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Regulation of *Vibrio anguillarum empA* Metalloprotease Expression and Its Role in Virulence

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Atlantic salmon (*Salmo salar*) were challenged with *Vibrio anguillarum* strains M93Sm and NB10 and *empA* null mutants M99 and NB12. Both wild types were virulent when administered by intraperitoneal (i.p.) injection or anal intubation. NB12 was avirulent via either route of infection. M99 virulence was attenuated when delivered by intubation, but fully virulent by i.p. injection. Northern blot analysis revealed *empA* expression in M93Sm and NB10 cells incubated in mucus, while incubation in Luria-Bertani broth plus 2% NaCl (LB20) induced *empA* expression only in NB10. Nucleotide differences between M93Sm and NB10 *empA* sequences were found in regions located 207 and 229 bp upstream of the *empA* translational start. Reverse transcription-PCR and 5′ rapid amplification of cDNA ends revealed the *empA* transcriptional start site 85 bp upstream of the translational start for both strains. A putative σ^S-dependent promoter was identified upstream of the transcriptional start in both strains. Site-directed mutagenesis was used to create *rpoS* mutants of M93Sm and NB10. Neither *rpoS* mutant exhibited protease activity. Since *empA* is expressed during stationary phase, the effects of conditioned medium on protease activity were examined. M99 conditioned LB20 supernatants stimulated protease activity in NB10 while allowing M93Sm to produce protease in LB20. Neither acyl homoserine lactones nor AI-2 induced protease activity. Conditioned LB20 supernatant from a *V. anguillarum luxS* mutant caused a more rapid induction of protease activity in wild-type cells. Our data show that expression of *empA* is differentially regulated in *V. anguillarum* strains NB10 and M93Sm and requires σ^S, quorum-sensing molecules, and gastrointestinal mucus.

Vibrio anguillarum is the causative agent of vibriosis in fish, bivalves, and crustaceans (3, 8, 10). Vibriosis has been a major problem for the aquaculture industry around the world. Large economic losses due to this fish pathogen are sustained by the aquaculture industry (3). Vibriosis in fish is observed as a hemorrhagic septicemia (3). Infected fish display skin discoloration and erythema around the base of the fins, vent, and mouth. Necrotic lesions are observed in the abdominal muscle. The intestinal tract and rectum become distended and filled with fluid. Mortalities within infected fish farm stocks range from 30 to 100% (3, 19, 30).

Olsson et al. (34) demonstrated that the gastrointestinal (GI) tract of fish can serve as the port of entry for *V. anguillarum*. Additionally, the GI tract of fish appears to be a site of colonization and amplification for pathogenic *Vibrio* species (36, 37). Once in the GI tract, the pathogen grows rapidly, utilizing intestinal mucus as a nutrient. Garcia et al. (15) have shown that salmon GI mucus is an excellent growth medium for *V. anguillarum*. Additionally, both *V. anguillarum* and *Vibrio alginolyticus* exhibit strong chemotaxis toward fish mucus (9). When growing in GI mucus, *V. anguillarum* specifically expresses a number of different proteins, including several outer membrane proteins (15) and EmpA protease (this study). Previously, we demonstrated that the *empA* metalloprotease is expressed during the stationary phase when *V. anguillarum* cells are incubated in mucus (13).

Quorum sensing (QS) is a term used to describe how bacteria can sense or measure their cell density. Bacteria monitor their cell density by using diffusible signal molecules termed autoinducers. This phenomenon was first studied by Hastings et al. (14, 32) in the marine symbiont *Vibrio fischeri*. This luminescent marine bacterium is found living in light organs of squid and fish (23, 38). Autoinducers in many gram-negative bacteria, such as *V. fischeri*, include acyl homoserine lactones (AHLs). These compounds, specifically 3-oxo-C_6-homoserine lactone (3-oxo-C_6-HSL), regulate light production (via the expression of *luxICDABEGH*) in *V. fischeri* (39). The AHL acts with the transcriptional activator LuxR to activate transcription of the AHL synthase, *luxI* and the *luxCDABEGH* genes in a positive feedback loop. Similarly, the opportunistic pathogen *Pseudomonas aeruginosa* regulates the production of the metalloprotease virulence factor elastase (a homologue of EmpA), using AHL-dependent QS (35, 46). Milton et al. (24, 25) have demonstrated that *V. anguillarum* mutants unable to produce C_6-HSL and 3-OH-C_6-HSL and mutants that fail to produce 3-oxo-C_{10}-HSLs still exhibit normal production of the EmpA metalloprotease. In addition to AHLs, many gram-negative and some gram-positive organisms produce a non-AHL signal molecule called autoinducer 2 (AI-2), which has been suggested to be a furanone borate diester (11). The synthesis of AI-2 in *S*-adenosylmethionine metabolism requires a functional *luxS* gene (40). LuxS is the AI-2 synthase, and its substrate is *S*-ribosylhomocysteine, which is cleaved to yield homocysteine and AI-2. AI-2 has been shown to regulate by QS the expression of the *luxCDABEGH* in *Vibrio harveyi* (5). LuxS-mediated QS has also been linked to the

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and description	Source or reference
Strains		
V. anguillarum		
M93Sm	Spontaneous Sm^r mutant of M93 (serotype J-O-1)	Denkin & Nelson (13)
M99	Sm^r Cm^r; M93Sm *empA* mutant	This study
M01	Sm^r Cm^r; M93Sm *luxS* mutant	This study
M03	Sm^r Cm^r; M93Sm *rpoS* mutant	This study
NB10	Wild type (serotype O1)	Milton et al. (26)
NB12	Cm^r; NB10 *empA* mutant	Milton et al. (26)
NB03	Sm^r Cm^r; NB10 *rpoS* mutant	This study
V. harveyi		
BB120	Wild type	Bassler et al. (43)
BB152	*luxMN* (AI-1$^-$ AI-2$^+$)	Bassler et al. (43)
BB170	*luxN*::Tn*5* (sensor 1$^-$ sensor 2$^+$)	Bassler et al. (43)
E. coli		
SM10	*thi thr leu tonA lacY supE recA* RP4-2-Tc::Mu::Km (λ *pir*)	Milton et al. (26)
JB525	Tc^r; MT102(pJBA132)	Andersen et al. (2)
JM109L	Ap^r; JM109(pSB1075)	Winson et al. (47)
Plasmids		
pNQ705-1	Cm^r; suicide vector with R6K origin	Milton et al. (26)
pempA	Cm^r Tc^r; pSUP202 with 4-kbp *empA*	Milton et al. (26)
pJBA132	*luxR*-P$_{luxI}$-RBSII-*gfp* (ASV) in pME6031 (detects C$_6$- to C$_8$-AHLs)	Andersen et al. (2)
pSB1075	*luxCDABE* fused to *lasRI′* in pUC18 (detects C$_{10}$- to C$_{12}$-AHLs)	Winson et al. (47)
pNQEmpA	Cm^r; 440-bp *empA* in pNQ701-1	This study
pNQLuxS	Cm^r; 270-bp *luxS* in pNQ705-1	This study
pNQRpoS	Cm^r; 250-bp *rpoS* in pNQ705-1	This study

expression of the genes in enterohemorrhagic *Escherichia coli* O157:H7 and enteropathogenic *E. coli* during attaching and effacing in the intestine (42).

It is hypothesized that the EmpA metalloprotease of *V. anguillarum* is important for virulence during infection of Atlantic salmon (*Salmo salar*). Extracellular proteases are secreted during infection in many pathogenic bacteria. The host tissue damage observed in infected fish suggests the production of proteases by *V. anguillarum*. The elastolytic protease of *P. aeruginosa* is required for tissue destruction during opportunistic infections (33). The VVP metalloprotease of *V. vulnificus* causes hemorrhagic damage by digesting type IV collagen after being injected intradermally into dorsal skin of guinea pigs (29). These proteases, which both result in destructive tissue damage, are highly homologous to EmpA metalloprotease of *V. anguillarum*.

In this study, wild-type strains (NB10 and M93Sm) and *empA* mutants (NB12 and M99) of *V. anguillarum* were tested for virulence in Atlantic salmon when introduced by intraperitoneal (i.p.) injection and by anal intubation (AIB). The two wild-type strains were chosen because they have different serotypes and we had previously examined the induction of protease activity in M93Sm (13), while Milton and her coworkers had examined *empA* in NB10 (26). We examined and characterized the differences in expression of *empA* in NB10 and M93Sm cells incubated in LB20 and mucus, using Northern blot analysis. Differences in EmpA activity in cell supernatants were also demonstrated by the azocasein protease assay. The *empA* genes in NB10 and M93Sm were sequenced and compared to determine and characterize the promoter and other possible upstream regulatory regions. Additionally, the effect of an *rpoS* mutation on EmpA expression was determined. The possibility of extracellular QS-directed expression of *empA* was also examined with regard to AHLs and AI-2.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All bacterial strains and plasmids used in this report are listed in Table 1. *V. anguillarum* strains were routinely grown in Luria-Bertani broth plus 2% NaCl (LB20) (15, 44), supplemented with the appropriate antibiotic, on a rotary shaker at 27°C. *V. harveyi* strains were grown in autoinducer bioassay (AB) broth (16) on a rotary shaker at 30°C. Experimental media included LB20, marine minimal medium (3 M) (15), and nine-salt solution (NSS; a carbon-, nitrogen-, and phosphorus-free salt solution) plus 200 μg of mucus protein/ml (NSSM) (15). Gastrointestinal mucus was harvested from Atlantic salmon as previously described by Garcia et al. (15). Overnight cultures of *V. anguillarum* were grown in LB20 and centrifuged (9,000 × *g*, 10 min), and pelleted cells were washed twice with NSS (15). Washed cells were resuspended to appropriate cell densities in experimental media. Specific conditions are described in the text for each experiment. Cell densities were determined by serial dilution and plating on LB20 agar plates or by measuring optical density at 600 nm (OD_{600}). Antibiotics were used at the following concentrations: streptomycin, 200 μg/ml; chloramphenicol, 5 μg/ml; kanamycin, 40 μg/ml; ampicillin, 100 μg/ml; and tetracycline, 200 μg/ml.

Bacterial matings. Plasmids were introduced into *V. anguillarum* M93Sm from *E. coli* SM10 by conjugation by the procedure described by Milton et al. (27, 28). Briefly, overnight cultures of *V. anguillarum* M93Sm and *E. coli* SM10 were prepared and mixed at ratios of 1:1 or 3:1 (recipient to donor) in NSS plus 10 mM $MgSO_4$. The cell suspension was vacuum filtered onto a 0.22-μm-pore-diameter nylon membrane, which was placed on an LB15 agar plate and allowed to incubate overnight at 27°C. Following incubation, the cells were removed from the filter by vigorous mixing in NSS plus 10 mM $MgSO_4$. The cell suspension (100 μl) was plated on LB20 with 200-μg/ml streptomycin and 5-μg/ml chloramphenicol and allowed to incubate at 27°C until *V. anguillarum* colonies were observed (usually 16 to 24 h).

Site-directed mutagenesis of *empA*, *luxS*, and *rpoS*. Site-directed mutagenesis was used to create gene interruptions within the structural genes of *empA*, *luxS*, and *rpoS*. Primers (Table 2) were generated based on the *empA* sequence for *V. anguillarum* NB10 (accession no. L02528) and M93Sm (accession no. AY428808), the *luxS* sequence for *V. harveyi* (accession no. AF120098),

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TABLE 2. Primers used in this study

Primer	Sequence[a]
pNQ705-R	5′-GCGTAACGGCAAAAGCACCGCCGGACATCA-3′
empA-F	5′-GCTATTCATGTACCGACGCG-3′
empA-R	5′-CGGAAGGATTTGAAAATGTCGC-3′
*empA*USP1	5′-GTTATGTGCACTATTAAATGTAC-3′
*empA*DSP1	5′-GACGCTCGTTTTGCACTATTTTTC-3′
*empA*USP2	5′-GATCCATTTTCAATGAGCGAATC-3′
*empA*DSP2	5′-GGATTGAGAAATGAAAAAAGTAC-3′
*empA*P2–1	5′-GCTGGTGGTTGATTGGATGTAAG-3′
*empA*P2–2	5′-GGATGTAAGTCGTCAGCGGATGC-3′
*empA*P2–3	5′-GCGGATGCTAATAATAACTCTC-3′
*empA*P2–4	5′-TAATAATAACTCTCTAGGATTGAG-3′
*empA*TXNST	5′-GAATCATTCGCAGTCACGTC-3′
empA-GSP1	5′-GCGTAAGTGCAATACTAA-3′
empA-GSP2	5′-GGTGAAGTACTTACCACATCAGAA-3′
5′RACE-AAP	5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′
5′RACE-AP	5′-GGCCACGCGTCGACTAGTACAAAAAAAAAAAAAAA-3′
SD*empA*-F	5′-GCTAGGAGCTCAAACCATGCAGAGGCG-3′
SD*empA*-R	5′-GCTAGTCTAGACCAAAGGTCATGGACG-3′
SD*luxS*-F2	5′-GCTAGGAGCTCCGTATGAATGCACC**R**GCGG-3′
SD*luxS*-R	5′-GCTAGTCTAGAGGCG**Y**ACCAATCAAGCTCATG-3′
SD*rpoS*-F	5′-GCTAGGAGCTCCGTGG**Y**GATGAAGCSGC**W**CG-3′
SD*rpoS*-R	5′-GCTAGTCTAGACGCA**G**ATAAATGTTCAGCTC-3′

[a] Restriction sites for SacI (GAGCTC) and XbaI (TCTAGA) are underlined, and mixed bases are in bold (**R** = A or G, **S** = G or C, **W** = T or A, and **Y** = C or T).

and *Vibrio cholerae* (accession no. AE004141), and the *rpoS* sequence for *V. harveyi* (accession no. AF321124), *Vibrio parahaemolyticus* (accession no. AF144608), and *V. cholerae* (accession no. AF000945). Sequences from multiple organisms were aligned, and primers were synthesized by using highly homologous regions. A 440-bp region from *empA* was PCR amplified with QIAGEN Taq DNA polymerase and cloned into the suicide vector pNQ705 by using SacI and XbaI restriction endonucleases to yield pNQSD8. The presence of the 440-bp *empA*-derived insert was confirmed by both PCR amplification and restriction analysis with SacI and XbaI. The mobilizable suicide vector containing *empA* was transferred into *V. anguillarum* by conjugation with *E. coli* SM10. *E. coli* SM10 contains the λ *pir* protein that is required for replication of pNQ705. Chloramphenicol-resistant colonies were selected and screened for insertion within *empA*. PCR and southern blot analysis were used to confirm the incorporation of pNQSD8. For PCR analysis, a primer described previously by Milton et al. (26) complementary to the pNQ705 vector was utilized (Table 2). The forward primer, SD*empA*-Forward (Table 2), is complementary to a region upstream of the insertion. PCR products were analyzed by electrophoresis through a 1.0% agarose gel in Tris-acetate EDTA (TAE) (4) buffer containing 0.2-μg/ml ethidium bromide. The gene was interrupted within the 440-bp region of *empA*, rendering the mutants resistant to chloramphenicol at 5 μg/ml. The resulting *V. anguillarum empA* mutant was designated as M99 (Table 1). For creating *luxS* and *rpoS* mutants, a 270-bp fragment and a 250-bp fragment, respectively, were PCR amplified separately and used in the same protocol as for the *empA* gene interruption.

Fish infections. *V. anguillarum* cells were prepared by growing in LB20 for 16 h on a rotary shaker at 27°C to a density of ~2×10^9 CFU/ml. Cells were harvested by centrifugation, washed twice in NSS, resuspended in NSS, and diluted to the appropriate inoculation concentrations. Inoculum dosages were determined by serial dilution and plating on LB20 agar plates. Juvenile Atlantic salmon were infected with wild-type or mutant strains via either of two routes of administration: i.p. or AIB. In each case, fish were anesthetized in water supplemented with tricaine methane sulfonate (75 mg/liter) before challenge. Fish were inoculated i.p. or AIB with equal volumes (50 μl) of cells (ranging from ~10^4 to 10^7 CFU/ml) in NSS or with NSS alone (control fish). Death due to vibriosis was determined by the observation of gross clinical signs and confirmed by the recovery and isolation of *V. anguillarum* cells that were resistant to the appropriate antibiotics from infected organs of dead fish. Observations for clinical signs of vibriosis were continued for 21 days.

Detection and quantification of protease activity. Culture supernatants were assayed for proteolytic activity according to our previously described modification of the method by Windle and Kelleher (13, 45). Briefly, culture supernatant was incubated with azocasein (5 mg/ml) dissolved in Tris-HCl (50 mM, pH 8.0) containing 0.04% NaN_3. Culture supernatant was prepared by centrifugation of 1 ml of cells at 12,000 × *g* (10 min, 20°C). Supernatant was removed and filtered through a 0.22-μm-pore-size cellulose-acetate filter. Filtered supernatant (100 μl) was incubated at 30°C with 100 μl of azocasein solution. An incubation time of 30 min was sufficient for assays of supernatants from cell suspensions of ≥5×10^8 cells/ml. Reactions were terminated by addition of trichloroacetic acid (TCA) (10% [wt/vol]) to a final concentration of 6.7% (wt/vol). The mixture was allowed to stand for 1 to 2 min and centrifuged (12,000 × *g*, 4 min) to remove unreacted azocasein, and supernatant containing azopeptides was suspended in 700 μl of 525 mM NaOH (45). Absorbance of the azopeptide supernatant was measured at 442 nm with a Pharmacia Ultrospec 4000 spectrophotometer. A blank control was prepared by boiling *V. anguillarum* M93Sm supernatant (100°C, 10 min). TCA was added to the blank control supernatant immediately after the addition of azocasein. The mucus used was also boiled (10 min) to destroy any inherent protease activity. Protease activity units were calculated with the following equation: 1 protease activity unit = [1,000 (OD_{442})/CFU] × (10^9) (13).

Northern blot analysis. Samples (1 ml) of cells were centrifuged (12,000 × *g*, 2 min, at 4°C) and cell pellets were collected and stored at −75°C. Total RNA was isolated from *V. anguillarum* M93Sm and NB10 cell pellets by using the Purescript RNA isolation kit (Gentra Systems, Inc., Minneapolis, Minn.) or using the RNeasy purification kit (QIAGEN). RNA samples were treated with RQ1 (RNase-free) DNase (1 U/μl) according to the manufacturer's specifications (Promega, Madison, Wis.). RNA (5 μg) was prepared according to the method of Ausubel et al. (4) and electrophoresed at 80 V for 90 min through a 1% agarose gel containing 1.1% formaldehyde in a mixture containing 1× MOPS [0.4 M 3-(*N*-morpholino)propanesulfonic acid], 0.2 M sodium acetate (pH 7.0), and 0.01 M Na_2EDTA (1 M Na_2EDTA stock solution to pH 8.0). All buffers, reagents, and solutions were made with 0.1% diethylpyrocarbonate-treated water (Sigma). After electrophoresis, the RNA gel was rinsed in distilled water twice and then washed in 10× sodium chloride-sodium citrate (SSC; 3 M NaCl, 0.3 M sodium citrate, pH 7.0) at room temperature for 45 min. RNA was transferred to a nylon membrane (Magna Graph; Osmonics, Inc.) using Turboblotter, a rapid downward neutral transfer system according to the directions of the manufacturer (Schleicher & Schuell, Keene, N.H.). RNA was bound to the nylon membrane by using a UV cross-linker (UVC-500; Hoefer Pharmacia Biotech, Inc., San Francisco, Calif.). The blot was probed with a digoxigenin (DIG)-dUTP labeled probe. The *empA* gene probe was constructed by purifying the pempA plasmid from *E. coli* DH1 by using a Promega Wizard plus miniprep DNA purification system (Promega) according to the manufacturer's instructions. The *empA* gene was amplified by PCR and labeled with DIG by using a PCR-DIG probe synthesis kit (Roche). The primers used to amplify *empA* were *empA*-F and *empA*-R (Table 2). A pempA (Table 1) sample (60 ng) was amplified with Taq polymerase (3.5 U/100 μl; Gibco BRL Life Technologies, Bethesda, Md.) on a Perkin-Elmer GeneAmp model 9700 Thermocycler (Perkin-Elmer). Gene probes for *vanM* and *vanI* were PCR amplified and DIG labeled with a PCR-DIG probe synthesis kit (Roche) from total genomic DNA. Genomic DNA was prepared with a QIAGEN Dneasy tissue kit. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa). The PCR cycle conditions were 94°C for 1 min, 51°C for 2 min, and 72°C for 3 min. The reaction was run for 35 cycles and then held at 4°C until collected. The primers used for PCR amplification, derived from the *empA* gene sequence (26), were *empA*-F and *empA*-R.

Solvent extraction of conditioned supernatant. A modification of the ethyl acetate extraction method described by Holden et al. (18) was used to obtain AHLs from culture supernatants. Briefly, conditioned cell culture supernatants were prepared from bacterial cultures grown (rotary shaker at 27°C, 20 h) in the experimental media described above unless otherwise noted. Cells were removed by centrifugation (7,000 × *g*, 10 min, 20°C). The cell supernatant was transferred to a screw-cap glass test tube and extracted twice for 30 to 60 s with acidified (100 μl of glacial acetic acid/liter) ethyl acetate (3 parts acidified ethyl acetate to 2 parts supernatant). Organic and aqueous phases were separated by centrifugation (2,000 × *g*, 5 min, 20°C). The organic phase was transferred to a 20-ml glass test tube and dried under nitrogen gas in a 40°C water bath. The dried samples were dissolved in 50 to 100 μl of 100% ethanol and then concentrated to a final volume of 10 μl.

AHL detection with *gfp*- and *lux*-based reporter *E. coli strains*. *V. anguillarum* strains were grown overnight and resuspended under the appropriate experimental condition. Cell-free conditioned supernatant was prepared at indicated

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TABLE 3. Virulence of *V. anguillarum* wild-type strains M93Sm and NB10 and *empA* mutants M99 and NB12 in Atlantic salmon by i.p. injections[a]

Strain	Dose/fish (CFU)	Mortality (no. dead/no. injected)	Day of death (no. dead/no. injected)
M93Sm	2.20×10^4	1/5	3 (1/5)
	3.03×10^5	3/5	2 (1/5), 3 (2/5), 4 (3/5)
	2.93×10^6	4/5	1 (1/5), 2 (3/5), 3 (4/5)
NB10	3.83×10^4	2/5	2 (1/5), 4 (2/5)
	3.27×10^5	2/5	3 (1/5), 5 (2/5)
	3.73×10^6	4/5	3 (3/5), 4 (4/5)
M99	2.63×10^4	1/5	3 (1/5)
	2.87×10^5	3/5	3 (1/5), 4 (3/5)
	2.60×10^6	5/5	1 (1/5), 2 (5/5)
NB12	4.43×10^4	0/5	NA[b]
	3.87×10^5	0/5	NA
	4.03×10^6	0/5	NA
Control (NSS)		0/15	NA

[a] Fish were injected with 50 μl of washed cells suspended in NSS (experimental) or NSS alone (control) at the indicated dosage amount. The experiment continued for 21 days. Death due to vibriosis was confirmed by observing clinical signs and isolation of streptomycin-resistant *V. anguillarum* cells.

[b] NA, not applicable: no death due to vibriosis during the 21-day experiment.

time points and extracted twice with acidified ethyl acetate and then dried under nitrogen. Dried material from extracts was dissolved in 100 μl of 100% ethanol. Samples were further dried and resuspended in 10 μl of 100% ethanol and then used in the *gfp*- or *lux*-based reporter assays. The *gfp*-based reporter strain *E. coli* JB525 (2) was used for detection of C_6- to C_8-AHLs. This reporter strain contains a *gfp* fusion to the *rhlI* promoter from *P. aeruginosa* PA01, allowing *gfp* expression in response to C_6- to C_8-AHLs. To detect longer-chain AHLs (C_{10} to C_{14}), the *lux*-based reporter strain *E. coli* JM109 containing pSB1075 (47) was used. This second reporter strain contains the *lasRI* promoter region coupled to the entire *lux* structural operon. Fluorescence by green fluorescent protein (GFP) was measured with a spectrofluorimeter (Perkin-Elmer; LS55). Luminescence from the *lux* reporter was measured with a luminometer, (Turner Designs; model TD-20/20).

Preparation of cell-free conditioned medium for AI-2 assay. To prepare cell-free conditioned medium, *V. anguillarum* strains were grown overnight in LB20 without antibiotics on a rotary shaker at 27°C. Cells were centrifuged at 9,000 × *g* for 10 min, and the cell pellets were washed twice in NSS. Cells were diluted 1:100 (~2×10^7 to 3×10^7 CFU/ml) into LB20 or NSSM and allowed to incubate with shaking at 27°C for 10 h. *V. anguillarum* cells were removed by centrifugation (9,000 × *g*, 10 min), and the resulting supernatant was passed through a 0.22-μm-pore-size acetate filter. Samples were stored at −20°C. *V. harveyi* BB152 conditioned medium was prepared as described by Surette and Bassler (43).

***V. harveyi* luminescence bioassay for AI-2.** The assay for AI-2 production was performed as described by Surette and Bassler (43). *V. harveyi* BB170 was grown overnight and diluted 1:5,000 into fresh AB medium. Prepared cell-free conditioned medium was added at 10% (vol/vol) (100 μl) to the diluted sensor strain (900 μl) and allowed to incubate for 3 h with aeration at 30°C. As a control, conditioned supernatant from *V. harveyi* BB152 was added to the AI-2 sensor strain BB170 and luminescence was measured. Aliquots (500 μl) were taken, and luminescence was measured with a luminometer (Turner Designs; model TD-20/20).

Identification of the transcriptional start site for *empA*. Total RNA was isolated from *V. anguillarum* cells incubated in either LB20, NSSM, 3 M, or NSS as described above for Northern blot analysis. The OneStep reverse transcription (RT)-PCR system from QIAGEN was used to approximate the *empA* transcriptional start. Primers used in RT-PCR are listed in Table 2. To identify the transcriptional start site, RNA was subjected to 5′ rapid amplification of cDNA ends (RACE) using the Invitrogen 5′ RACE kit. Briefly, 5 μg of RNA was used to generate specific first-strand cDNA from *empA* mRNA in a reverse transcriptase reaction with an *empA* primer (empA-GSP1). A poly(dC) or poly(dA) tail was added to the 3′ cDNA end with dCTP or dATP and terminal deoxynucleotidyl transferase. A PCR product was amplified from the tailed cDNA by using a 5′ RACE-abridged anchor primer (AAP) or a 5′ RACE anchor primer (AP) (Table 2) and a nested *empA* primer (empA-GSP2). The PCR product was purified with a PCR Qiaquick spin kit (QIAGEN) and sequenced. DNA sequences were aligned by using the Baylor College of Medicine search launcher multiple sequence alignment program ClustalW 1.8 (http://searchlauncher.bcm.tmc.edu).

DNA sequencing. DNA sequencing was performed by the HHMI Biopolymer/Keck Foundation Biotechnology Resource Laboratory at Yale University (New Haven, Conn.) and by the University of Rhode Island Genomics and Sequencing Center (Kingston, R.I.). Sequencing was performed on Applied Biosystems 377 and 3100 DNA analysis instruments (Keck) and on a Beckman-Coulter CEQ 8000 (URI). Fluorescent-labeled dideoxynucleotide Big Dye terminators with Taq FS DNA polymerase (Keck) and the Dye Terminator Cycle Sequencing (DTCS) quick start kit (URI) in thermal cycling programs were used in the sequence reactions. DNA samples were mixed with the appropriate primer (Table 2) and then submitted for sequencing.

RESULTS

Role of EmpA as a virulence factor during infection of Atlantic salmon. In order to examine the role of EmpA as a virulence factor, juvenile Atlantic salmon were infected in separate experiments by i.p. injection or by AIB with *V. anguillarum* wild-type strains M93Sm and NB10 or *empA* mutant strains M99 and NB12. The results of i.p. infections shown in Table 3 reveal that both *V. anguillarum* wild-type strains (M93Sm and NB10) were equally virulent. Infection with 3×10^6 to 4×10^6 CFU by the i.p. route resulted in 80% mortality by 3 to 4 days. The *empA* mutant strain *V. anguillarum* M99 exhibited virulence equal to that of the parental wild-type strain (M93Sm). In contrast, i.p. inoculation of salmon with 4×10^6 CFU of the *empA* mutant strain *V. anguillarum* NB12 resulted in no salmon mortalities over the 21-day experiment.

In a second series of experiments, salmon were infected with wild-type (M93Sm and NB10) and *empA* mutant strains (M99 and NB12) of *V. anguillarum* by AIB (Table 4). *V. anguillarum* wild-type strains M93Sm and NB10 administered by AIB at doses of 7.15×10^6 and 6.50×10^6 CFU, respectively, resulted in 80 to 100% mortality after 10 to 11 days. Both *empA* mutant strains exhibited attenuated virulence compared to their wild-type parental strains. AIB infections of salmon with strain M99 using 1.04×10^7 CFU resulted in 40% mortality to the fish. AIB infection of salmon with strain NB12 using 8.15×10^7 CFU caused no deaths in the infected salmon over the 21-day experiment. Control fish inoculated with NSS only by either the i.p. or AIB route did not show any signs of vibriosis during the 21-day experiment. These data suggest that EmpA is a virulence factor that is important during the infection of the GI tract of Atlantic salmon by *V. anguillarum*.

Northern blot analysis of *empA* expression in LB20 and NSSM. We have previously demonstrated that salmon GI mucus is a specific inducer of the EmpA metalloprotease in *V. anguillarum* M93Sm when cells are at high density (13). The expression of *empA* was examined by Northern blot analysis in M93Sm and NB10 (Fig. 1A). Since maximum protease activity was observed at high cell density (≥1×10^9 CFU/ml) (13), *empA* expression was examined in cells suspended at 2×10^9 CFU/ml. When cells of strain NB10 were incubated in LB20, *empA* mRNA was first detected 90 min after the start of incubation. Maximum levels of transcription were observed at 120 and 180 min. In contrast, no detectable *empA* message was detected in cells of strain M93Sm incubated in LB20 under

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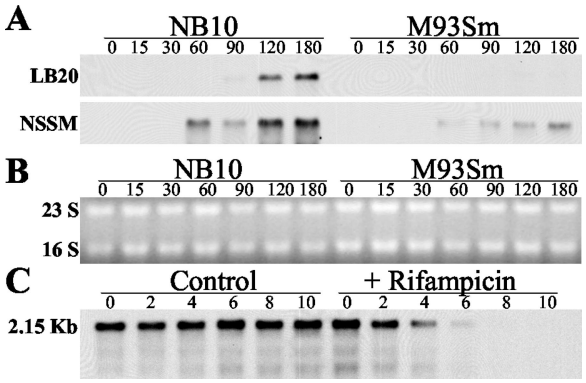

FIG. 1. Northern blot analysis of *empA* expression in LB20 and NSSM. (A) *V. anguillarum* strains NB10 and M93Sm were grown overnight in LB20 to stationary phase. Cells were washed twice in NSS and resuspended under the experimental conditions at 2×10^9 CFU/ml. Cells were incubated (27°C) with shaking. Samples (1 ml) were taken at 0, 15, 30, 60, 90, 120, and 180 min. Total RNA was isolated and separated on a formaldehyde agarose gel (5 μg of RNA/sample). RNA was transferred from the gel to a nylon membrane and probed with a DIG-dUTP-labeled *empA* probe for 16 h at 51°C. Panel B shows the RNA gel from panel A stained with ethidium bromide to demonstrate equal loading of all samples. (C) mRNA half-life for *empA* was determined in M93Sm cells incubated in NSSM for 3 h. Rifampin was added to the cell suspension, and cell samples (1 ml) were taken at 0, 2, 4, 6, 8, and 10 min. Total RNA was prepared and analyzed as described above for panel A.

identical conditions. When cells of either strain NB10 or M93Sm were incubated in NSSM, *empA* message was detected by 60 min, with maximum levels of expression observed at 180 min. Comparison of the amounts of *empA* transcript produced by each strain revealed that NB10 produced approximately threefold more than M93Sm at 180 min. The calculated size for the *empA* transcript of each strain was 2.15 kb. To determine the *empA* message half-life, cells were incubated in NSSM for 3 h. Rifampin was added to an aliquot of NSSM-incubated cells to block further mRNA synthesis, and samples were taken every 2 min for RNA preparation. The half-life of *empA* mRNA from M93Sm cells induced in mucus was 2.5 min (Fig. 1C). In both experiments, equal amounts of RNA (5 μg) were loaded into each lane. The half-life of the *empA* transcript in NB10 was similar to that of M93Sm (data not shown).

TABLE 4. Virulence of *V. anguillarum* wild-type strains M93Sm and NB10 and *empA* mutants M99 and NB12 in Atlantic salmon by AIB[a]

Strain	Dose/fish (CFU)	Mortality (no. dead/no. intubated)	Day of death (no. dead/no. intubated)
M93Sm	9.15×10^5	3/5	5 (1/5), 10 (3/5)
	7.15×10^6	4/5	2 (1/5), 6 (2/5), 9 (3/5), 11 (4/5)
	2.77×10^7	5/5	2 (2/5), 3 (3/5), 4 (4/5), 6 (5/5)
NB10	1.42×10^6	4/5	3 (1/5), 5 (2/5), 10 (3/5), 11 (4/5)
	6.50×10^6	5/5	2 (2/5), 5 (3/5), 8 (4/5), 10 (5/5)
	5.35×10^7	5/5	2 (1/5), 7 (2/5), 8 (3/5), 9 (5/5)
M99	9.50×10^5	1/5	2 (1/5)
	3.47×10^6	1/5	2 (1/5)
	1.04×10^7	2/5	4 (1/5), 6 (2/5)
NB12	1.68×10^6	0/5	NA[b]
	1.04×10^7	0/5	NA
	8.15×10^7	0/5	NA
Control (NSS)		0/15	NA

[a] Fish were anally intubated with 50 μl of washed cells suspended in NSS (experimental) or NSS alone (control) at the indicated dosage amount. A small amount of phenol red was added to the cell suspension to ensure there was no leakage. The experiment continued for 21 days. Death due to vibriosis was confirmed by observing clinical signs and isolation of streptomycin-resistant *V. anguillarum* cells.

[b] NA, not applicable: no death due to vibriosis during the 21-day experiment.

Protease activity in LB20 and NSSM. In addition to examining *empA* expression at the transcriptional level, cell-free supernatant was assayed with azocasein (13) to determine the amount of protease activity. Cell supernatant was prepared from cells that were used to examine *empA* expression in Fig. 1. When NB10 cells were incubated in LB20, significantly increased protease activity was observed at 120 min (Fig. 2). While a low level of *empA* message could be detected at 90 min in LB20-incubated NB10 cells (Fig. 1), *empA* was strongly expressed by 120 min. In contrast, protease activity was not detected in LB20-incubated M93Sm cells. When NB10 or M93Sm cells were incubated in NSSM, protease activity was first detected at 60 and 90 min, respectively. Protease activity for both wild-type cells continued to increase during the 180-min experiment, with NB10 cells exhibiting 1.5-fold-greater protease activity than M93Sm in NSSM at 180 min.

Analysis of *empA* DNA sequence from M93Sm and NB10. In order to determine whether upstream regulatory sequences in the *empA* gene could account for the differences in *empA* expression between strains M93Sm and NB10, the *empA* gene of M93Sm was cloned, sequenced (accession no. AY428808), and compared to that of NB10. The sequence alignment of *empA* genes from M93Sm to NB10 is shown in Fig. 3. The structural gene was >99% identical at the nucleotide level to *empA* in NB10. Three (out of 14) different base pair changes in the structural gene of *empA* resulted in a changed amino acid

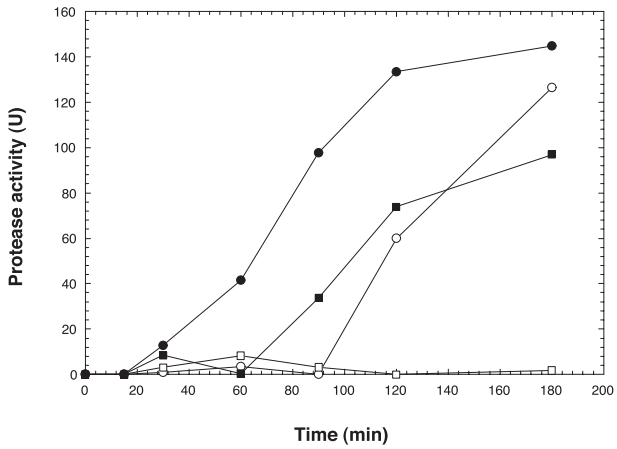

FIG. 2. Induction of protease activity in *V. anguillarum* M93Sm (squares) and NB10 (circles) cells incubated in LB20 or NSSM. Cells were grown for 16 h in LB20, harvested by centrifugation (10 min at 9,000 × *g*, 4°C), washed twice in NSS, and resuspended at 2×10^9 CFU/ml in either LB20 (open symbols) or NSSM (solid symbols). Samples were taken at the indicated times, and the cell supernatant was assayed for protease activity.

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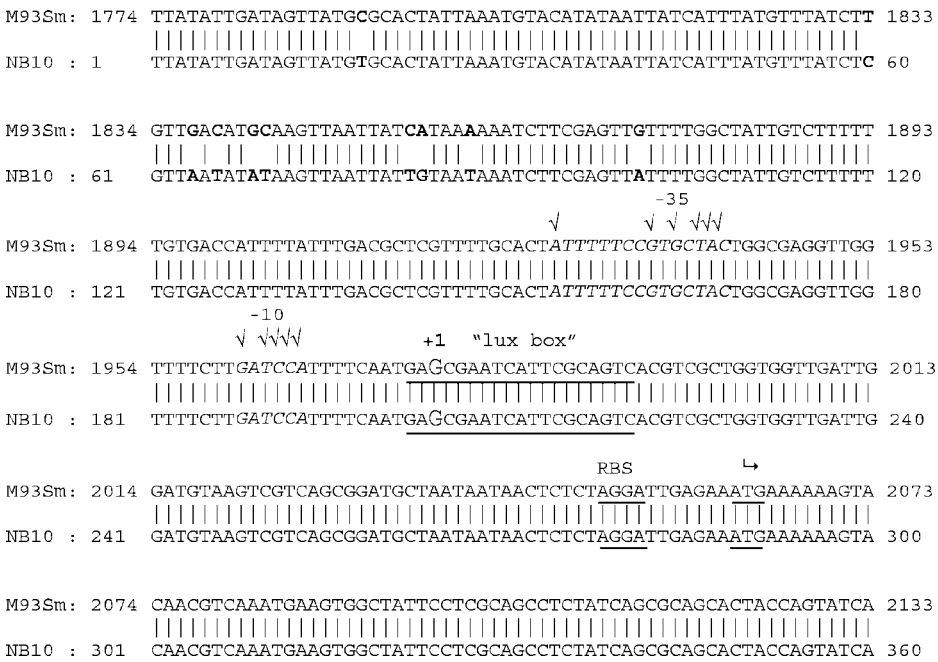

FIG. 3. DNA sequence alignment of the *empA* regulatory and 5′ regions in *V. anguillarum* M93Sm and NB10. The −10 and −35 promoter regions are italicized, and check marks indicate matches with consensus. The transcriptional start site is labeled +1. The ribosomal binding site is labeled RBS and is underlined. The translational start site (AtG) is denoted with a bent arrow and is underlined. The differences observed between the two sequences are in bold. The potential *lux* box region is underlined.

(data not shown). The methionines at amino acids 57 and 102 in M93Sm are a valine and leucine, respectively, in NB10. The valine at amino acid 237 in M93Sm is a methionine in NB10. However, ten base pair differences were detected between 188 and 228 bp upstream of the translational start site. There were no other differences detected between the two sequences. A putative *lux* box reported (26) between 69 and 87 bp upstream of the translational start in NB10 was found in the same position in M93Sm. The differences detected upstream of *empA* and within the structural gene were confirmed by sequencing the pempA clone from NB10.

Identification of the *empA* transcriptional start. To determine the approximate transcriptional start region for *empA*, RT-PCR using several sense-strand primers encompassing the predicted start (Table 2) in combination with a single antisense primer (empA-R; Table 2) was initially carried out. RT-PCR products were observed only with sense strand primers designed within 79 bp upstream of the translational start for *empA* (Fig. 4A). These results suggested that *empA* transcription begins between 53 and 91 bp upstream of the ATG start translation codon. 5′ RACE was then used to determine the transcriptional start for the *empA* promoter. Total RNA was extracted from protease-producing cells incubated in LB20 (NB10) or in NSSM (NB10 and M93Sm) and used in the 5′ RACE determination of the transcriptional start. A 480-bp 5′ RACE product was detected by using RNA from M93Sm and NB10 cells in NSSM (Fig. 4B). The 5′ RACE products were sequenced, and all cases revealed that the *empA* transcriptional start is a C or G located 84 to 85 bp upstream of the translational start (Fig. 4C). To determine if transcription begins at the C or G, 5′ RACE was repeated by using dA tailing. The 5′ RACE product obtained by dA tailing also yielded a 480-bp product (data not shown) that upon sequencing revealed the transcriptional start base as a G (Fig. 4D). Analysis of the −10 and −35 sequences (Fig. 3) showed high similarity to a σ^S promoter (17).

Effects of conditioned LB20 cell supernatants on expression of *empA*. Previously, we demonstrated that induction of EmpA activity required high cell density (13). The presence of a putative *lux* box suggested the possibility of cell density regulation of *empA*. In order to determine whether *empA* expression is induced by a secreted cell density-dependent factor, conditioned LB20 cell supernatant prepared from the *empA* mutant, M99, was added to stationary-phase LB20-grown cells of *V. anguillarum* strains M93Sm and NB10 (Fig. 5A). Protease activity in M93Sm cells resuspended in conditioned LB20 (at 10^9 CFU/ml) was induced by 140 min and reached a maximum activity by 180 min. No protease activity was observed in M93Sm cells resuspended in fresh LB20 (at 10^9 CFU/ml). Additionally, when NB10 cells were resuspended in conditioned LB20 (at 10^9 CFU/ml), EmpA protease activity was detected after 60 min of incubation. In contrast, NB10 cells suspended in fresh LB20 did not exhibit protease activity until 140 min. Furthermore, maximum levels of protease activity for NB10 cells in conditioned LB20 were about 55% greater than in NB10 cells incubated in fresh LB20. To confirm that EmpA was the protease induced, M99 was incubated in conditioned media and no activity was observed.

To demonstrate the dose-dependent nature of the inducer of protease activity in conditioned media, M93Sm cells were incubated in various concentrations of conditioned LB20 ranging from 100% to 0% (Fig. 5B). When M93Sm cells were incubated in undiluted (100%) conditioned LB20, protease activity was first observed at 120 min. However, protease activity was

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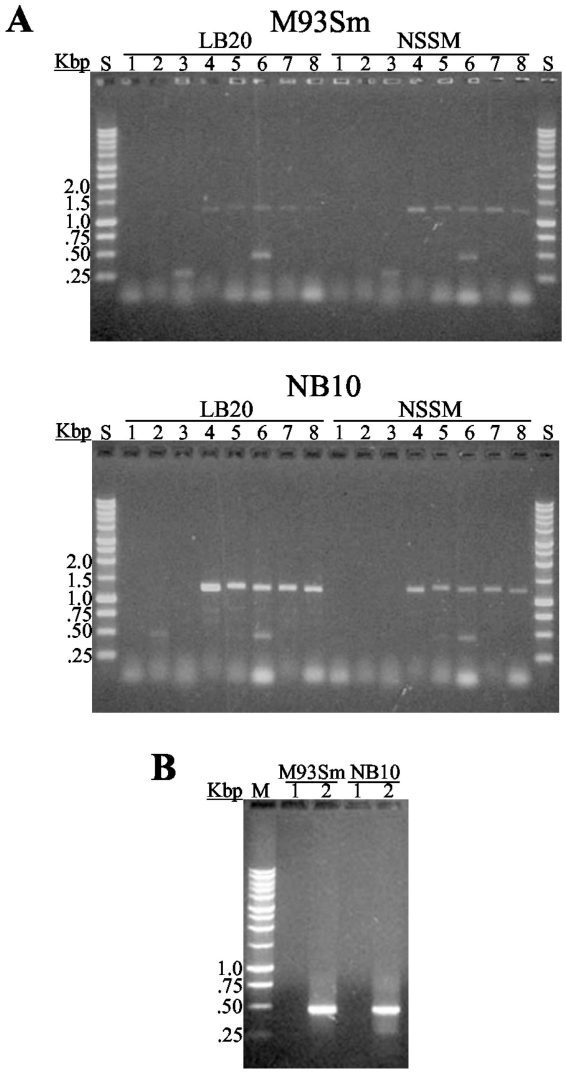

FIG. 4. Determination of *empA* transcriptional start by RT-PCR and 5′ RACE analysis. (A) Eight different *empA* sense-strand primers (lanes 1 to 8, primers *empA*USP1, *empA*DSP1, *empA*USP2, *empA*DSP2, *empA*P2-1, *empA*P2-2, *empA*P2-3, and *empA*P2-4, respectively) were used with an *empA* antisense strand primer (empA-R) to determine the approximate transcriptional start for *empA*. RNA from LB20- and NSSM-grown M93Sm (top panel) or NB10 (bottom panel) cells was prepared and used in the RT-PCRs. The RT-PCR products were visualized on a 1% agarose TAE gel containing ethidium bromide. Molecular weight standards (indicated in kilobase pairs) are in lanes S. (B) One percent agarose TAE gel showing 5′ RACE products (even-numbered lanes) from M93Sm and NB10 in NSSM. As a negative control, the tailing enzyme, terminal deoxynucleotidyl transferase, was left out of the reaction (odd-numbered lanes). A 1-kbp DNA ladder was used as the size standard (lane S). (C) DNA sequence alignments of dC-tailed and (D) dA-tailed 5′ RACE products showing the *empA* transcriptional start site (underlined) for NB10 in LB20 and NSSM and M93Sm under NSSM growth conditions.

not detected until 180 min for cells incubated in 87.5, 75, and 62.5% conditioned LB20. Maximum EmpA activity (measured at 240 min) declined as the conditioned medium was diluted with fresh LB20. At concentrations below 62.5% conditioned LB20, protease activity was not detected (data not shown).

Production of AHLs in LB20 and mucus. The production of AHLs in *V. anguillarum* M93Sm and NB10 was examined during growth in LB20 or NSSM by using *gfp*-based (for C_6-AHLs) and *lux*-based (for C_{10}-AHLs) reporter systems. Ethyl acetate extracts from cells grown in either LB20 or NSSM were added

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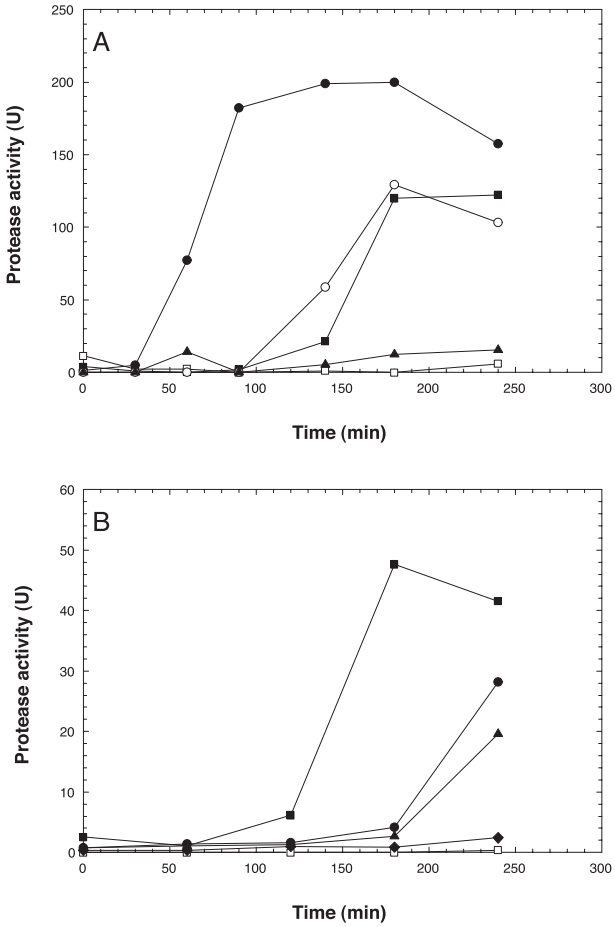

FIG. 5. (A) Induction of protease activity in *V. anguillarum* M93Sm (squares), NB10 (circles), and M99 (triangles) by conditioned LB20 cell supernatants. *V. anguillarum empA* mutant strain M99 was grown for 20 h in LB20, and conditioned supernatant was prepared as described in Materials and Methods. *V. anguillarum* cells were grown for 16 h to 2×10^9 CFU/ml in LB20, prepared as described in the legend to Fig. 1, and resuspended in conditioned LB20 supernatant (solid symbols) or fresh LB20 (open symbols). (B) Conditioned LB20 supernatant was prepared from M99 as described and diluted with fresh LB20. *V. anguillarum* M93Sm was grown overnight and resuspended at 2×10^9 CFU/ml in the diluted conditioned LB20: 100% (solid squares), 87.5% (solid circles), 75% (solid triangles), 62.5% (solid diamonds), and 0% (open squares).

to the *gfp* biosensor strain, and fluorescence intensities were monitored (Fig. 6A and B, respectively). No fluorescence was detected until the cells of either ethyl acetate-extracted strain reached densities of 4×10^8 to 5×10^8 CFU/ml in LB20 (Fig. 6A) or 1×10^9 to 1.5×10^9 CFU/ml in NSSM. Since no C_{10}-AHL production nor *vanI* message was detected until cells reached stationary phase (data not shown), ethyl acetate extracts from supernatants of cells incubated in either LB20 or NSSM at 2×10^9 CFU/ml were added to the *lux* biosensor strains to detect C_{10}-AHL production. Production of the C_{10}-AHL was detected within 1 h of incubation in LB20 in both M93Sm and NB10 (Fig. 6C). Maximum luminescence was detected by 2 h of incubation and declined thereafter. Additionally, M93Sm produced only ~67% of the C_{10}-AHL that NB10 produced in LB20. When cells were incubated in NSSM, much lower levels of C_{10}-AHL were detected for both strains (Fig. 6D). While C_{10}-AHL production could be detected in NB10 cells after 1 h of incubation, no significant increase in luminescence was detected in M93Sm cells until 3 h of incubation. Further, C_{10}-AHL production in NB10 cells incubated in NSSM was >2.4-fold higher than in M93Sm cells.

Effects of ethyl acetate extracts containing AHLs on protease activity. Since both M93Sm and NB10 produced AHLs when grown or incubated in LB20, conditioned supernatant from a stationary-phase culture of M99 cells was extracted for AHLs. The resulting ethyl acetate extracts, which contained levels of AHLs found in M93Sm (data not shown), were added to cells in order to determine whether the rate of EmpA induction or level of expression would be affected. No protease activity was observed when ethyl acetate extracts from either M99 conditioned LB20 or fresh LB20 was added to M93Sm cells suspended in LB20 (Fig. 7). Since protease activity is usually induced in NB10 cells in LB20 by 3 h (Fig. 2), ethyl acetate extracts from M99 conditioned LB20 were added to NB10 cells in LB20 to determine whether the rate of induction or level of expression would be affected. No increase in protease activity was observed compared to that with in incubation in ethyl acetate extract from fresh LB20. These data suggest that AHLs do not affect *empA* expression in either M93Sm or NB10 cells.

Effects of a *luxS* mutation on AI-2 production and protease activity. A *luxS* mutant, M01, of *V. anguillarum* M93Sm was created by site-directed mutagenesis and tested for AI-2 production. Conditioned media from M01 and M93Sm were assayed for AI-2 by using the *V. harvey*i BB170 sensor strain. The *luxS* mutant, M01, did not produce AI-2 when grown in LB20 or NSSM, whereas M93Sm did (data not shown). To determine if AI-2 had any effect on protease activity, M93Sm cells (2×10^9 CFU/ml) were resuspended in conditioned LB20 supernatant from M01 (Fig. 8A). Incubation in conditioned LB20 from M01 resulted in an earlier induction of protease activity in M93Sm compared to incubation in M99 conditioned LB20. Also, the levels of protease activity in M93Sm cells incubated in M01 conditioned LB20 for 240 min was 2.2-fold higher than the protease activity in M99 conditioned LB20. Additionally, when the *luxS* mutant, M01, was incubated in M01 conditioned LB20, protease activity was induced faster than when M01 was incubated in M99 conditioned LB20 (Fig. 8A). *V. anguillarum* M01 and M93Sm were compared for protease activity when incubated in NSSM at high cell density (2×10^9 CFU/ml). Both the *luxS* mutant and M93Sm exhibited the same rate of induction and amount of protease activity in mucus (Fig. 8B).

Effects of an *rpoS* mutation on protease activity in LB20 and NSSM. Since the −10 and −35 promoter regions for *empA* exhibited a strong similarity to the consensus sequence for a σ^S-regulated promoter, we searched for *rpoS* in *V. anguillarum*. Cells were grown, prepared, and assayed for protease activity as described in the legend to Fig. 2. PCR primers were designed based on the *rpoS* sequences from *V. cholerae*, *V. harveyi*, and *V. parahaemolyticus* (Table 2) and used to amplify a 250-bp product from *V. anguillarum* genomic DNA. Sequencing revealed 82% identity to *rpoS* of *V. cholerae* and *V. parahaemolyticus* and 83% identity to *rpoS* of *V. harveyi*. The two *rpoS* mutants (M03 and NB03) created by site-directed mu-

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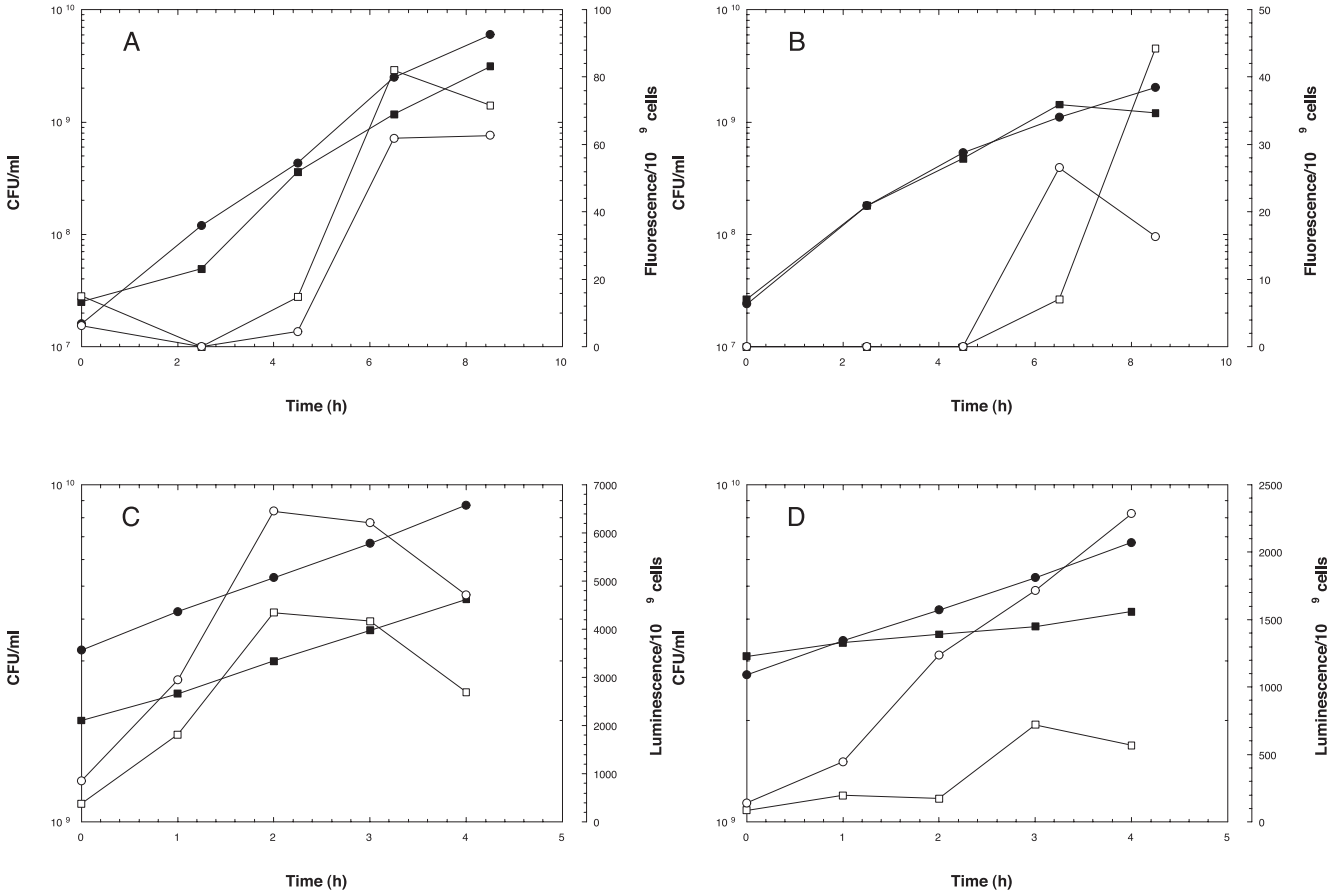

FIG. 6. AHL production measured with *E. coli* JB525 *gfp*-based (A and B) and *E. coli* JM109L *lux*-based (C and D) AHL sensor strains. Ethyl acetate extracts prepared from M93Sm (squares) and NB10 (circles) growing in LB20 (A and C) or NSSM (B and D) were added to *gfp*-based (which responds to C_6-C_8 AHLs) and *lux*-based (which responds to C_{10}-C_{12} AHLs) sensor strains and observed for stimulation of luminescence or fluorescence, respectively, was measured. The number of CFU per milliliter (solid symbols) was determined at each time point and is indicated in addition to *lux* or *gfp* activity (open symbols).

tagenesis were derived from the wild-type strains M93Sm and NB10, respectively. These mutants did not exhibit any protease activity when incubated in NSSM at 2×10^9 CFU/ml for 0 or 4 h. There was no protease activity at 0 h for M93Sm and NB10, whereas at 4 h, the levels of protease activity were 96.9 U for M93Sm and 133.4 U for NB10. No protease activity was observed in NB03 cells when incubated in LB20 (data not shown). These data strongly suggest that the regulation of *empA* metalloprotease in LB20 and NSSM is controlled by σ^S.

DISCUSSION

We have previously demonstrated that *empA* metalloprotease is strongly induced when cells at high density are incubated in Atlantic salmon GI mucus (13). These findings suggested a role for EmpA during colonization and amplification of the bacteria while in the salmon GI tract. In this study, we present data that demonstrate that *empA* is important for virulence by *V. anguillarum* in Atlantic salmon. Wild-type strains NB10 and M93Sm of *V. anguillarum* kill 60 to 80% of salmon when administered by i.p. injection or by AIB at doses of $\sim 1 \times 10^6$ total CFU/fish. The *empA* mutant strain, NB12 (derived from NB10), is completely avirulent in fish by either i.p. injec-

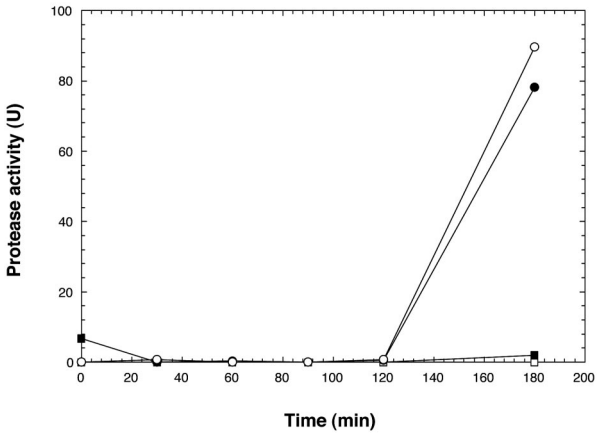

FIG. 7. Induction of protease activity in *V. anguillarum* M93Sm and NB10 by ethyl acetate extracts from conditioned LB20 supernatants. Conditioned supernatants from the *V. anguillarum empA* mutant, M99, were extracted with ethyl acetate. M93Sm (squares) and NB10 (circles) cells were suspended at 2×10^9 CFU/ml in fresh LB20 containing ethyl acetate extract from either M99 (closed symbols) or fresh LB20 (open symbols). As a control, M93 and NB10 cells were suspended in ethyl acetate extract from fresh LB20 (open symbols) and observed for normal induction of protease activity.

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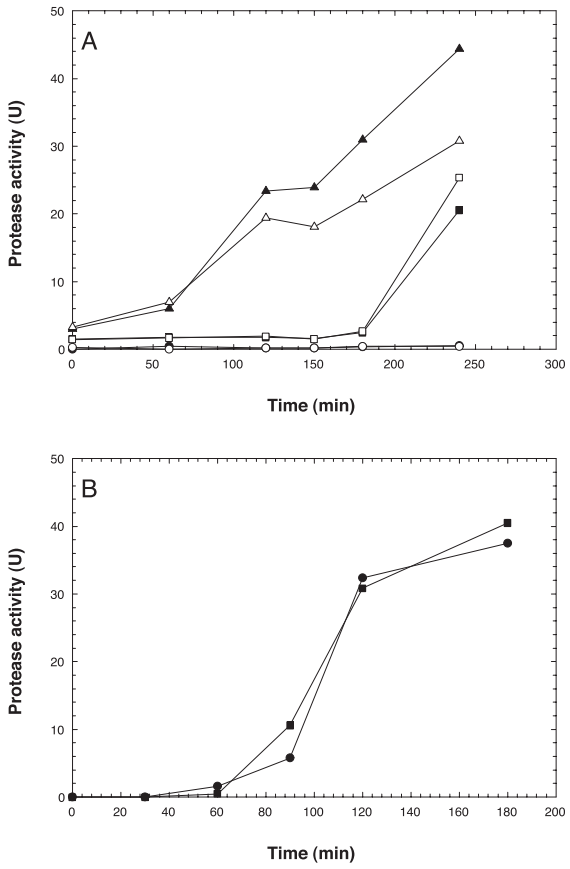

FIG. 8. (A) Induction of protease activity in *V. anguillarum* by conditioned LB20 supernatants from M01 (*luxS* mutant). *V. anguillarum* M93Sm (open symbols) and M01 (closed symbols) were grown for 16 h and resuspended at 2×10^9 CFU/ml in conditioned LB20 supernatant from M01 (triangles), M99 (squares), or fresh LB20 (circles). (B) Induction of protease activity in *V. anguillarum* M93Sm (squares) and M01 (circles) in Atlantic salmon GI mucus. Cells were grown overnight in LB20, washed twice in NSS, and resuspended at 2×10^9 CFU/ml in 200 μg of mucus protein/ml of NSS (NSSM).

tion or AIB. However, the *empA* mutant M99 (derived from M93Sm) exhibits wild-type virulence when i.p. injected, but is attenuated in virulence when administered by AIB. While these data show that *empA* is required for pathogenesis by *V. anguillarum* NB10 in Atlantic salmon, differences in virulence observed for M99 when administered by i.p. injection and AIB suggest a role for *empA* during colonization and amplification within the fish intestine. It should be emphasized that the *empA* mutations in NB12 and M99 are nearly identical, with insertions within the same 200- to 300-bp region in the first third of the coding sequence. While Milton et al. (26) demonstrated that the *empA* mutant, NB12, exhibited somewhat attenuated virulence when introduced into rainbow trout by i.p. injection or immersion (20- and 50-fold, respectively), it should be noted that the host species used in the two studies are different. Atlantic salmon show much greater resistance to *V. anguillarum* infection than rainbow trout. In Atlantic salmon, the 50% lethal dose (LD_{50}) of i.p.-injected *V. anguillarum* is $\sim 2 \times 10^5$ CFU, while in rainbow trout, the LD_{50} is 10^0 CFU.

The observation that the *empA* mutants M99 and NB12 exhibit differential virulence suggested that *empA* might be differentially regulated in the parental wild-type strains M93Sm and NB10. This was confirmed: M93Sm expresses *empA* only when at high cell density when incubated in GI mucus, while NB10 expressed *empA* when at high cell density in all growth media tested (NSSM, LB20, or 3 M). Further, both Northern blot analysis and protease activity assays show that *empA* is expressed at higher levels in NB10. Examination of the *empA* structural gene in M93Sm and NB10 sequences reveals 99.2% identity at the nucleotide level with three amino acid differences (amino acids 57, 102, and 237). The three amino acid differences are conserved and are located in the leader sequence for EmpA, not within the mature protein. Therefore, differences in protease activity in M93Sm and NB10 are not linked to the activity of EmpA. Further, the 187 nucleotides upstream of the start translation are identical. Additionally, 5′ RACE analysis shows that the transcriptional start site is identical in both strains under all conditions of *empA* expression. Both strains regulate transcription by identical σ^S-dependent promoters. This raises the question of why these two strains display differential regulation of *empA*. Since *vanT* (the *V. harveyi luxR* homolog) mutants do not produce protease (12; this study), it can be hypothesized that the regulation of *empA* involves VanT binding to a *lux* box. However, identical putative *lux* box sequences are found in each strain, which makes it unlikely that differences in *empA* expression are due to differences in binding efficiencies of VanT. We hypothesize that differences in *empA* expression may result from differences in nucleotide sequences of M93Sm and NB10 beginning 100 bp upstream of the promoter site.

Toxin-coregulated pilus (*tcpA*) is differentially expressed in classical and El Tor strains of *V. cholerae* (22, 31). Classical *V. cholerae* exhibits *tcpA* expression under many growth conditions, similar to *V. anguillarum* NB10 *empA* expression under multiple growth conditions and starvation at high density (data not shown). In contrast, *tcpA* expression in El Tor strains of *V. cholerae* occurs only under bicarbonate-containing (e.g., AKI media) growth conditions. In a similar manner, *V. anguillarum* M93Sm only produces protease when cells are incubated at high density in mucus. The nucleotide differences in potential upstream *empA* regulatory sequences of M93Sm and NB10 are very similar to the nucleotide differences observed upstream of the *tcpA* promoter in classical and El Tor strains of *V. cholerae*. In *V. cholerae*, there is a single nucleotide difference (A versus G) between classical and El Tor strains in the *tcpA* regulatory region located 65 to 66 bp upstream of the transcriptional start. This difference (A versus G) between NB10 and M93Sm sequences was detected 104 bp upstream of the transcriptional start. In *V. cholerae*, when the nucleotide difference is changed from A to G and G to A for classical and El Tor strains, respectively, the opposite phenotype of *tcpA* expression is observed (22). Additional base pair differences found 60 bp upstream of the initial difference are conserved between the two strains of *V. cholerae* and *V. anguillarum*. Further studies will be necessary to prove that these nucleotide differences upstream of *empA* account for the expression patterns observed in the two wild-type strains.

The contribution of metalloproteases to virulence in *P. aeruginosa* has been studied with the rat chronic lung infection model (49). The elastase mutant was less virulent than the parental strain in the rat lung model. The *lasB* gene of *P. aeru-*

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ginosa encodes the elastase metalloendopeptidase (7), which is highly homologous to *empA* metalloprotease of *V. anguillarum*. Comparison of the amino acid sequences of LasB elastase to EmpA by BLASTP (1) reveals that the proteins exhibit 48% identity and 65% similarity. Expression of *lasB* is cell density dependent and involves QS using AHLs (35). Additionally, Zhu et al. (50) suggested that expression of the hemagglutinin protease (encoded by *hapA*) in *V. cholerae* is positively regulated by QS signals. They showed that *hapR*, the positive regulator of *hapA*, is negatively regulated by *luxO*. They also suggest that *luxO* is negatively regulated by QS signals. Thus, at high cell density, AHLs and/or AI-2 would repress *luxO* and allow the induction of *hapR*, which would activate *hapA* transcription. In contrast, we show that in *V. anguillarum* neither AHLs nor AI-2 induces protease activity. We have previously shown that *empA* expression is cell density dependent (13). *V. anguillarum* produces three AHLs: 3-oxo-C_{10}-AHL, C_6-AHL, and 3-OH-C_6-AHL (24, 25). The production of C_6-AHL and 3-OH-C_6-AHL was detected in M93Sm and NB10 when the cells reached late log phase in LB20 and NSSM. The *lux*-based reporter for detection of long-chain AHLs showed production of 3-oxo-C_{10}-AHL by both strains in LB20 and NSSM when the cells reached stationary phase and increased for 3 h. In this study, we demonstrated that ethyl acetate extracts containing AHLs produced by *V. anguillarum* had no effect on protease production. Our result correlated with the findings of Milton et al. (24, 25). They cloned and made null mutations in the *V. anguillarum* AHL synthase genes *vanI* and *vanM*. The *vanI* and *vanM* mutants still exhibited *empA* protease activity.

While ethyl acetate extracts containing AHLs did not activate *empA* expression, NB10 and M93Sm cells incubated in conditioned cell LB20 supernatant from a stationary-phase culture of *V. anguillarum* M99 exhibited earlier and greater expression of protease activity. We tested LB20 supernatants of *V. anguillarum luxS* mutants and found no AI-2 activity when using the *V. harveyi* BB170 strain. Conditioned LB20 supernatant from the *luxS* null mutant (M01) allowed for a more rapid induction of protease activity in both wild-type and *luxS* mutant cells compared to incubation in either M99 conditioned LB20 or fresh LB20 (Fig. 8). One possibility is that AI-2 represses the expression of *empA* metalloprotease. Another possibility is the buildup of intermediates in the pathway for synthesis of AI-2 (48) in the *luxS* mutant (M01) somehow causes an earlier induction of protease activity when the conditioned supernatant is added to cells. Microarray analysis of an *E. coli* O157:H7 *luxS* mutant revealed over 400 genes up- or down-regulated in comparison to the parental strain (41). However only two observable phenotypes were restored when in vitro-synthesized AI-2 was added to *luxS* mutant cells (42). This suggested the possibility of a third unidentified signal, AI-3, which is synthesized by LuxS (42). Recently *vanT* of *V. anguillarum*, the *luxR* homologue of *V. harveyi* that regulates the *lux* operon, has been cloned and sequenced (12). A *vanT* mutant of *V. anguillarum* did not exhibit protease activity. Although this gene has been demonstrated to control *empA*, the autoinducer that may regulate the activity of this transcriptional regulator is not an AHL or AI-2 and has not been identified. In *V. cholerae*, the LysR homolog, AphB, regulates differential activation of the *tcpPH* promoter in classical and El Tor biotypes (22). Since the nucleotide differences between M93Sm and NB10 correspond to that observed in the *tcpPH* regulatory region, it could be hypothesized that *empA* of *V. anguillarum* is regulated by a similar mechanism. Finally, the requirement of stationary-phase conditions for protease activity and the sequence of the promoter region of *empA* suggested that *empA* expression is dependent upon σ^S encoded by *rpoS*. This was shown to be correct when EmpA expression was abolished in each of the *rpoS* mutant strains constructed. The expression of the *empA* homologues *vvpE* in *V. vulnificus* and *hapA* in *V. cholerae* has also been shown to be dependent upon *rpoS* (6, 20, 21). In summary, our data demonstrate that *empA* is regulated by at least three factors: (i) σ^S-dependent expression during stationary phase, (ii) gastrointestinal mucus as an inducer, and (iii) QS.

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