*I, AHL negative, Cm^r	This study
pAHP9	1.38-kb *Xho*I-*Pst*I fragment of pAHP2 cloned into pT7blue, AHL positive, Ap^r	This study
pAHP13	2.75-kb *Pst*I-*Eco*RI fragment from pAHP2 cloned into pUC19, AHL positive, Ap^r	This study
pAHP14	213-bp (encoding the N-terminal 71 amino acids) *Nde*I fragment deleted from *ahyR* in pAHP13, Ap^r	This study
pAHR1	AHL biosensor-activating *A. hydrophila* clone containing a 10-kb *Eco*RI fragment in pBluescriptSK+II, Ap^r	This study
p*ahyRI′*::*lux*	*luxCDABE Pst*I cassette from pSB390 cloned into the *Pst*I site of pAHP13, AHL positive, Ap^r	This study
p*ahyRI″*::*lux*	*luxCDABE Bam*HI cassette from pSB390 cloned into the *Bam*HI site of *ahyI* in pAHP13, AHL negative, Ap^r	This study
p*ahyR*Δ*NI″*::*lux*	*luxCDABE Bam*HI cassette from pSB390 cloned into *ahyI* on pAHP14, AHL negative, Ap^r	This study
pASP3.7	3.7-kb *Pst*I fragment from p7B9 cloned into pUC19, AHL positive, Ap^r	This study
pASE9	9-kb *Eco*RI fragment from p7B9 cloned into pUC19, AHL positive, Ap^r	This study
pBluescript SK+II	pUC-derived multicloning site phagemid, Ap^r	Stratagene
pSB390	*luxCDABE* cassette containing vector; pACYC184 derived, Tc^r	62
pSB401	AHL biosensor *luxR luxI* (*Photobacterium fischeri* [ATCC 7744])::*luxCDABE* (*Photorhabdus luminescens* [ATCC 29999]) fusion; pACYC184 derived, Tc^r	62
pSB403	Broad-host-range AHL biosensor *luxR luxI* (*Photobacterium fischeri* [ATCC 7744])::*luxCDABE* (*Photorhabdus luminescens* [ATCC 29999]) fusion; pRK415 derived, Tc^r	62
pSB406	AHL biosensor *vsmR vsmI* (*Pseudomonas aeruginosa* PAO1)::*luxCDABE* (*Photorhabdus luminescens* [ATCC 29999]) fusion; pBR322 derived, Ap^r	62
pSU8	Multicloning site vector, pACYC184 derived, Cm^r	4
pSU20	Multicloning site vector, pACYC184 derived, Cm^r	4
pT7blue	High-copy-number cloning vector, pBR322 derived, Ap^r	Novagen
pUC18 and pUC19	High-copy-number pBR322-derived cloning vectors, Ap^r	63
pUC18*Not*	pUC18 with *Not*I sites flanking multicloning site, Ap^r	11
Cosmids		
p4E7	pLAFR3 cosmid clone containing *A. salmonicida exe* region, Tc^r. The ends of the cloned DNA are downstream of *tyrS* and *cdpB* homologs.	28
p4E7 *exeL*::*lacZ*	p4E7 containing Tn*B20* (48) *lacZ* fusion in *exeL*, Tc^r Km^r	This study
p5E3	pLAFR3 cosmid clone containing *A. salmonicida exe* and *asa* regions, AHL positive, Tc^r. The ends of the cloned DNA are downstream of *iciA* and within *uhpA* homologs.	28; this study
p7A3	pLAFR3 cosmid clone containing *A. salmonicida exe* and *asa* regions, AHL positive, Tc^r. The ends of the cloned DNA are within *mtlA* and *exeF* homologs.	28; this study
p7B9	pLAFR3 cosmid clone containing *A. salmonicida asa* region, AHL positive, Tc^r. The ends of the cloned DNA are within *mtlR* and *dppA* homologs.	28; this study

phospholipid-cholesterol acyltransferase (33, 34), lipase (2), and serine protease (58). The expression of several of these exoproducts is associated with high cell densities in the late exponential/stationary phase (2, 32–34, 58), and they therefore represent putative phenotypes for control by quorum sensing.

In this study, we report the identification of an AHL (*N*-butanoyl-L-homoserine lactone [BHL]) and the cognate LuxRI homologs AhyRI and AsaRI from *Aeromonas hydrophila* and *Aeromonas salmonicida*, respectively. We also present evidence for the quorum sensing-dependent regulation of *ahyI* in *A. hydrophila* and serine protease in *A. salmonicida*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Table 1 describes the bacterial strains, plasmids, and cosmids used in this study. Growth was on tryptone soya medium (Oxoid) containing the appropriate antibiotics at the given temperature, except

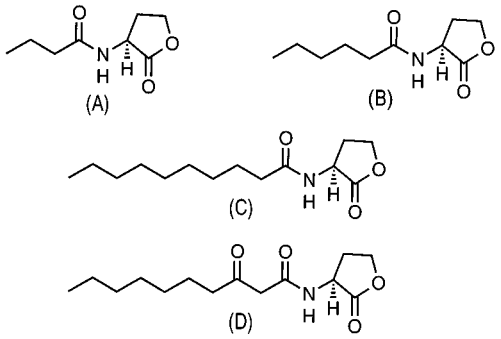

FIG. 1. *N*-Acylhomoserine lactone structures. (A) BHL; (B) HHL; (C) DHL; (D) ODHL.

where tryptone soya buffered with Davis minimal medium (37) (TSD), M9 minimal medium (46) supplemented with 0.2% acid hydrolyzed casein (ACH; Oxoid), or modified Griffin's liquid medium (MGLM) (39) is specified. Agar plates and top layer agars contained 1.5 and 0.7% no. 1 agar (Oxoid), respectively.

DNA manipulation. Genomic DNA was purified as described by Swift et al. (52); plasmid DNAs were isolated by alkaline lysis as described by Sambrook et al. (46) and further purified by using cesium chloride gradients (46) or Qiagen plasmid preparation columns. Restriction enzyme digestions and DNA ligations were performed as instructed by the manufacturer (Promega, Boehringer Mannheim, or Pharmacia). Southern hybridizations were carried out as described previously (55). DNA sequencing was performed by the University of Nottingham Automated Sequencing Facility or as described by Karlyshev and MacIntyre (28). Oligonucleotides were synthesized by the Biopolymer Synthesis and Analysis Unit, University of Nottingham, or by GenoSys. PCR products for sequencing were purified from agarose gels by using Qiaquick PCR purification kits (Qiagen). Standard PCR amplifications were performed as described previously (44). Long-range PCRs were performed by using the Expand Long Template PCR system (Boehringer Mannheim) with amplimers specific for *exeC* (5′GTCTGGAGCACTACTTCGCCC), *yrfH* (5′GTCTGGAGCACTACTTCGCCC), and *asaI* (5′AAGACGCGATTGCGAAAGCG). RNA was isolated from 10^9 bacterial cells by using an RNeasy kit (Qiagen). DNA contamination was removed by treatment with RNase-free DNase (Boehringer Mannheim). For reverse transcriptase (RT)-mediated PCR (RT-PCR) (17), template cDNA was made by using total RNA extracted from cultures with 0.2 to 0.3 A_{600} increments in the range of 0.2 to 2.1 U, reverse transcribed with avian myeloblastosis virus RT (Promega) with an *aspA* (5′GTGCCTACGCTGGAGTAGGAAG)- or *exeD* (5′AGCCCAGTTGCCATGGTAG)-specific primer, and subjected to 25 cycles (nonsaturating conditions) of standard PCR (44) with amplimers specific to *aspA* (5′GATGACAACATCCAGCAGTTC and 5′GTGCCTACGCTGGAGTAGGAAG) or *exeD* (5′CATCAGCCGAGATGGCCAGC and 5′CGATCAGGAAGTGGACATCATC). No PCR product was detected with negative controls (RNA without RT treatment), indicating the absence of interference from possible DNA contamination of RNA. PCR products were quantified by agarose gel electrophoresis (46).

Analysis of DNA sequences. Comparisons of DNA sequences and deduced protein primary sequences were performed by using the Biotechnology and Biological Sciences Research Council-funded Seqnet. The OWL nonredundant protein database searched by using NEWSWEEP 2.00 (developed by Alan Bleasby) was used to analyze protein sequences. FASTA (12) was used to analyze DNA sequences.

AHL bioassays. AHL production was detected by cross-streaking against biosensor strains (Table 1). Positive assays were judged as induction of the purple pigment violacein in the *C. violaceum* CV0blu reporter (35) or the induction of bioluminescence in *E. coli* JM109 carrying plasmid pSB401 or pSB406 (52, 61), detected with a Hamamatsu Argus 100 Vim3 (Hamamatsu Photonics U.K. Ltd., Enfield, England) or a Berthold LB980 (E.G. & G. Berthold U.K. Ltd., Milton Keynes, England) photon video camera. The presence of AHLs in filter (0.25-μm pore size)-sterilized conditioned media or in high-pressure liquid chromatography (HPLC) fractions was detected by a 1:1 mixing of the biosensor strain (as a 1:10 dilution of an overnight culture with fresh broth) with the test culture supernatant. Pigment production by *C. violaceum* CV0blu was scored after overnight incubation at 30°C. Bioluminescence was determined after 4 h of incubation at 30°C, with readings taken with either a Berthold LB980 photon video camera or an Amerlite microplate luminometer (Kodak Ltd., Hertfordshire, England). Recombinant *E. coli* libraries were screened for the presence of a LuxI homolog either by toothpicking into *C. violaceum* CV0blu overlays or by cross-streaking CV0blu against 24-h growth on plates of *E. coli* JM109 transformants. Alternatively, plasmid-based libraries were introduced into *E. coli* JM109 (pSB401) by electroporation (24), and the recombinant clones obtained were examined for bioluminescence. In both cases, incubation was at 30°C.

Purification and characterization of AHLs. Spent supernatants (6 liters) from stationary-phase cultures of *A. hydrophila* A1 (Table 1; grown in M9 plus ACH), *A. salmonicida* NCIMB 1102 (Table 1; grown in MGLM), and *E. coli* JM109 transformed with the recombinant plasmid (grown in M9 plus ACH) pAHH1 (which carries the *A. hydrophila ahyI* gene [Table 1]) or pASP3.7 (which carries the *A. salmonicida asaI* gene [Table 1]) were extracted three times with dichloromethane (700:300 supernatant/dichloromethane). The dried extract was reconstituted in acetonitrile, and then samples were subjected to analytical and preparative thin-layer chromatography (TLC) and preparative HPLC. TLC analysis was carried out essentially as developed by Shaw et al. (47a) on C_{18} reverse-phase chromatography plates (catalogue no. 4803 800; Whatman), using 60% (vol/vol) methanol in water as the mobile phase. AHLs were identified by overlaying the chromatograms with a thin layer of LB top layer agar (142.5 ml) seeded with CV0blu (7.5-ml overnight culture) (35). Plates were incubated at 30°C overnight and examined for purple spots. Tentative identification of AHLs can be made by comparing the R_f values of the positive sample spots with those of synthetic AHL standards. For preparative TLC, samples were separated as described above and the silica matrix at the relevant R_f was collected. AHLs were extracted from the TLC matrix three times with 2 ml of acetone and evaporated to dryness. For preparative HPLC, samples were separated by using a Kromasil KR100-5C8 (250 by 8 mm) reverse-phase column (Hichrom, Reading, England) with an isocratic mobile phase of 70% (vol/vol) acetonitrile in water at a flow rate of 2 ml per min and monitored at 210 nm. Fractions showing activity in the CV0blu reporter assay were pooled and rechromatographed by using 60% (vol/vol) acetonitrile in water; the procedure was repeated, using a final chromatographic separation employing 35% (vol/vol) acetonitrile in water. Active fractions with the same retention times were pooled and analyzed by mass spectrometry (MS) on a V.G. 70-SEQ instrument (Fisons Instruments, VG Analytical, Manchester, England). Samples were ionized by positive-ion fast atom bombarbment (FAB), and the molecular ion (M + H) peaks recorded by FAB-MS were further analyzed by tandem MS (MS-MS).

Synthesis of AHLs. The general method described by Chhabra et al. (9) was used to synthesize BHL (Fig. 1A), *N*-hexanoyl-L-homoserine lactone (HHL) (Fig. 1B), *N*-decanoyl-L-homoserine lactone (DHL) (Fig. 1C), and *N*-(3-oxodecanoyl)-L-homoserine lactone (ODHL) (Fig. 1D). Each compound was purified to homogeneity by semipreparative HPLC, and its structure was confirmed by MS and proton nuclear magnetic resonance spectroscopy (3, 9, 61).

Bioluminescent reporter assays. The relationship between light emission and growth phase was determined as described by Swift et al. (52). Where indicated, BHL was added to a final concentration of 1 μM.

Serine protease assays. *A. salmonicida* strains were diluted 1:100 after overnight growth in TSD broth at 25°C (NCIMB 1102) or 20°C (MT1326) into TSD broth and TSD broth containing 10 μM BHL, DHL, or ODHL. Aliquots of culture supernatant were assayed for serine protease activity, using azocasein as the substrate (6), at increasing culture optical densities, with product accumulation measured at 436 nm (6). Serine protease activity was expressed in units as the change in absorbance at 436 nm per minute per 0.1 ml of culture supernatant. To ensure that the activity of the AspA serine protease was being measured, an *aspA*::*kan* mutant created by allelic replacement (57) was assayed and found to have no measureable activity.

Assay of *exe* expression. The activity of an *exeL*::*lacZ* transcriptional fusion (Table 1) was monitored through β-galactosidase reporter activity as described previously (37).

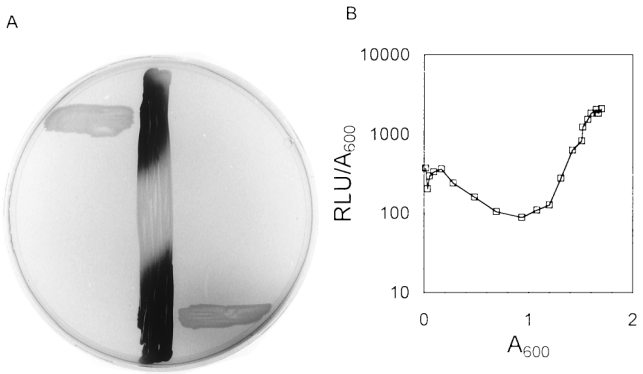

FIG. 2. *Aeromonas* produces AHLs. (A) Cross-streaks of *A. hydrophila* A1 (top) and *A. salmonicida* NCIMB 1102 (bottom) against *C. violaceum* CV0blu (center). Diffusible AHL production by the *Aeromonas* species induces the reporter to produce a purple pigment. (B) Cell density-dependent AHL production by *A. hydrophila*. Light emission from *A. hydrophila*(pSB403) is indicative of AHL production and is presented relative to increasing cell density represented by A_{600}. Light emission is depicted as relative light units (RLU) per unit of A_{600}.

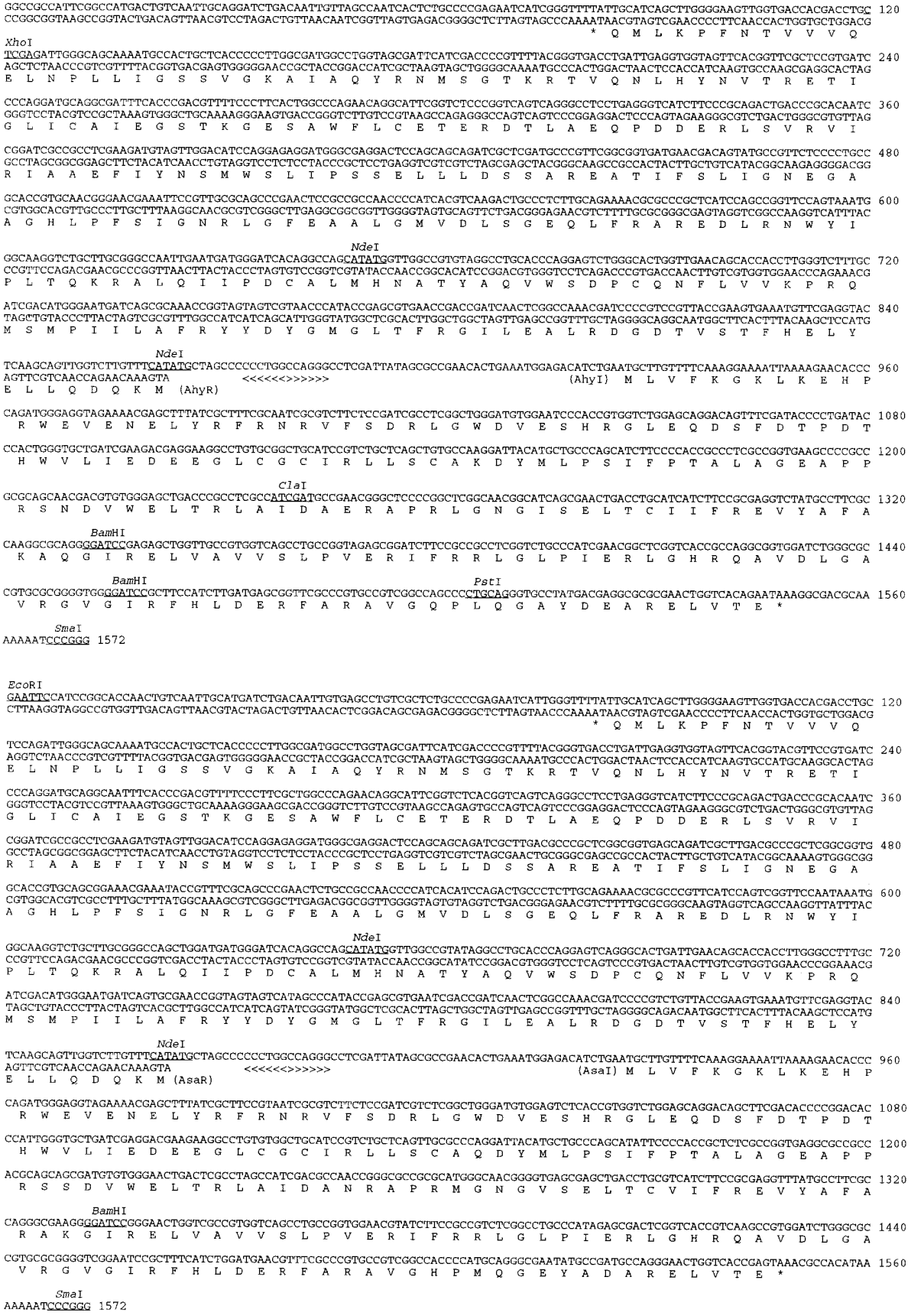

FIG. 3. DNA and deduced protein sequences of *luxRI* homologs from *A. hydrophila* (A; sequences of the *ahyRI* genes and AhyRI proteins are shown) and *A. salmonicida* (B; sequences of the *asaRI* genes and AsaRI proteins are shown). Restriction sites described in the text and a region of dyad symmetry, ><, not homologous to the *lux* box consensus sequence (13, 30) are shown. (C) Protein homology between AhyR, AsaR, and LuxR. (D) Protein homology between AhyI, AsaI, and LuxI. |, identical residues; :, similar residues. These data were generated by using the PILEUP program of the University of Wisconsin Genetics Computer Group package (12).

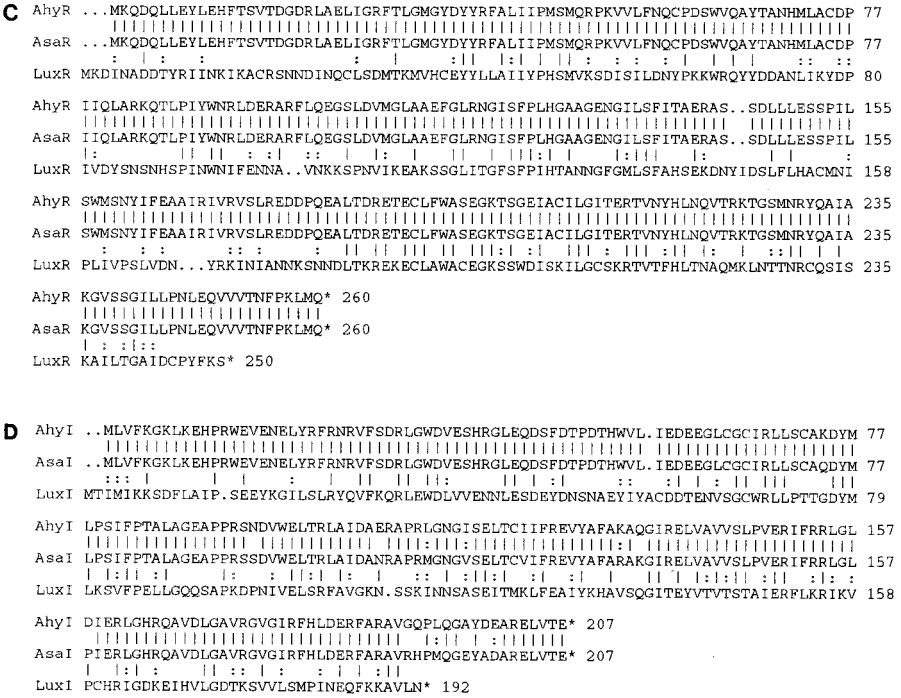

FIG. 3—*Continued.*

Nucleotide sequence accession numbers. The nucleotide sequences for the *luxRI* homologs described in this report and flanking regions have been deposited with GenBank under accession no. X89469 (*A. hydrophila ahyRI*), U65741 (*A. salmonicida asaRI*), and X96968 (*A. salmonicida yrfH*).

RESULTS

***Aeromonas* species produce AHLs.** Cross-streaking experiments with both *A. salmonicida* and *A. hydrophila* at 25°C against the *C. violaceum* CV0blu (Fig. 2A), *E. coli* JM109(pSB401), and *E. coli* JM109(pSB406) AHL biosensors (Table 1) indicated that both *Aeromonas* species produce diffusible AHLs. Furthermore, *A. hydrophila* transformed with the broad-host-range AHL biosensor plasmid pSB403 (Table 1) demonstrates cell density-dependent induction of bioluminescence. In this construct, the induction of reporter gene expression at high cell density is likely to be due to the production of a signal molecule (AHL) complementing the signal generator mutation in the biosensor (Fig. 2B).

Cloning of the *luxRI* homologs *ahyRI* and *asaRI*. Plasmid libraries of the *A. hydrophila* genome digested with either *Eco*RI, *Hin*dIII, or *Pst*I were screened for AHL production by using either the *E. coli*JM109(pSB401) or *C. violaceum* CV0blu AHL biosensor. All positive clones derived from this screen contained a common region of insert DNA, as determined by restriction enzyme mapping and Southern hybridization analysis (data not shown). A 2.8-kbp *Pst*I fragment from pAHP2 (Table 1) hybridizes with a corresponding 2.8-kbp *Pst*I fragment and an 11-kbp *Eco*RI fragment from the *A. hydrophila* chromosome. This *A. hydrophila* 2.8-kbp *Pst*I fragment also hybridizes at high stringency with a number of fragments from the *A. salmonicida* chromosome, namely, a 3.9-kbp *Pst*I fragment and both a 9- and a 1-kbp *Eco*RI fragment. Sequencing of this 2.8-kbp region, using deletion (pAHP3, -8, -13, and -14 [Table 1]) and primer walking strategies, identified the open reading frames (ORFs) designated *ahyR* and *ahyI* (Fig. 3A) with putative gene products showing homology to LuxR (51% similarity and 27% identity [Fig. 3C]) and LuxI (59% similarity and 32% identity [Fig. 3D]), respectively. Deletion analysis showed that AHL biosynthesis requires the plasmid-encoded *ahyI* gene as far as the *Pst*I site proximal to the 3′ end.

Using the same screening strategy, we identified no *A. salmonicida* clones, although in excess of 20,000 recombinants were screened. Nevertheless, as indicated above, Southern analysis indicates that a region homologous to the *ahyRI* locus exists on the *A. salmonicida* genome. PCR analysis using amplimers within the deduced *ahyRI* genes identified products from the putative *asaRI* which were of the expected size. DNA sequence analysis of these PCR products demonstrated a 96% identity between *ahyRI* and *asaRI* over this 390-bp region (nucleotides 592 to 982 in Fig. 3A and B). A previously generated cosmid library of the *A. salmonicida* genome (28) was therefore screened. Toothpicking into the *C. violaceum* CV0blu overlay did not initially identify any AHL production; however, cross-streaking of *C. violaceum* CV0blu against overnight cosmid library colonies was successful. PCR confirmed that the *C. violaceum* CV0blu positive cosmid clones p7B9, p7A3, and p5E3 (Table 1) contain the *ahyRI* homologous region. The *asaRI*-containing region was subcloned from cosmid p7B9 into pUC19 as 9-kbp *Eco*RI (pASE9) and 3.9-kbp *Pst*I (pASP3.7) fragments (Table 1), as indicated by Southern hybridization, and subjected to DNA sequencing (Fig. 3B). The pUC19-based clones did not require preincubation to elicit pigment production by *C. violaceum* CV0blu in cross-streak assays.

DNA sequence analysis of the regions adjacent to the *ahyRI* and *asaRI* loci identified a number of additional ORFs that have homologs in the database (Fig. 4). DNA sequence analysis of the extremities of the cloned fragments and cosmid clones allowed further mapping of *ahyRI* and *asaRI* regions. In addition to *asaRI*, the cosmid clones p7A3 and p5A3 (Table 1) from *A. salmonicida* contain the *exe* genes, which constitute a type II secretion system for the export of, e.g., serine protease (28). Long-range PCR using these cosmids as template DNA

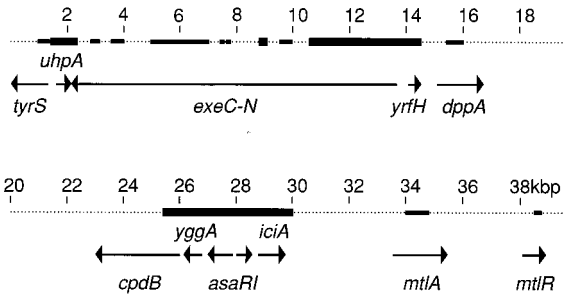

FIG. 4. Physical map of the *asaRI* region. ORFs in the vicinity of *asaRI* with homologs in the database are shown and identified with their closest *E. coli* homologs. Genes identified are the *tyrS* homolog (tyrosyl tRNA synthetase; the *E. coli* homolog [Swissprot P00951] shows 77% similarity over the N-terminal 55 amino acids of the predicted TyrS homolog from *A. salmonicida*; TyrS from *E. coli* is a 423-amino-acid polypeptide); *uhpA* homolog (transcriptional regulator; the *E. coli* homolog [Swissprot P10940] shows 49% similarity to the predicted UhpA homolog [Swissprot P45785] from *A. salmonicida*); *exeC-N* (type II secretion apparatus; GenBank accession no. X80505, X80506, and X84310 [28]); *yrfH* homolog (function unknown; the *E. coli* homolog [Swissprot P45802] shows 69% similarity to the predicted YrfH homolog from *A. salmonicida*; submitted to GenBank under accession no. X96968); *dppA* homolog (dipeptide binding protein; the *E. coli* homolog [Swissprot P23847] shows 100% similarity to a 38-amino-acid region of the predicted DppA homolog from *A. salmonicida*; DppA from *E. coli* is a 536-amino-acid polypeptide); *cpdB* homolog (2′,3′-cyclic nucleotide phosphodiesterase; the *E. coli* homolog [Swissprot P08331] shows 58% similarity over the N-terminal 212 amino acids of the predicted CpdB homolog from *A. salmonicida*; CpdB from *E. coli* is a 646-amino-acid polypeptide); *yggA* homolog (function unknown; the *E. coli* homolog [Swissprot P11667] shows 50% similarity to the predicted YggA homolog from *A. salmonicida*); *asaR* (quorum sensing response regulator; submitted to GenBank under accession no. U65741); *asaI* (quorum sensing signal generator; submitted to GenBank under accession no. U65741); *iciA* homolog (inhibitor of initiation of chromosomal replication at *oriC*; the *E. coli* homolog [Swissprot P24194] shows 72% similarity with the predicted IciA homolog from *A. salmonicida*, which is 123 amino acids longer at the C terminus); *mtlA* homolog (mannitol permease, enzyme II of the phosphotransferase system; the *E. coli* homolog [Swissprot P00550] shows 66% similarity to the predicted *A. salmonicida* homolog over a 261-amino-acid region; MtlA from *E. coli* is a 638-amino-acid polypeptide); and *mtlR* homolog (mannitol repressor; the *E. coli* homolog [Swissprot P36563] shows 74% similarity to the predicted *A. salmonicida* protein homolog over a 54-amino-acid region; MtlR from *E. coli* is a 195-amino-acid polypeptide). Regions that have been submitted to the database are shown as thick lines. Regions identified tentatively through single-stranded DNA sequencing are shown as thin lines. Unsequenced regions are represented by dotted lines. Genes are shown to scale, and distances indicated are kilobase pairs. The distance between *exeC* and *asaI* (approximately 11 kbp) was determined by long-range PCR. The distance between *asaR* and *mtlA* (approximately 8 kbp) was determined by restriction enzyme analysis of pASE9. Analysis of the *A. hydrophila ahyRI* region has revealed similarities in layout between *mtlA* and *cdpB*.

allowed mapping of the relative distances between, and the orientations of, *asa* and *exe* (Fig. 4). The distance between *asaI* and *exeC* was determined to be approximately 11 kbp, with these genes transcribed divergently (Fig. 4).

The *Aeromonas* signal molecules. Comparison of the relative activities of supernatants from *A. hydrophila* A1, *A. salmonicida* NCIMB 1102, *E. coli* JM109(pAHH1) (Table 1), and *E. coli* JM109(pASP3.7) (Table 1) in the AHL biosensor assays with *E. coli*(pSB401), *E. coli*(pSB406), and *C. violaceum* CV0blu suggested that the AHLs produced by *Aeromonas* possess a short N-linked acyl chain of either four or six carbons. For both *A. hydrophila* and *E. coli* JM109(pAHH1), TLC overlay analysis with CV0blu revealed a major AHL ($R_f = 0.64 \pm 10\%$) and a minor AHL ($R_f = 0.37 \pm 10\%$) which corresponded to the R_f values obtained for synthetic BHL and HHL, respectively (Fig. 5A). Similar results were obtained for the *A. salmonicida* wild type, although an additional spot migrating between the BHL and HHL spots was observed for the *E. coli asaI* clone (Fig. 5B). The migration of this additional spot did not correspond with any available AHL standard, and therefore we are unable to make a suggestion as to its chemical identity.

To identify unequivocally the *Aeromonas* AHLs, cell-free supernatants from *E. coli* JM109(pAHH1) and *E. coli* JM109 (pASP3.7) were fractionated by HPLC. We identified two active fractions with retention times corresponding to synthetic BHL and HHL and which were present in a ratio of approximately 70:1. These fractions were collected and subjected to high-resolution MS-MS. From the MS-MS spectra obtained, we confirmed the major *Aeromonas* AHL as BHL and the minor component as HHL (50).

To further demonstrate that BHL is the major AHL produced by AhyI and AsaI in the *Aeromonas* genetic background, solvent extractions of spent culture media were separated by TLC, and AHLs were recovered and subjected to MS (Fig. 6) and high-resolution MS-MS (Fig. 7). From the MS-MS spectra obtained, the major *Aeromonas* AHL was confirmed as BHL (Fig. 7).

Transcription of *ahyI* depends on BHL. Two *luxCDABE* transcriptional reporters of *ahyI* were constructed (Fig. 8A). p*ahyRI′*::*lux* (Table 1; Fig. 8A) has the *Photorhabdus luminescens luxCDABE* operon inserted at the *Pst*I site of *ahyI* in pAHP13 (Table 1) and retains AHL biosynthetic activity. p*ahyRI″*::*lux* (Table 1; Fig. 8A) has *luxCDABE* inserted at the *Bam*HI site of pAHP13 and exhibits no detectable AHL biosynthetic activity. In *E. coli* JM109, transcription from p*ahyRI′*::*lux* is essentially constitutive (Fig. 8A). The further deletion of *ahyI*, which abolishes BHL biosynthesis, leads to a 100-fold reduction in reporter gene activity (Fig. 8B). The provision of 1 μM BHL at the time of inoculation fully restores the transcription of *ahyI″*::*lux* to *ahyI′*::*lux* levels, indicating that *ahyI* expression is BHL dependent (Fig. 8B), a situation analogous to that of *luxI* expression in *V. fischeri* (16). Conceivably the BHL effect could be through an *E. coli* protein; however, a transcriptional fusion at the *Bam*HI site with a deletion of the N-terminal 71 amino acids of AhyR (pAHP14 [Table 1]) was BHL independent, indicating that AhyR is required for the BHL-dependent response (data not shown).

Evidence for the AHL-dependent regulation of *A. salmonicida* serine protease. The identification of a LuxRI regulon in *Aeromonas* led us to investigate potential phenotypes con-

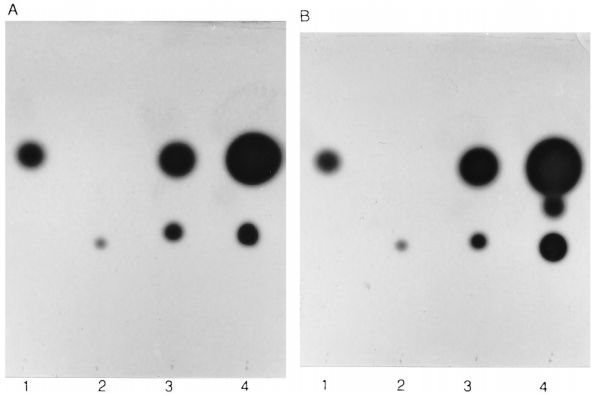

FIG. 5. Analysis of signal molecules produced by AhyI and AsaI by TLC. AHLs are visualized as the induction of pigment production by CV0blu after overnight incubation. (A) Lanes: 1, 1.8×10^{-9} mol of synthetic BHL standard; 2, 10^{-11} mol of synthetic HHL standard; 3, AHL extracted from 20 ml of *E. coli*(pAHH1) supernatant; 4, AHL extracted from 200 ml of *A. hydrophila* A1 supernatant for *A. hydrophila*. (B) Lanes: 1, 1.8×10^{-9} mol of synthetic BHL standard; 2, 10^{-11} mol of synthetic HHL standard; 3, AHL extracted from 20 ml of *A. salmonicida* NCIMB 1102 supernatant; 4, AHL extracted from 12 ml of *E. coli*(pASP3.7) supernatant for *A. salmonicida*.

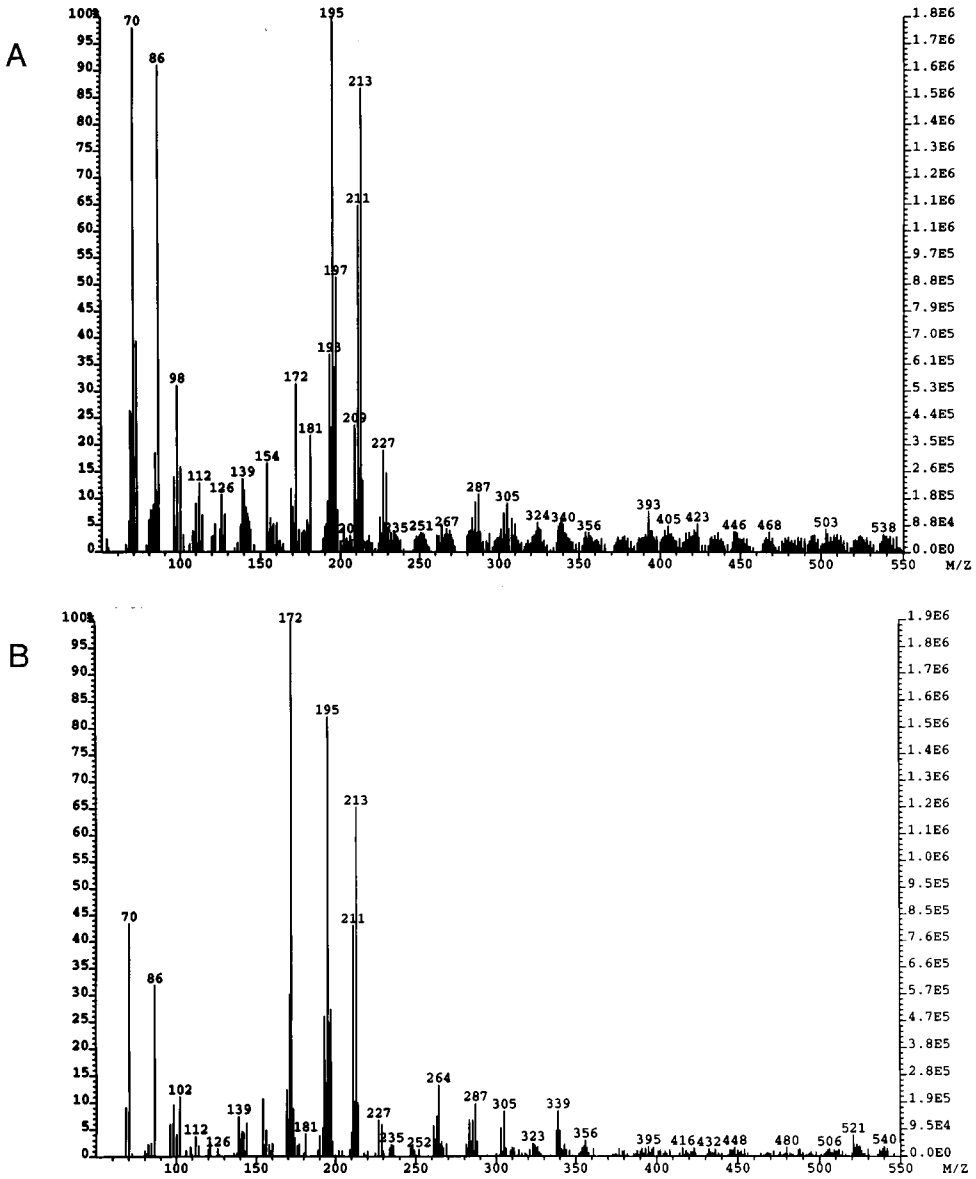

FIG. 6. MS of AHL recovered from the presumptive BHL spot after TLC of AHLs extracted from *A. hydrophila* A1 (A) and *A. salmonicida* NCIMB 1102 (B). The peak at 172 indicates that BHL is present.

trolled through quorum sensing. In other gram-negative bacterial pathogens, virulence determinants and secondary metabolites are regulated by AHLs (18, 45, 51). We therefore focused on the possibility that expression of the *A. salmonicida* AspA serine protease and/or the protease secretory apparatus (the *exe* locus) were BHL dependent. *exe* expression in particular was studied because of the proximity of the *exe* locus to the *luxRI* homologs on the *Aeromonas* genome (Fig. 4) and the report of stationary-phase synthesis of *exe* homologs in *P. aeruginosa* (*xcp* [1]) and *Erwinia chrysanthemi* (*out* [31]). In the case of *A. salmonicida*, we hypothesized that addition of BHL to early-exponential-phase cultures of the wild-type organism should give induction, whereas the addition of AHLs known to antagonize the BHL-dependent activation of violacein in *Chromobacterium*, i.e., DHL and ODHL, should reduce or abolish expression (35, 38).

The effect of exogenous BHL, added at the time of inoculation, on *exe* expression in *A. salmonicida* was investigated by using an *exeL::lacZ* fusion (Table 1). No early induction of *exeL* expression was observed, implying that the export apparatus is not subject to quorum sensing. Using RT-PCR analysis of mRNA production for *aspA* and *exeD* mRNAs in *A. salmonicida* NCIMB 1102 (data not shown), we confirmed *aspA* transcription to be induced in the stationary phase, but in contrast to the cases for *Erwinia* and *Pseudomonas* (1, 31), *exeD* transcription appeared to be constitutive. However, it is important to note that the possibility of a burst in *exeD* transcription over a narrow range of A_{600} (within 0.2- to 0.3-U increments) cannot be excluded. In contrast, we observed that the addition of exogenous AHLs influenced serine protease production in *A. salmonicida* (Fig. 9). When exogenous BHL is added to the *A. salmonicida* wild type at the time of inoculation, there is only a small stimulation of the production of serine protease in comparison to the control culture. When

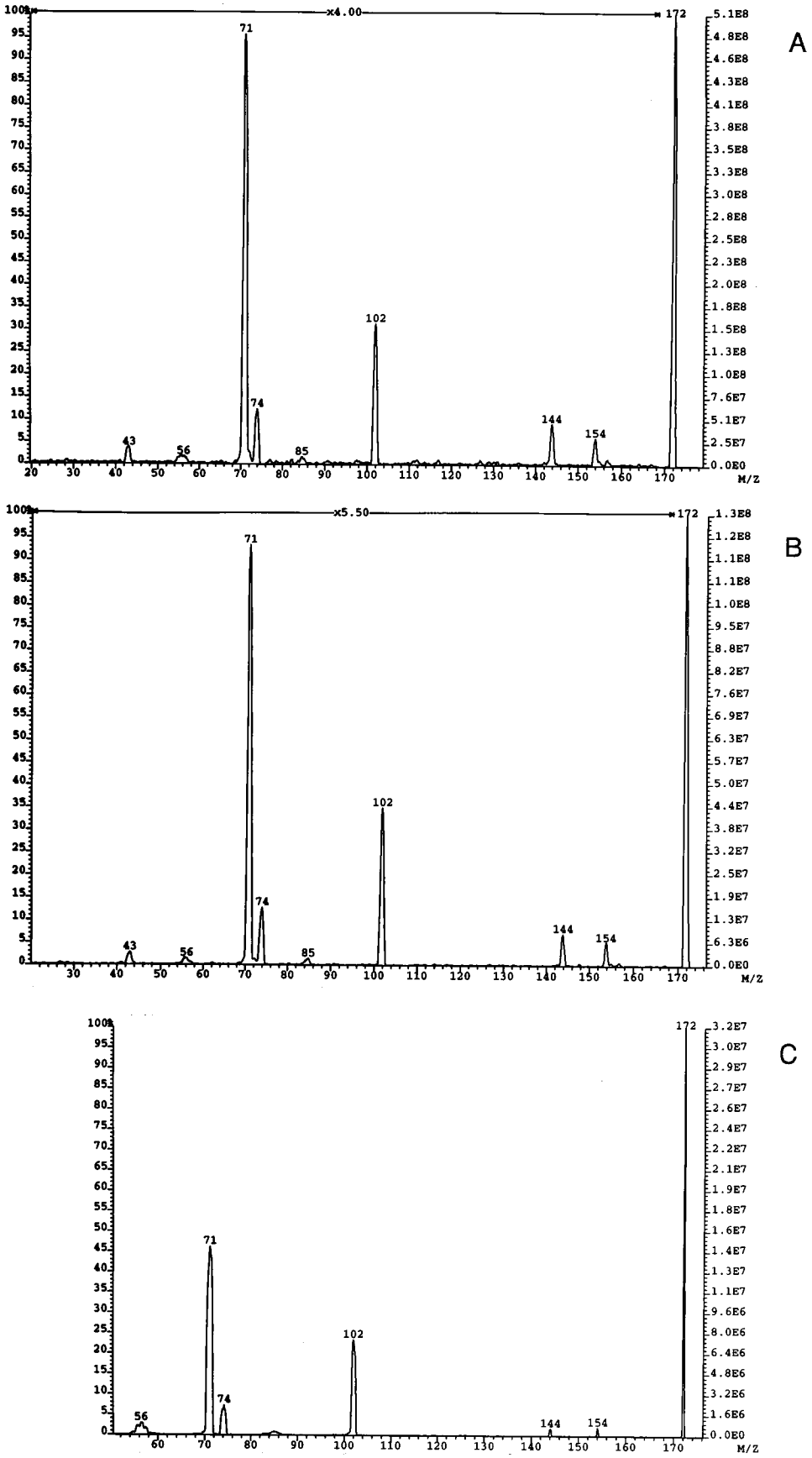

FIG. 7. MS-MS profiles of the peaks at 172 identified in *A. hydrophila* (A) and *A. salmonicida* (B) supernatants, with a synthetic BHL standard (C) for comparison. The identical traces for extracts and the synthetic BHL standard confirm BHL as the major signal molecule present.

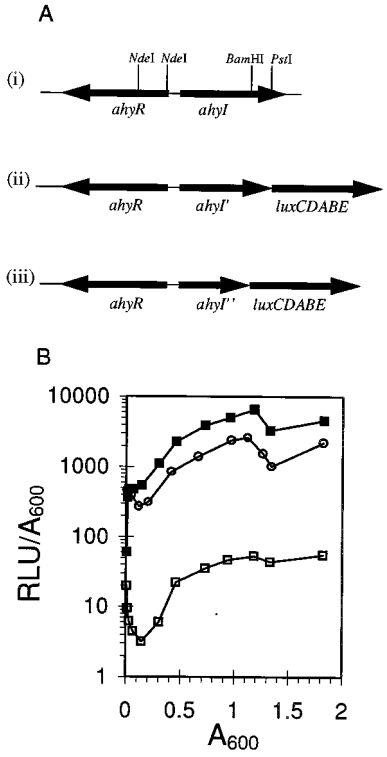

FIG. 8. *ahyI*::*lux* reporters of *ahyI* expression. (A) Construction of *ahyI*::*lux* reporters for the evaluation of *ahyI* expression in *E. coli*. (i) The *ahyRI* region contained on pAHP2; (ii) the p*ahyRI'*::*lux* construct with *luxCDABE* cloned into the *Pst*I site at the 3′ end of *ahyI*; (iii) the p*ahyRI''*::*lux* construct with *luxCDABE* cloned into the *Bam*HI site at the 3′ end of *ahyI*. (B) Evaluation of *ahyI* expression in *E. coli*. The variation in *lux* reporter gene expression relative to increasing culture A_{600} is presented by plotting A_{600} against light emission, depicted as relative light units (RLU) per unit of A_{600}. Data for *E. coli* JM109 (p*ahyRI'*::*lux*) (○), *E. coli* JM109(p*ahyRI''*::*lux*) (□), and *E. coli* JM109(p*ahyRI''*::*lux*) to which 1 μM BHL was added at the time of inoculation (■) are shown.

either DHL or ODHL is added, the appearance of the serine protease in the culture supernatant is delayed and the final activity attained is reduced. The effect with DHL is small; however, ODHL is a more effective inhibitor of protease production in the culture supernatant, reducing the final levels by approximately 50% and delaying the appearance from an A_{650} of 0.9 in the control to an A_{650} of 1.2 in the test (Fig. 9).

DISCUSSION

The complementation of signal generator mutations has facilitated the cloning of AHL signal generator genes (*luxI* homologs) from many different gram-negative bacteria (15, 30, 38, 52). The close linkage of a response regulator (*luxR* homolog) to the *luxI* homolog has allowed the cocloning of the transcriptional activator. This study further highlights the power of this approach to clone *luxRI* homologs from bacteria known to produce a signal complementing the *luxI* homolog mutation in the biosensor. Importantly, the necessity of allowing cosmid clones of the *asaI* signal generator at least an overnight incubation to produce sufficient AHL before application of the biosensor gives a refinement of the protocol.

AhyRI and AsaRI have homology with the other proteins in the LuxRI family, and two features are highlighted by homology studies. The first shows the apparent lack of any consensus sequence associated with the production or response to given AHL signal molecules (19, 29, 30). The homologs from *Aeromonas* spp. described here do not exhibit any obviously greater or lesser similarities to other LuxRI homologs which would aid the identification of the cognate AHL from sequence analysis alone. Second, we see a very high homology between AhyRI and AsaRI (Fig. 3D), which is perhaps not surprising given the close relatedness of *A. hydrophila* and *A. salmonicida* (28). However, given the sequence divergence between the two *P. aeruginosa* LuxRI pairs (LasRI and VsmRI (RhlRI [30, 39a, 39b]) and between the quorum sensing systems of other closely related species (30, 55), this is interesting. Furthermore, like VsmI, AhyI and AsaI both direct the synthesis of the same major AHL, BHL even though the *Aeromonas* LuxI homologs are not particularly closely related to VsmI (33% identity [AsaI-VsmI] and 35% identity [AhyI-VsmI] [30, 61]). BHL is also produced via the LuxI homolog SwrI from *Serratia liquefaciens* (15), and all four LuxI homologs also direct the synthesis of low levels of HHL in both the homologous and heterologous (*E. coli*) genetic backgrounds (15, 61). Thus, BHL has now been identified as a quorum sensing signal molecule in four different gram-negative bacteria. In *P. aeruginosa*, BHL is involved in the regulation of multiple exoproducts, including elastase, hemolysin, chitinase, alkaline protease, cyanide, lectins, staphylolytic activity, pyocyanin, and the alternative stationary-phase sigma factor RpoS (29, 30, 61). In *S. liquefaciens*, the initiation of swarmer cell differentiation and the production of a phospholipase both depend on BHL (16). For *A. salmonicida*, our observation that ODHL in particular both delays the induction of and reduces final yields of the protease suggests that in this fish pathogen, the AspA exoprotease is subject to quorum sensing control. We are currently following a directed gene knockout approach to verify this finding. In addition, our data derived from RT-PCR and an *exeL*::*lacZ* transcriptional fusion suggest that the growth phase-dependent appearance of serine protease in the culture supernatant (58) is due to regulation of *aspA* transcription rather than regulation of the Exe secretion apparatus. Other growth phase-associated phenotypes which may also be under quorum sensing control include α-hemolysin (32), glycerophospholipid-cholesterol acyltransferase (33, 34), and lipase (2). We are currently determining the spectrum of BHL-dependent phenotypes in *Aeromonas* to determine whether, as in *P. aeruginosa* and *Erwinia carotovora*, quorum sensing is a global regulator of virulence determinants and secondary metabolites. In addition, the close genetic linkage of the AhyRI locus to a homolog of a

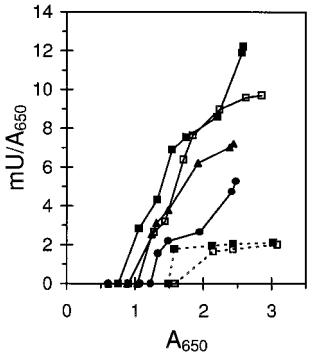

FIG. 9. Influence of AHLs on serine protease production in *A. salmonicida*. Serine protease activity per unit of A_{650} of *A. salmonicida* NCIMB 1102 (solid line) and MT1326 (broken line) relative to increasing cell density represented by A_{650} is shown. The effect on protease production of exogenous BHL (■), DHL (●), or ODHL (▲), each added at a concentration of 10 μM and compared with a control culture to which no exogenous AHL (□) had been added at the time of inoculation, is shown.

gene known to modulate cell replication in *E. coli*, i.e., *iciA* (Fig. 4; reference 54), offers the intellectually attractive hypothesis of a role for AHLs in the control of cell division. This is particularly interesting given the suggested role for quorum sensing in the control of cell division via SdiA and FtsQAZ in *E. coli* (20, 49).

A feature of many genes controlled by LuxRI homologs is the presence of a consensus DNA sequence for LuxR homolog binding, the *lux* box (13, 19, 30), in the upstream region of the target gene. No such consensus sequence is present in the region upstream of *ahyI*, *asaI*, or *aspA*; however, upstream of *ahyI* and *asaI* there is a region of dyad symmetry (Fig. 3A and B) which may be involved in the binding of activated LuxR homologs such as AhyR and AsaR. We have not, however, identified this sequence upstream of any other *Aeromonas* genes putatively under quorum sensing control. Our data do, however, suggest that in *A. hydrophila*, *ahyI* is part of the quorum sensing regulon controlled by BHL (Fig. 8). For *A. salmonicida*, the same is almost certainly true, given the 100% identity of AsaR and AhyR, the 100% identity of the intergenic regions of *ahyRI* and *asaRI*, and the use of BHL as the signal molecule.

When *ahyI* expression is uncoupled from the *A. hydrophila E. coli*, *ahyI*, expression is constitutive in the presence of BHL (Fig. 8B). This finding suggests that a negative-acting regulatory element may be involved in controlling *ahyI* expression in *Aeromonas*. Furthermore, the addition of BHL to early exponential cultures does not result in the immediate induction of serine protease expression, suggesting that when other, as yet unidentified factors are favorable, serine protease expression occurs immediately because BHL is present. This contrasts with the production of carbapenem antibiotic in *Erwinia*, where the addition of exogenous OHHL at the time of inoculation overrides the cell density dependency and results in the immediate synthesis of antibiotic (59). However, negative regulatory factors have been identified in *Agrobacterium tumefaciens* and in *C. violaceum*, where they influence the AHL-dependent conjugal transfer of the Ti plasmid (25) and the production of the purple pigment violacein (60), respectively. Furthermore, in *Erwinia carotovora*, the *csrA* (43) homolog *rsmA*, which appears to act at the posttranscriptional level probably by binding to mRNA, is another example of such a negative regulator influencing expression of quorum sensing-dependent phenotypes, in this case, plant cell wall-degrading exoenzymes (7, 8). Work in our laboratory has already demonstrated the presence of this regulator in a number of organisms, including *A. hydrophila* and *A. salmonicida* (56). We are currently evaluating the role of this gene product and looking for additional regulators. Furthermore, the increased serine protease activity seen in response to exogenous BHL may be due to amplification of this signal by quorum sensing. Here it is conceivable that while expression of certain virulence determinants is repressed at low cell densities to reduce the risk of premature detection by host defenses, it can be amplified rapidly at high bacterial population densities to overwhelm the host before an appropriate response can be mounted.

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REFERENCES

1. **Akrim, M., M. Bally, G. Ball, J. Tommassen, H. Teerink, A. Filloux, and A. Lasdunski.** 1993. Xcp-mediated protein secretion in *Pseudomonas aeruginosa*: identification of two additional genes and evidence for regulation of *xcp* gene expression. Mol. Microbiol. **10:**431–443.
2. **Anguita, J., L. B. Rodriguez-Aparicio, and G. Naharro.** 1993. Purification, gene cloning, amino acid sequence analysis, and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate. Appl. Environ. Microbiol. **59:**2411–2417.
3. **Bainton, N. J., P. Stead, S. R. Chhabra, B. W. Bycroft, G. P. C. Salmond, G. S. A. B. Stewart, and P. Williams.** 1992. *N*-(3-Oxohexanoyl)-L-homoserine lactone regulated carbapenem antibiotic production in *Erwinia carotovora*. Biochem. J. **288:**997–1004.
4. **Bartolomé, B., Y. Jubete, E. Martinez, and F. de la Cruz.** 1991. Construction and properties of a family of pACYC-derived cloning vectors compatible with pBR322 and its derivatives. Gene **103:**75–78.
5. **Bassler, B. L., M. Wright, and M. R. Silverman.** 1994. Sequence and function of LuxO, a negative regulator of luminescence in *Vibrio harveyi*. Mol. Microbiol. **12:**403–412.
6. **Braun, V., and G. Schmitz.** 1980. Excretion of a protease by *Serratia marcescens*. Arch. Microbiol. **124:**55–61.
7. **Cui, Y., A. Chatterjee, Y. Liu, C. K. Dumenyo, and A. K. Chatterjee.** 1995. Identification of a global repressor gene, *rsmA*, of *Erwinia carotovora* subsp. *carotovora* that controls extracellular enzymes, *N*-(3-oxohexanoyl)-L-homoserine lactone, and pathogenicity in soft-rotting *Erwinia* spp. J. Bacteriol. **177:**5108–5115.
8. **Chatterjee, A., Y. Cui, Y. Liu, C. K. Dumenyo, and A. K. Chatterjee.** 1995. Inactivation of *rsmA* leads to overproduction of extracellular pectinases, cellulases, and proteases in *Erwinia carotovora* subsp. *carotovora* in the absence of the starvation cell density-signal, *N*-(3-oxohexanoyl)-L-homoserine lactone. Appl. Environ. Microbiol. **61:**1959–1967.
9. **Chhabra, S. R., P. Stead, N. J. Bainton, G. P. C. Salmond, P. Williams, G. S. A. B. Stewart, and B. W. Bycroft.** 1993. Autoregulation of carbapenem biosynthesis in *Erwinia carotovora* ATCC 39048 by analogues of *N*-(3-oxohexanoyl)-L-homoserine lactone. J. Antibiot. **46:**441–454.
10. **Choi, S. H., and E. P. Greenberg.** 1992. Genetic dissection of DNA binding and luminescence gene activation by the *Vibrio fisheri* LuxR protein. J. Bacteriol. **174:**4064–4069.
11. **de Lorenzo, V., M. Hessero, U. Jakubzik, and K. N. Timmis.** 1990. Mini-Tn*5* transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. J. Bacteriol. **172:**6568–6572.
12. **Devereux, J., P. Haerberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
13. **Devine, J. H., G. S. Shadel, and T. O. Baldwin.** 1989. Identification of the operator of the *lux* regulon from the *Vibrio fischeri* strain ATCC7744. Proc. Natl. Acad. Sci. USA **86:**5688–5692.
14. **Eberhard, A., A. L. Burlingame, G. L. Kenyon, K. H. Nealson, and N. J. Oppenheimer.** 1981. Structural identification of autoinducer of *Photobacterium fischeri* luciferase. Biochemistry **20:**2444–2449.
15. **Eberl, L., M. K. Winson, C. Sternberg, G. S. A. B. Stewart, G. Christiansen, S. R. Chhabra, B. W. Bycroft, P. Williams, S. Molin, and M. Giskov.** 1996. Involvement of *N*-acyl-L-homoserine lactone autoinducers in controlling the multicellular behaviour of *Serratia liquefaciens*. Mol. Microbiol. **20:**127–136.
16. **Engebrecht, J., K. Nealson, and M. Silverman.** 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. Cell **32:**773–781.
17. **Frohman, M. A., M. K. Dush, and G. R. Martin.** 1988. Rapid production of full length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85:**8998–9002.
18. **Fuqua, C., S. C. Winans, and E. P. Greenberg.** 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing regulators. Annu. Rev. Microbiol. **50:**727–751.
19. **Fuqua, W. C., S. C. Winans, and E. P. Greenberg.** 1994. Quorum sensing in bacteria-the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol. **176:**269–275.
20. **Garcia-Lara, J., L. H. Shang, and L. I. Rothfield.** 1996. An extracellular factor regulates expression of *sdiA*, a transcriptional activator of cell division genes in *Escherichia coli*. J. Bacteriol. **178:**2742–2748.
21. **Gilson, L., A. Kuo, and P. V. Dunlap.** 1995. AinS and a new family of autoinducer synthesis proteins. J. Bacteriol. **177:**6946–6951.
22. **Hanzelka, B. L., and E. P. Greenberg.** 1995. Evidence that the N-terminal region of the *Vibrio fischeri* LuxR protein constitutes an autoinducer-binding domain. J. Bacteriol. **177:**815–817.
23. **Hastings, J. W., and K. H. Nealson.** 1977. Bacterial bioluminescence. Annu. Rev. Microbiol. **31:**549–595.
24. **Hill, P. J., S. Swift, and G. S. A. B. Stewart.** 1991. PCR based gene engineering of the *Vibrio harveyi lux* operon and the *Escherichia coli trp* operon provides for biochemically functional native and fused gene products. Mol. Gen. Genet. **226:**41–48.
25. **Hwang, I., D. M. Cook, and S. K. Farrand.** 1995. A new regulatory element modulates homoserine lactone-mediated autoinduction of Ti plasmid con-

jugal transfer. J. Bacteriol. **177:**449–458.
26. **Janda, J. M.** 1991. Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with genus *Aeromonas*. Clin. Microbiol. Rev. **4:**397–410.
27. **Jones, S., B. Yu, N. J. Bainton, M. Birdsall, B. W. Bycroft, S. R. Chhabra, A. J. R. Cox, P. Golby, P. J. Reeves, S. Stephens, M. K. Winson, G. P. C. Salmond, G. S. A. B. Stewart, and P. Williams.** 1993. The lux autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*. EMBO J. **12:**2477–2482.
28. **Karlyshev, A. V., and S. MacIntyre.** 1995. Cloning and study of the genetic organisation of the exe gene cluster of *Aeromonas salmonicida*. Gene **158:** 77–82.
29. **Latifi, A., M. Foglino, T. Tanaka, P. Williams, and A. Lazdunski.** 1996. A hierarchical quorum sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and VsmR to expression of the stationary phase sigma factor RpoS. Mol. Microbiol. **21:**1137–1146.
30. **Latifi, A., M. K. Winson, M. Fogilino, B. W. Bycroft, G. S. A. B. Stewart, A. Lazdunski, and P. Williams.** 1995. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PA01. Mol. Microbiol. **17:**333–343.
31. **Linderberg, M., and A. Collmer.** 1992. Analysis of eight *out* genes in a cluster required for pectic enzyme secretion by *Erwinia chrysanthemi*: sequence comparison with secretion genes from other gram-negative bacteria. J. Bacteriol. **174:**7385–7397.
32. **Ljung, A., B. Wretlind, and R. Molby.** 1981. Separation and characterisation of enterotoxin and two haemolysins from *Aeromonas hydrophila*. Acta Pathol. Microbiol. Scand. Sect. B **89:**387–397.
33. **MacIntyre, S., and J. T. Buckley.** 1978. Presence of glycerophospholipid cholesterol acyltransferase and phospholipase in culture supernatants of *Aeromonas hydrophila*. J. Bacteriol. **135:**402–407.
34. **MacIntyre, S., T. J. Trust, and J. T. Buckley.** 1979. Distribution of glycerophospholipid cholesterol acyltransferase in selected bacterial species. J. Bacteriol. **139:**132–136.
35. **McClean, K. H., M. K. Winson, L. Fish, A. Taylor, S. R. Chhabra, M. Camara, M. Daykin, S. Swift, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** Quorum sensing and *Chromobacterium violaceum*: exploitation and violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Submitted for publication.
36. **Merino, S., X. Rubires, S. Knøchel, and J. M. Tomas.** 1995. Emerging pathogens: *Aeromonas* spp. Int. J. Food Microbiol. **28:**157–168.
37. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 352–355. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Milton, D. L., A. Hardman, M. Camara, S. R. Chhabra, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1997. Quorum sensing in *Vibrio anguillarum*: characterization of the *vanI*/*vanR* locus and identification of the autoinducer *N*-(3-oxodecanoyl)-L-homoserine lactone. J. Bacteriol. **179:**3004–3012.
39. **O'Leary, W. M., C. Panos, and G. E. Helz.** 1956. Studies on the nutrition of *Bacterium salmonicida*. J. Bacteriol. **72:**673–676.
39a. **Oschner, U. A., and J. Reiser.** 1995. Autoinducer-mediated regulation of rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:**6424–6428.
39b. **Oschner, U. A., A. K. Koch, A. Fiechter, and J. Reiser.** 1994. Isolation and characterisation of a regulatory gene affecting rhamnolipid biosurfactant synthesis in *Pseudomonas aeruginosa*. J. Bacteriol. **176:**2044–2054.
40. **Passador, L., J. M. Cook, M. J. Gambello, L. Rust, and B. H. Iglewski.** 1993. Expression of *Pseudomonas aeruginosa* virulence genes requires cell-to-cell communication. Science **260:**1127–1130.
41. **Pearson, J. P., L. Passador, B. H. Iglewski, and E. P. Greenberg.** 1995. A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:**1490–1494.
42. **Pirhonen, M., D. Flego, R. Heikinheimo, and E. T. Palva.** 1993. A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production by the plant pathogen *Erwinia carotovora*. EMBO J. **17:**2467–2476.
43. **Romeo, T., M. Gong, M. Y. Liu, and A. M. Brunzinkernagel.** 1993. Identification and molecular characterisation of *csrA*, a pleiotropic gene from *Escherichia coli* that affects glycogen biosynthesis, gluconeogenesis, cell size, and surface properties. J. Bacteriol. **175:**4744–4755.
44. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
45. **Salmond, G. P. C., B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1995. The bacterial enigma: cracking the code of cell-cell communication. Mol. Microbiol. **16:**615–624.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Schweizer, H. P.** 1991. *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. Gene **97:**109–112.
47a. **Shaw, P., S. L. Daly, C. Cha, J. E. Cronan, Jr., K. L. Rinehart, and S. K. Farrand.** Detecting and characterising acyl-homoserine lactone signal molecules by thin layer chromatography. Proc. Natl. Acad. Sci. USA, in press.
48. **Simon, R., J. Quandt, and W. Klipp.** 1989. New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. Gene **80:**161–169.
49. **Sitnikov, D. M., J. B. Schineller, and T. O. Baldwin.** 1996. Control of cell division in *Escherichia coli*: regulation of transcription of *ftsQA* involves both *rpoS* and SdiA-mediated autoinduction. Proc. Natl. Acad. Sci. USA **93:**336–341.
50. **Swift, S., L. Fish, M. Camara, M. K. Winson, B. W. Bycroft, P. Williams, and G. S. A. B. Stewart.** 1996. Quorum sensing in *Aeromonas*: identification and characterisation of the LuxRI homologous cassette, AhyRI in *Aeromonas hydrophila*, abstr. H-208, p. 520. *In* Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
51. **Swift, S., J. P. Throup, G. P. C. Salmond, P. Williams, and G. S. A. B. Stewart.** 1996. Quorum sensing: a population-density component in the determination of bacterial phenotype. Trends Biochem. Sci. **21:**214–219.
52. **Swift, S., M. K. Winson, P. F. Chan, N. J. Bainton, M. Birdsall, P. J. Reeves, C. E. D. Rees, S. R. Chhabra, P. J. Hill, J. P. Throup, B. W. Bycroft, G. P. C. Salmond, P. Williams, and G. S. A. B. Stewart.** 1993. A novel strategy for the isolation of luxI homologues: evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. Mol. Microbiol. **10:**511–520.
53. **Taylor, A., K. H. McClean, S. R. Chhabra, M. Camara, M. K. Winson, M. Daykin, B. W. Bycroft, G. S. A. B. Stewart, and P. Williams.** 1996. Exploitation of violacein production and inhibition in *Chromobacterium violaceum* for the detection of *N*-acylhomoserine lactones, abstr. H-206, p. 519. *In* Abstracts of the 96th General Meeting of the American Society for Microbiology 1996. American Society for Microbiology, Washington, D.C.
54. **Thöny, B., D. S. Hwang, L. Fradkin, and A. Kornberg.** 1991. *iciA*, an *Escherichia coli* gene encoding a specific inhibitor of chromosomal initiation of replication *in vitro*. Proc. Natl. Acad. Sci. USA **88:**4066–4070.
55. **Throup, J. P., M. Camara, G. S. Briggs, M. K. Winson, S. R. Chhabra, B. W. Bycroft, P. Williams, and G. S. A. B. Stewart.** 1995. Characterisation of the *yenI*/*yenR* locus from *Yersinia enterocolitica* mediating the synthesis of two *N*-acyl-homoserine lactone signal molecules. Mol. Microbiol. **17:**345–356.
56. **Throup, J. P., K. P. Francis, S. Swift, T. Romeo, P. Williams, and G. S. A. B. Stewart.** Unpublished data.
57. **Vipond, R.** 1995. Ph.D. thesis. University of Reading, Reading, England.
58. **Whitby, P. W., M. Landon, and G. Coleman.** 1992. The cloning and nucleotide sequence of the serine protease gene (*aspA*) of *Aeromonas salmonicida* spp. *salmonicida*. FEMS Microbiol. Lett. **99:**65–72.
59. **Williams, P., N. J. Bainton, S. Swift, M. K. Winson, S. R. Chhabra, G. S. A. B. Stewart, G. S. A. B., G. P. C. Salmond, and B. W. Bycroft.** 1992. Small molecule mediated density dependent control of gene expression in prokaryotes: bioluminescence and the biosynthesis of carbapenem antibiotics. FEMS Microbiol. Lett. **100:**161–168.
60. **Winson, M. K., N. J. Bainton, S. R. Chhabra, B. W. Bycroft, G. P. C. Salmond, P. Williams, and G. S. A. B. Stewart.** 1994. Control of *N*-acylhomoserine lactones-regulated expression of multiple phenotypes in *Chromobacterium violaceum*, abstr. H-71, p. 212. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
61. **Winson, M. K., M. Camara, A. Latifi, M. Foglino, S. R. Chhabra, M. Daykin, M. Bally, V. Chapon, G. P. C. Salmond, B. W. Bycroft, A. Lazdunski, G. S. A. B. Stewart, and P. Williams.** 1995. Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **92:**9427–9431.
62. **Winson, M. K., S. Swift, J. P. Throup, B. W. Bycroft, P. Williams, and G. S. A. B. Stewart.** 1996. Unpublished data.
63. **Yanisch-Peron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–109.