Aeromonas hydrophila AH-3 Type III Secretion System Expression and Regulatory Network[▽]

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The *Aeromonas hydrophila* type III secretion system (T3SS) has been shown to play a crucial role in this pathogen's interactions with its host. We previously described the genetic organization of the T3SS cluster and the existence of at least one effector, called AexT, in *A. hydrophila* strain AH-3. In this study, we analyzed the expression of the T3SS regulon by analyzing the activity of the *aopN-aopD* and *aexT* promoters (T3SS machinery components and effector, respectively) by means of two different techniques: promoterless *gfp* fusions and real-time PCR. The expression of the *A. hydrophila* AH-3 T3SS regulon was induced in response to several environmental factors, of which calcium depletion, a high magnesium concentration, and a high growth temperature were shown to be the major ones. Once the optimal conditions were established, we tested the expression of the T3SS regulon in the background of several virulence determinant knockouts of strain AH-3. The analysis of the data obtained from *axsA* and *aopN* mutants, both of which have been described to be T3SS regulators in other species, allowed us to corroborate their function as the major transcription regulator and valve of the T3SS, respectively, in *Aeromonas hydrophila*. We also demonstrated the existence of a complicated interconnection between the expression of the T3SS and several other different virulence factors, such as the lipopolysaccharide, the PhoPQ two-component system, the *ahyIR* quorum sensing system, and the enzymatic complex pyruvate deshydrogenase. To our knowledge, this is the first study of the *A. hydrophila* T3SS regulatory network.

Aeromonas hydrophila, a ubiquitous waterborne bacterium, is an opportunistic pathogen of animals such as amphibians, reptiles, and fish, as well as humans (4). *A. hydrophila* causes motile aeromonad septicemia, which is a major freshwater disease affecting aquaculture worldwide. In humans, *A. hydrophila* strains belonging to hybridization groups 1 (HG1) and HG3, *Aeromonas veronii* biovar sobria (HG8/HG10), and *Aeromonas caviae* (HG4) have been associated with gastrointestinal and extraintestinal diseases, such as wound infections and, less commonly, septicemias in immunocompromised patients (29). The pathogenesis of the genus *Aeromonas* is multifactorial, having been linked to different virulence determinants: toxins, proteases, outer membrane proteins, lipopolysaccharide (LPS), S-layer, capsules, and flagella.

Many gram-negative pathogens utilize the type III secretion system (T3SS) to inject virulence determinants, the so-called effectors, into the cytosol of host cells (28). These bacterial effector proteins directly interfere with and alter host processes (15). The presence of a T3SS has been reported in different *A. hydrophila* strains (42, 46) and in the fish pathogen *Aeromonas salmonicida* (11). In addition, two *A. hydrophila* (AexT and AexU) and four *A. salmonicida* (AexT, AopP, AopH, and AopO) effector proteins have been identified so far (10, 16, 19, 39, 43).

Transcription of type III secretion genes is controlled by regulatory networks that integrate diverse environmental signals, probably to ensure the efficient use of this energy-con-

suming machinery. Whatever these regulatory pathways are, they usually converge in the regulation of an AraC-like transcriptional activator (28). Achieving the appropriate combination of signals in vitro to induce the production and functionality of the type III secretion machinery is one of the main aims for researchers (21). Recently there have been some attempts to decipher T3SS gene regulation in the genus *Aeromonas* by using the reverse transcription (RT)-PCR and proteomic approach, as well as Western and Northern blot analysis (18, 39, 47). To better understand and study T3SS gene regulation in *A. hydrophila*, we combined two powerful techniques. We developed a reporter system using a promoterless *gfp* gene that allowed in vivo reporting of differential gene expression. The promoters chosen for this purpose were those for the *aopN-aopB* operon (T3SS) and the *aexT* gene (T3SS effector). The *gfp* gene fusion constructs obtained were evaluated using various mutants and growth conditions, with this being the first study to explore the relationship between the T3SS and different virulence determinants in *A. hydrophila*. Furthermore, we used the real-time PCR technology as a second way to evaluate the activities of these promoters.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown on Luria-Bertani (LB) Miller broth and LB Miller agar at 37°C, while *Aeromonas* strains were grown in either tryptic soy broth (TSB) or swarm agar (1% tryptone, 0.5% NaCl, 0.5% agar) at temperatures ranging from 20 to 37°C. Calcium-depleted conditions were obtained by adding 10 mM EGTA. When required, ampicillin (50 µg/ml), kanamycin (50 µg/ml), rifampin (rifampicin) (100 µg/ml), chloramphenicol (Cm) (25 µg/ml), spectinomycin (50 µg/ml), MgCl₂ (20 mM), or NaCl (200 mM) was added to the medium.

DNA techniques. DNA manipulation was carried out essentially as described previously (38). DNA restriction endonucleases, T4 DNA ligase, *E. coli* DNA

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TABLE 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/phenotype ^a	Source/reference
Strains		
<i>A. hydrophila</i>		
AH-3	O34; wild type	35
AH-405	AH-3; spontaneous Rif ^r	35
AH-1114	AH-405; <i>aopN</i> in-frame deletion mutant	43
AH-3:: <i>axsA</i>	AH-405; <i>axsA</i> ::Km ^r	This study
AH-5502	AH-405; <i>rpoN</i> ::Km ^r	13
AH-5503	AH-405; <i>lafK</i> ::Km ^r	13
AH-3:: <i>flrA</i>	AH-405; <i>flrA</i> ::Km ^r	Our laboratory
AH-2325	AH-405; <i>flgL</i> defined insertion mutant, Km ^r	2
AH-3::wzy	AH-405; <i>wzy</i> ::Km ^r	30
AH-3:: <i>waaL</i>	AH-405; <i>waaL</i> ::Km ^r	30
AH-3:: <i>phoP</i>	AH-405; <i>phoP</i> defined insertion mutant; Km ^r	This study
AH-3:: <i>ahyI</i>	AH-405; <i>ahyI</i> defined insertion mutant; Km ^r	This study
AH-3:: <i>ahyR</i>	AH-405; <i>ahyR</i> defined insertion mutant; Km ^r	This study
AH-3:: <i>aceA</i>	AH-405; <i>aceA</i> defined insertion mutant; Km ^r	This study
AH-1206	AH-405; <i>aopN</i> promoter- <i>gfp</i> fusion; <i>cis</i> configuration; Cm ^r	This study
AH-1199	AH-405; <i>aexT</i> promoter- <i>gfp</i> fusion; <i>cis</i> configuration; Cm ^r	This study
<i>E. coli</i>		
XL1-Blue	<i>recA1 lacI^qΔM15 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 Tn10; Tc^r</i>	Stratagene
DH5α	<i>F⁻ endA hsdR17 (r_k⁻ m_k⁺) supE44 thi-1 recA1 gyrA96 80lacZ</i>	23
MC1061	<i>thi thr-1 leu6 proA2 his4 argE2 lacY1 galK2 ara14 xyl5 supE44 λ pir</i>	37
Plasmids		
pGEM-Teasy	PCR cloning vector; Amp ^r	Promega
pAcGFP1	Expression vector; <i>lacZ-acgfp1</i> fusion; Amp ^r	Clontech
pFS100	pGP704 suicide plasmid; <i>λpir</i> dependent; Km ^r	37
pFS-PhoP	pFS100 with internal fragment of AH-3 <i>phoP</i> gene; Km ^r	This study
pFS-AhyI	pFS100 with internal fragment of AH-3 <i>ahyI</i> gene; Km ^r	This study
pFS-AhyR	pFS100 with internal fragment of AH-3 <i>ahyR</i> gene; Km ^r	This study
pFS-AceA	pFS100 with internal fragment of AH-3 <i>aceA</i> gene; Km ^r	This study
pDM4	Suicide plasmid; <i>pir</i> dependent with <i>sacAB</i> genes; oriR6K; Cm ^r	34
PDM4-AxsA	pDM4 with AH-3 <i>axsA</i> ::Km; Km ^r	This study
pCM100	pGP704 suicide plasmid; <i>λpir</i> dependent; Cm ^r	46
pRK2073	Helper plasmid; Spc ^r	37
pAcGFP1- <i>aopN</i> _P	pAcGFP1 with <i>aopN</i> promoter- <i>gfp</i> fusion; Amp ^r	This study
pAcGFP1- <i>aexT</i> _P	pAcGFP1 with <i>aexT</i> promoter- <i>gfp</i> fusion; Amp ^r	This study
pCM- <i>aopN</i> _P	pCM100 with <i>aopN</i> promoter- <i>gfp</i> fusion; Cm ^r	This study
pCM- <i>aexT</i> _P	pCM100 with <i>aexT</i> promoter- <i>gfp</i> fusion; Cm ^r	This study
PUC4-K1XX	Source of Tn5-derived <i>nptII</i> gene; Km ^r	Stratagene

^a Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Rif^r, rifampin resistant.

polymerase Klenow fragment, and alkaline phosphatase were used as recommended by the suppliers. The PCR was carried out using *Taq* DNA polymerase (Invitrogen) in a Gene Amplifier PCR System 2400 Perkin Elmer thermal cycler.

Plasmid and mutant construction. To obtain single defined insertion mutations in the genes *phoP*, *ahyI*, *ahyR*, and *aceA* (AH-3::*phoP*, AH-3::*ahyI*, AH-3::*ahyR*, AH-3::*aceA*), we used a method based on the suicide plasmid pFS100 (37). Briefly, an internal fragment of the selected gene was amplified by PCR using *A. hydrophila* AH-3 genomic DNA, ligated into pGEM-Teasy (Promega), and transformed into *E. coli* XL1-Blue. The oligonucleotides *phoP*4F (5'-CGACAAGCTGCTCAATCAC-3') and *phoP*4R (5'-TCACCTCTATGGT GTTGGG-3') were used to amplify a 532-bp internal fragment of the *phoP* gene; *ahyI*F2 (5'-CACGGGCAAAACGTTTCATC-3') and *ahyI*R1 (5'-ACGAGCTTT ATCGTTTCCG-3') amplified a 213-bp internal fragment of the *ahyI* gene; *ahyR*F1 (5'-ATATGCTGGCCTGTGATCC-3') and *ahyR*R3 (5'-AACGACCT GCTCAGATTG-3') were used to obtain a 541-bp internal fragment of the *ahyR* gene; *aceA*F1 (5'-CAAGGTCATCAACGAGCTG-3') and *aceA*R1 (5'-C GTGCAGTACTTGTGCTC-3') amplified a 542-bp internal fragment of the *aceA* gene. The DNA inserts were recovered by EcoRI restriction digestion and ligated into the EcoRI-digested and phosphatase-treated pFS100 plasmid vector. The ligation product was transformed into *E. coli* MC1061 (*λpir*) and selected for kanamycin resistance (Km^r). Triparental mating with the mobilizing strain HB101/pRK2073 was used to transfer the recombinant plasmid into the *A. hydrophila* rifampin-resistant (Rif^r) strain AH-405 to obtain defined insertion mutants, selecting for Rif^r and Km^r (13).

To obtain the *axsA* (GenBank accession no. AY528667) nonpolar mutant

(AH-3::*axsA*), a 1,349-bp DNA fragment containing the *axsA* gene was amplified by PCR from *A. hydrophila* AH-3 genomic DNA using the oligonucleotides *axsA*F1 (5'-CACGCAGATTCAGGTTTCAG-3') and *axsA*R1 (5'-GATGCAA AGGGTGAAGGAC-3'), ligated into the vector pGEM-Teasy (Promega), and transformed into *E. coli* XL1-Blue. The Tn5-derived kanamycin resistance cassette (*nptII*) from pUC4-K1XX was inserted, using the endonuclease BamHI, in the gene according to its orientation. The Km^r cassette contains an outward-reading promoter that ensures the expression of downstream genes when inserted in the correct orientation (9). Constructs containing the mutated genes were ligated into the suicide vector pDM4 (34), electroporated into *E. coli* MC1061 (*λpir*), and plated on LB with Cm at 30°C. The plasmid with the mutated *axsA* gene was transferred into the *A. hydrophila* rifampin-resistant (Rif^r) strain AH-405 by triparental mating using *E. coli* MC1061 (*λpir*) containing the insertion constructs and the mobilizing strain HB101/pRK2073. Transconjugants were selected on plates containing Cm, kanamycin, and rifampin. PCR analysis confirmed that the vector had integrated correctly into the chromosomal DNA. To complete the allelic exchange, the integrated suicide plasmid was forced to recombine out of the chromosome by addition of 5% sucrose to the agar plates. The pDM4 vector contains the *sacB* gene, which produces an enzyme that converts sucrose into a product that is toxic to gram-negative bacteria. Transconjugants surviving on plates with 5% sucrose (Rif^r, Km^r, and Cm-sensitive [Cm^s]) were chosen and confirmed by PCR.

Promoterless *gfp* gene fusions' construction. The putative promoter regions of the genes *aopN* and *aexT* (GenBank accession no. AY528667 and EF442031, respectively) were amplified from *A. hydrophila* AH-3 genomic DNA using the

oligonucleotides PscNF1 (5'-AACTGCAGGAAATAGGCTGGGTGATGC-3') and BaopNR5 (5'-CGGGATCCTT GAGCTCTTCGCCATATC-3') and BaexTF11 (5'-CGGGATCCCTGTGGTTGC ATCAGTGT-3') and AexTR10 (5'-GCAGCTGGCTCATCGCCTC-3'), respectively. The BamHI site is underlined, and the PstI site is doubly underlined. Amplified bands containing the *aopN-aopD* or *aexT* promoter and its Shine-Dalgarno sequence and the N-terminal region of *aopN* or *aexT*, respectively, were ligated independently into pGEM-Teasy and transformed into *E. coli* XL1-Blue. The DNA inserts were recovered by BamHI/PstI restriction double digestion and ligated into the BamHI/PstI-digested pAcGFP1 plasmid vector, which codes for a very stable green fluorescent protein (GFP). The ligation product was transformed into *E. coli* XL1-Blue, and recombinants containing pAcGFP1-*aopN* or pAcGFP1-*aexT* were selected for ampicillin resistance (Amp^r). Recombinants containing *gfp* fusions were checked by DNA sequence. *aopN-gfp* and *aexT-gfp* fusions were recovered by HindIII/SpeI double digestion, made blunt ended, and ligated into the EcoRV-digested and phosphatase-treated pCM100 (46) plasmid vector. The ligation products were transformed into *E. coli* MC1061 (λ pir) and selected for Cm^r. Triparental mating with the mobilizing strain HB101/pRK2073 was used to transfer the recombinant plasmid into the different *A. hydrophila* strains to obtain the insertion of the constructs in the chromosome. Transconjugants were chosen according to the antibiotic resistance pattern expected and confirmed by PCR.

Analysis of GFP expression by spectrofluorometry. Five-ml cultures of the different strains harboring the *gfp* fusion constructs were grown overnight under various environmental conditions, harvested by centrifugation, rinsed in PBS (1 $\times$) and centrifuged again, and resuspended in PBS (1 $\times$) to a cell density in the range of 10⁹ cells/ml, and aliquots were added to a 96-well plate (200 μ l/well). Fluorescence was assayed in an FL600 microplate fluorescence reader (BioTek). Spectrofluorometer analysis and settings were as follows: 475-nm excitation, 505-nm emission, and data recorded in relative fluorescence units. All strains were analyzed in duplicate in at least three independent experiments. Specific fluorescence was calculated as the quotient of relative fluorescence units (average of repetitions) and the optical density at 600 nm (OD₆₀₀), being then designated specific relative fluorescence units (relative fluorescence units/OD₆₀₀). All the values obtained were referenced to a that of a control (strain AH-3 without the *gfp* fusion) in order to eliminate the fluorescence emission of this strain at 505 nm.

Total RNA extraction, DNase treatment, and RT. For each culture condition, 1-ml aliquots with an OD₆₀₀ of 0.6 (containing approximately 5 $\times$ 10⁸ CFU/ml) were collected and mixed with RNeasy lysis reagent (Qiagen) according to the manufacturer's protocol. Cell lysis was performed with 0.4 mg/ml lysozyme for 5 min at room temperature, and total RNA was isolated using the Qiagen RNeasy mini kit. RNA samples were treated with Ambion's DNA-free DNase treatment and removal reagents. After precipitation, the RNA concentration and quality were determined by using a Bioanalyzer 2100 (Agilent Technologies).

First-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (New England Biolabs), containing (for each reaction) 2 μ g RNA and 50 ng random oligonucleotides (Promega). The reaction mixtures were incubated at 25°C for 10 min, 37°C for 120 min, and 75°C for 15 min. Control reactions lacking reverse transcriptase were carried out to confirm that RNA samples were not contaminated with genomic DNA (RT negative controls).

Real-time PCR. Quantitative PCR (qPCR) was performed using an Applied Biosystems 7700HT real-time PCR system with 13- μ l reaction mixtures containing 6.5 μ l Power SYBR green PCR master mix (Applied Biosystems), 5 μ M of each primer, and various quantities of template (ranging from 70 pg to 37 ng). The thermal program included a 10-min incubation at 95°C to activate AmpliTaq Gold polymerase, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. During each extension step, SYBR green fluorescence was monitored, providing the real-time quantitative measurements of the fluorescence. A no-template control and an RT negative control were included in each real-time run. Dissociation curves were performed after each PCR run to ensure that a single PCR product had been amplified. The products were also analyzed by gel electrophoresis.

Oligonucleotides were designed for the *aexT* (aexTF16 [5'-GATGCTGTTCA GTTCGCTTG-3'] and aexTR16 [5'-GTGGCCATCAAGAGTGGAT-3']) and *aopN* (aopNF6mod [5'-GGGTAGTGCCATCGTGTGG-3'] and aopNR6mod [5'-TCTCGCAGATCCTGCTACC-3']) genes using the Primer3 software program (36). The lengths of the PCR cDNA amplification products were between 50 and 200 bp, according to the optimum length for SYBR green use. For the comparative qRT-PCR, the internal normalizer gene used was the 16S rRNA gene *rrsA* (amplified with the oligonucleotides rrsAF2 [5'-GCCTAACACATG CAAGTCGAG-3'] and rrsAR2 [5'-GCGGTATTAGCAGTCGTTCC-3']), and all mRNA expression data were normalized to 16S rRNA and corrected using the reference dye (ROX).

Statistical analysis. One-way analysis of variance was used for statistical analysis of the data using the Statgraphics plus for Windows software program, version 5.1, (Statpoint Technologies Inc., Warrenton, VA). Differences were considered significant at *P* values of <0.05.

RESULTS

Expression conditions of *A. hydrophila* AH-3 *aopN* and *aexT* genes. To determine the specific conditions to activate the *A. hydrophila* AH-3 T3SS component production, both the *aopN*- and *aexT-gfp* gene fusions were cloned into the mobilizable suicide vector pCM100, rendering the plasmids pCM-*aopN*_p and pCM-*aexT*_p, respectively. Both were independently transferred by mating into strain AH-405 (AH-3 Rif^r [35]), obtaining the strains AH-1206 and AH-1199, respectively, both with the gene fusion cloned in the *cis* configuration. These strains were grown at 30°C in TSB medium containing (or not) 10 mM EGTA, 20 mM MgCl₂, or 200 mM NaCl and all the possible combinations, assessing promoter expression by measuring the fluorescence of each culture. The quantitative data confirmed that strains AH-1206 and AH-1199 showed at least a twofold increase in the amount of fluorescence observed when 20 mM MgCl₂ was added (Fig. 1B). No differences were detected between the fluorescence of the strains grown in TSB and that of those grown in TSB supplemented with 200 mM NaCl, although a slight increase was achieved by adding 20 mM MgCl₂ to cultures containing 200 mM NaCl. Under all conditions, the addition of 10 mM EGTA (Ca²⁺-specific chelator) gave rise to the largest changes in fluorescence (gene expression), ranging from 3- to 10-fold increases (Fig. 1B). We also analyzed the effect of the growing temperature on the expression of both promoters by culturing the strains in the different media previously mentioned, incubating them at 20°C and 37°C, and measuring the fluorescence (Fig. 1A and C). The growth rates of strains harboring the *gfp* fusion constructs were analyzed under the different culture conditions evaluated, and their growth rates did not show any differences (μ = 0.588 $\pm$ 0.02 h⁻¹). The maximum fluorescence emission corresponded to the addition of 20 mM MgCl₂ and 10 mM EGTA to the medium at all temperatures tested. The optimal temperature for expression of both type III secretion promoters was 37°C (Fig. 1C). Furthermore, the optimal expression of the *aopN-aopD* and *aexT* genes related to oxygen tension was under strictly aerobic conditions (data not shown).

We also performed SYBR green-based real-time qRT-PCR analysis to detect the mRNA of *aopN* and *aexT* in *A. hydrophila* AH-3 grown under the optimal conditions observed for their expression (TSB plus 20 mM MgCl₂ plus 10 mM EGTA, 37°C) and noninducing conditions (TSB). The melting-curve analysis of the PCR products showed that both the 16S rRNA gene (*rrsA*) and the target genes (*aopN* and *aexT*) were specific for amplification, without dimers or other contaminations, indicating that the samples were suitable for subsequent quantitative PCR. The amount of target gene expression was calculated from the respective amplification plots and quantitative expression of the target genes normalized using the housekeeping gene *rrsA*. Consistent with the GFP assays, the real-time quantitative PCR results showed a fivefold increase in the amount of *aopN* and *aexT* mRNAs when 20

FIG. 1. Influence of temperature, 20°C (A), 30°C (B), or 37°C (C), and culture medium on *aopN* and *aexT* promoter activity as measured by the fluorescence emissions from strains AH-1206 and AH-1199, respectively. The values represent the means $\pm$ standard deviations from three independent experiments.

mM MgCl₂ and 10 mM EGTA were added to the culture medium (Fig. 2).

***aopN* and *aexT* expression on different *A. hydrophila* AH-3 mutants.** Once the conditions for the type III secretion component expression in strain AH-3 had been established, we decided to assess whether mutations in specific T3SS components or in other structures or proteins (polar/lateral flagella, σ^{54} factor RpoN, LPS, the PhoPQ two-component and quorum sensing systems, and the pyruvate dehydrogenase AceA subunit) would change the expression levels of the

aforementioned type III secretion promoters. We studied *aopN* and *aexT* expression under inducing conditions at 30°C by again measuring the GFP fluorescence emission in different AH-3 mutants containing the *gfp* fusions and quantifying the mRNA levels of *aopN* and *aexT* genes in these mutants. The growth rates of the different mutant strains harboring the *gfp* fusion constructs were analyzed, and they showed μ value differences lower than 5% compared with the wild-type strain ($\mu = 0.588 \pm 0.02 \text{ h}^{-1}$). The AH-3::*aceA* mutant showed a reduction in its growth rate that was com-

FIG. 2. mRNAs from AH-3 strains cultured under noninducing (TSB) or inducing (TSB plus 20 mM $MgCl_2$ plus 10 mM EGTA) conditions were subjected to quantitative real-time PCR. The maximum (max.) value of expression, which corresponded to *aopN* under inducing conditions, was set to 100%; the values indicated are the means $\pm$ standard deviations from three independent experiments. Standard deviations are shown. The threshold for detection of *aopN* and *aexT* expression is 37 ng of RNA equivalents or a 0.001 ratio of *aopN/aexT* to *rrsA*. The black asterisks indicate significant differences versus results under noninducing conditions (P values < 0.05).

compensated by the addition of 5 mM sodium acetate to the medium; its fluorescence emission did not change compared to that of a culture not containing sodium acetate.

***A. hydrophila* AH-3 T3SS genes.** To characterize the functions of some so-called T3SS-specific regulators, we performed mutagenesis of the *axsA* (putative T3SS master transcriptional regulator) and *aopN* (putative closing valve under noninducing conditions, previously described [43]) genes, obtaining the AH-3::*axsA* and AH-1114 (*aopN*) mutant strains, respectively. Next, we transferred the *aopN-gfp* and *aexT-gfp* fusions to these mutants by triparental mating and measured their fluorescence when grown in both TSB (noninducing conditions) and TSB supplemented with 20 mM $MgCl_2$ and 10 mM EGTA (optimal inducing conditions).

As shown in Fig. 3A, in calcium-depleted medium (under inducing conditions), the *axsA* mutant showed 65% and 85% decreases in fluorescence, i.e., the activities of the *aopN-aopD* and *aexT* promoters, respectively. ExsA-dependent promoters contain an ExsA consensus binding site (TNAAAANA) 50 bp upstream of the transcriptional start site (45). Computer analysis of the putative promoter regions of the AH-3 T3SS gene cluster and *aexT* showed putative ExsA binding sites among all of them (42, 43). Furthermore, a domain prediction of AH-3 *AxsA* showed an AraC-type DNA-binding domain in its N-terminal region and two helix-turn-helix motifs in its C-terminal region. All these data are in agreement with the likely fact of *AxsA* being the master regulator of the *A. hydrophila* AH-3 type III secretion genes. We found no significant differences at the expression level between the *aopN* mutant and the parental strain when these were grown under inducing conditions. However, when the *aopN* mutant was grown in TSB medium (non-inducing conditions), we detected a significant fluorescence increase (Fig. 3A), corresponding to an upregulation of the *aopN* and *aexT* promoters (320% and 550% increase, respectively). When the *axsA* mutant was grown under inducing conditions, quantitative RT-PCR analysis showed 60 and 85% decreases of *aopN* and *aexT* mRNAs, respectively, in comparison with results for the wild-type strain (Fig. 4A). Quantification of *aopN* and *aexT* mRNAs for the *aopN* mutant was

similar to that for the wild-type strain under inducing conditions, but increases of 300 and 500% were detected under noninducing conditions (Fig. 4A).

Cross-talking between T3SS and polar/lateral flagella. Sequence and ultrastructural similarities exist between T3SS components and those of the flagellar assembly machineries in prokaryotes (8). In order to establish any possible relationship between the flagellum and the T3SS in *A. hydrophila* AH-3, we analyzed the activities of both the *aopN-aopD* and *aexT* promoter regions in different AH-3 mutants affecting biosynthesis and/or correct assembly of the polar and lateral flagellar systems. Therefore, the fact that *A. hydrophila* AH-3 shows two flagellar systems, a constitutive polar flagellum and an induced lateral flagella, has allowed us to study for the first time both systems in relation to the T3SS (33). The *aopN-gfp* and *aexT-gfp* gene fusions were transferred, independently, into *fliA*, *flgL*, and *lafK* mutant strains (AH-3::*fliA*, AH-2325, and AH-5503, respectively) and the fluorescence under inducing conditions assessed. The *fliA* and *flgL* (GenBank accession no. DQ124697 and AY129558, respectively) mutants are devoid of the polar flagellum but still present lateral flagella, being able to swarm but not to swim. The *lafK* (GenBank accession no. DQ124694) mutant is devoid of the lateral flagella but still possesses a polar flagellum, being then able to swim but not to swarm (13). The *fliA* and *flgL* mutant strains containing *gfp* fusions were cultured in liquid medium, while *lafK* mutant strains harboring promoter-reporter constructs were cultured in swarm agar (lateral flagellum inducing conditions).

Both the *aopN*- and *aexT-gfp* fusions showed a decrease in fluorescence (approximately 65% and 50%, respectively) when introduced into *fliA* and *flgL* mutants (Fig. 3B). When the *A. hydrophila* strains were grown in semisolid medium, no fluorescence could be measured. By qRT-PCR analysis, the same pattern of differences was detected for *fliA* and *flgL* mutants: the two of them presented a clear decrease in the T3SS mRNA levels analyzed (Fig. 4B). Furthermore, no expression differences were observed when the wild-type strain AH-3 and the *lafK* mutant grown in semisolid medium were compared (Fig. 3C and 4C).

σ^{54} factor RpoN. The alternative sigma factor RpoN is responsible for recruiting core RNA polymerase to the promoters of genes required for diverse physiological functions in a variety of eubacterial species. We had previously described the implication of the RpoN protein in the control of both polar and lateral gene cluster expression in *Aeromonas* (13, 14). Therefore, we decided to assess any possible connection between RpoN and the T3SS components. The *aopN-gfp* and *aexT-gfp* gene fusions were transferred independently into the *rpoN* (GenBank accession no. DQ124695) mutant strain and the fluorescence under inducing conditions evaluated.

Both promoter fusions showed an approximately 40% decrease in fluorescence (Fig. 3B). Furthermore, the qRT-PCR results also showed a similar reduction in the *aopN* and *aexT* mRNA levels (Fig. 4B).

LPS affects type III secretion. Contact between the bacterium and the host cell seems to be essential for effective type III secretion. Therefore, it has been suggested that structural changes in the LPS could affect the expression of components of the T3SS and its functionality (3, 44). To address a possible association between the LPS and the T3SS in *A. hydrophila* AH-3, we studied *aopN* and *aexT* expression in *wzy* and *walL*

FIG. 3. Spectrofluorometric analysis of fluorescence from different mutant strains containing the *aopN_p-gfp* (white bars) or *aexT_p-gfp* (black bars) fusion. AH-3-1114 (*aopN*) and AH-3::axsA (A), AH-3::rpoN, AH-3::flrA, AH-3::flgL, AH-3::wzy, AH-3::waaL, AH-3::phoP, AH-3::ahyI, AH-3::ahyR, and AH-3::aceA (B), and AH-3::lafK (C) are analyzed. The values represent the means $\pm$ standard deviations from three independent experiments.

mutants (AH-3::wzy and AH-3::waaL, respectively). The *waaL* (GenBank accession no. EU296246) mutant is devoid of O34 antigen LPS, which is due to the lack of the O-antigen ligase that joins the LPS O-antigen to the LPS lipid A core. The *wzy* (GenBank accession no. EU274663) mutant shows only a single O34 antigen LPS repetition because it lacks the O-antigen polymerase.

As shown in Fig. 3B, both fusions showed 30% and 60% decrease in fluorescence when introduced into *wzy* and *waaL* mutants, respectively, in comparison to the wild-type strain. In agreement with these results, qRT-PCR analysis showed re-

ductions of 10 to 20% and 70 to 80% in the mRNA levels of *aopN* and *aexT*, respectively, in the aforementioned mutants (Fig. 4B).

PhoPQ two-component system. We also introduced, independently, the two *gfp* fusions into a *phoP* mutant (AH-3 sequences unpublished), which had been constructed previously as described in Materials and Methods. PhoP-PhoQ is a two-component system that mediates the adaptation to Mg^{2+} -limiting environments and transcriptionally regulates numerous virulence determinants in different bacteria.

In both cases, a slight repetitive increase (approximately

FIG. 4. *aopN* (white bars) or *aexT* (black bars) gene expression relative to that of 16S rRNA in different *A. hydrophila* AH-3 mutant strains. The *aopN* expression level was set to 100%. AH-3-1114 (*aopN*) and AH-3::axsA (A), AH-3::rpoN, AH-3::flrA, AH-3::flgL, AH-3::wzy, AH-3::waaL, AH-3::phoP, AH-3::ahyl, AH-3::ahylR, and AH-3::aceA (B), or AH-3::lafK (C) is analyzed. The values represent the means $\pm$ standard deviations from three independent experiments. The black asterisks indicate significant differences versus results for the control strain, AH-3 (P values < 0.05). Max., maximum.

10%) of fluorescence was observed, indicating that PhoP might have a negative regulatory effect, either direct or indirect, on the type III machinery and effectors (Fig. 3B). qRT-PCR studies showed 10 and 20% increases in the mRNA levels of *aopN* and *aexT*, respectively (Fig. 4B).

AhyIR quorum sensing system. We assessed whether mutations in the *A. hydrophila* AH-3 quorum sensing system may affect type III gene expression. The *ahyl* and *ahyR* mutants are devoid of the unique quorum sensing system found in *Aeromonas* (AH-3 sequences unpublished). The *ahyl* and *ahyR*

FIG. 5. *A. hydrophila* AH-3 proteins controlling T3SS and *aexT* transcription under inducing conditions. The black square shows the effect of AopN only under noninducing conditions. Black solid lines indicate a regulatory connection that controls both T3SS and *aexT* transcription. Black discontinuous lines indicate regulatory connections that control T3SS transcription only. Gray solid lines indicate hypothetical regulatory connections. See the text for the details of each system.

mutants containing the *aopN-gfp* gene fusion showed a 35% decrease in fluorescence emission, while the same mutants harboring the *aexT-gfp* gene fusion did not present significant differences in comparison with the wild-type strain, AH-3 (Fig. 3B). Again, qRT-PCR analyses allowed us to confirm the previous results, since *aopN* and *aexT* mRNA levels underwent a 20% decrease and no changes, respectively, in both mutants compared to the parental strain (Fig. 4B). Furthermore, when the same analysis was performed using RNA from overnight cultures, the results followed the same patterns (data not shown).

Pyruvate dehydrogenase AceA subunit. The *aceA* mutant (AH-3 sequences unpublished) is devoid of the enzymatic complex pyruvate dehydrogenase (PDHc) because it lacks the decarboxylase subunit of the system. On the basis of studies by Dacheux and coworkers (17), we finally analyzed the *aopN* and *aexT* promoters' activity in this mutant, resulting in 60% and 75% fluorescence decreases for the *aopN-gfp* and *aexT-gfp* fusions, respectively. An equivalent reduction in the mRNA quantification was observed (Fig. 3B and 4B).

DISCUSSION

To study the expression of T3SS in *A. hydrophila* AH-3, we chose to analyze the promoter regions of the *aopN-aopD* operon (as the model for production of T3SS components) and the *aexT* gene (as the model for production of effector proteins) by two different methods. First, we developed a reporter system using a *gfp* gene fusion that allows in vivo reporting of differential gene expression. A second technique, real-time PCR, was used to quantify the transcriptional levels of these genes and validate the results obtained with the *gfp* gene fusions.

Since many pathogens sense they have infected their host via

environmental indicators, we tested the following previously described conditions in TSB medium: addition of magnesium chloride, low calcium, high sodium chloride, different growth temperatures, and oxygen tension. In *A. hydrophila* AH-3, the production of T3SS components and the effector protein AexT was induced upon exposure to calcium-depleted medium and enhanced with the addition of magnesium chloride and the incubation temperature of 37°C (Fig. 5). However, no changes in expression were detected with a high concentration of sodium chloride. Recently the *A. salmonicida* A229 T3SS and AexT toxin have been shown to be upregulated upon growth at 24 to 28°C (18). Neither *A. salmonicida* A229 T3SS nor AexT nor *A. hydrophila* SSU AexU expression seems to be promoted upon calcium chelation (18, 39). This is in contrast with the *A. hydrophila* AH-3 T3SS and AexT and *A. salmonicida* JF2267 AexT (12), which are upregulated after growth in calcium-depleted media. All these data suggest the existence of regulatory differences among the different T3SS reported for *Aeromonas* spp., even at the intraspecies level.

Calcium depletion has been broadly referred to as the in vitro signal for T3SS activation, both transcriptionally and functionally, of several species, such as *Yersinia* spp. and *Pseudomonas* spp (28). The coupling of transcription to secretion is a common feature among several T3SSs. In *Pseudomonas aeruginosa*, this mechanism is mediated by a signaling cascade that ultimately regulates the activity of the T3SS master regulator, ExsA (45). Under T3SS-inducing conditions, we found a high decrease in the activity of both promoters when we assayed an AH-3 *axsA* mutant in comparison with results for the wild-type strain (Fig. 5). These data together with the existence of ExsA-like consensus binding sites and ExsA-like domain prediction suggest that AxsA is the master regulator of type III secretion

genes in *A. hydrophila* AH-3. Due to the fact that the T3SS gene cluster and *aexT* gene are located in different genomic regions, the AxsA control represents a common regulatory pathway. On the other hand, the protein YopN (the AopN-homologue) in *Yersinia* spp. is thought to form a complex with three other proteins, SycN, YscB, and TyeA, which would prevent Yop secretion in the presence of calcium or prior to contact with a host cell by blocking the type III secretion apparatus (20). The activity levels of both *aopN-aopD* and *aexT* promoters under noninducing conditions in an AH-3 *aopN* mutant were similar to those in the wild-type strain when grown under inducing conditions (Fig. 5). Moreover, we recently showed the AH-3 *aopN* mutant to be constitutive for type III secretion (43). AopN may have the valve function predicted for YopN, being involved in the type III secretion regulatory mechanism. Thus, the absence of AopN could provoke opening of the type III secretion channel, consequently activating the positive feedback mechanism, resulting in AxsA activation.

The flagellar export and type III secretion systems seem to be evolutionarily and functionally related (22). Both *fliA* and *fliG* polar flagellum mutants displayed a decrease in the activities of *aopN-aopD* and *aexT* promoters, which indicates a positive cross talk between the polar flagellum and the T3SS in *A. hydrophila* AH-3 (Fig. 5). One possible explanation for these results is that motility may aid the entry process by facilitating the contact between the bacteria and the host cells, which is required for delivery of effector proteins via the T3SS. It is remarkable that a negative cross talk between the flagellar and type III secretion systems has been described for the species *P. aeruginosa* and *Yersinia enterocolitica* (6, 40). We could not detect any difference in T3SS expression between strain AH-3 and the lateral flagellar mutant AH-3::*lafK* when grown in semisolid medium (lateral flagellum inducing conditions). Recently Yu et al. found that *A. hydrophila* AH-1 $\Delta aopN$ and $\Delta exsD$ mutants had reduced expression of the lateral flagellin genes but presented unaltered the transcriptional levels of the polar flagellins after growth in a protein-free culture medium (Dulbecco's modified Eagle medium) in a humidified atmosphere of 5% CO₂. Furthermore, deletion of *exsA* in either the $\Delta aopN$ and $\Delta exsD$ background restored the lateral flagellin secretion under this growth condition (47). It is remarkable that *A. hydrophila* AH-1 seems to be able to express lateral flagella after growth in a protein-free liquid culture medium in the presence of 5% CO₂ and *A. hydrophila* AH-3 only is able to express lateral flagella in highly viscous media, like *Vibrio parahaemolyticus*. Moreover, overexpression of AxsA in *A. hydrophila* AH-3 did not alter either the swimming or the swarming, and deletion of *axsA* was not associated with differences in its ability to swim and swarm (data not shown). These results suggest that environmental and regulatory factors involved in flagellar systems and T3SS regulation may be different at the intraspecies level.

When we analyzed an *rpoN* mutant for T3SS expression, we found a slight decrease in the activities of the *aopN-aopD* and *aexT* promoters. The difference observed at the reduced level of T3SS expression in *rpoN* and *fliA* mutants could be explained by the roles of the FliA and RpoN proteins. FliA is the master regulator of polar flagellum. RpoN, as a σ^{54} factor controlled by environmental signals, might positively regulate

some other system that has a repressing function with regard to the expression of type III secretion genes. It has been proved that RpoN is absolutely necessary to the synthesis of the polar and lateral flagella in *A. hydrophila* AH-3 (13) (Fig. 5). On the other hand, RpoN has been shown to be absolutely required for the expression of *hrp* genes encoding the components of a T3SS in *Pseudomonas syringae*, although its role in the regulation of cytotoxicity in *P. aeruginosa* is less clear and awaits further elucidation (24, 25, 32).

We also demonstrate a correlation between the LPS structure and T3SS and effector production (Fig. 5), as shown by the decrease in *aopN* and *aexT* expression in the *wzy* and *waaL* mutants. In contrast, recent studies of *P. aeruginosa* showed that the less complex the O antigen is, the more the expression of the type III secretion genes increases (3). One possible explanation for our results is that the O-antigen structural changes affect the hydrophilic properties of the bacterial surface, which could interfere in the signal perception involved in T3SS machinery activation. On the other hand, in *Y. enterocolitica* O:8, it has been found that the presence or absence of LPS O antigen affects the expression of virulence factors, suggesting its role in the regulation of gene expression (5).

We have also found a correlation at the expression level between the T3SS and the two-component PhoPQ system, the enzymatic complex PDHc, and the described quorum sensing system (Fig. 5). The mutant AH-3::*phoP* showed a slight increase in the activities of both promoters, which could correlate with a direct or indirect negative regulatory effect in the wild-type background. It is important to point out that the PhoPQ system responds to the extracytoplasmatic levels of Mg²⁺ and transcription of PhoP-activated genes is promoted at low Mg²⁺ levels and repressed at high Mg²⁺ levels, and so it is, as our results indicate, the *A. hydrophila* AH-3 T3SS regulon (Fig. 5). Furthermore, our results agree with those for *Salmonella* spp., for which it was shown that the PhoPQ system repressed the expression of the SPI-1 machinery by downregulating its transcription (1). However, most data reported so far suggest that PhoPQ is unlikely to play a direct role in the regulation of *Salmonella* SPI-2 (31). The mutation of the *aceA* gene in strain AH-3 provoked a decrease in the *aopN-aopB* and *aexT* promoters' activity, indicating that PDHc seems to be necessary for T3SS expression (Fig. 5), results that are in agreement with those reported by Dacheux and coworkers (17). The mutants AH-3::*ahyI* and AH-3::*ahyR* showed a decrease in *aopN-aopB* promoter activity compared to the wild-type strain. This means that the only currently known *A. hydrophila* quorum sensing system could be involved in the upregulation of T3SS component production in strain AH-3 (Fig. 5). A possible correlation between the T3SS and the quorum sensing system has been described for different bacterial species. Genes encoding the T3SS and the Tir and intimin intestinal colonization factors of enteropathogenic *E. coli* and enterohemorrhagic *E. coli* have been described as being activated by quorum sensing (41). In *P. aeruginosa*, two quorum sensing circuits arranged in a series have been described: quorum sensing system 1, LasI/LasR, seems to exert no regulatory effect on type three secretion regulon expression, whereas quorum sensing system 2, RhlI/RhlR, has been shown to negatively control it (27, 7). In *V. harveyi*, two quorum sensing

circuits, LuxM/LuxN and LuxS-LuxPQ, function in parallel to repress T3SS expression (26).

In conclusion, we have demonstrated that *A. hydrophila* AH-3 induces the expression of its T3SS in response to several environmental factors, of which calcium depletion and a high magnesium concentration are the most remarkable. Most importantly, we can assert that there are regulatory differences among the several T3SS reported for *Aeromonas* spp., even in the same bacterial species (18, 39). We also have shown the existence of a complicated regulatory network that correlates the T3SS production with several bacterial functions, which indicates that the precise timing and coordination for the expression of virulence determinants are essential to the infectious process.

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