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 aexT 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 dehydrogenase. 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-consuming 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 swam 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 (Col) (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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The ligation product was transformed into ligated into the EcoRI-digested and phosphatase-treated pFS100 plasmid vector.

To obtain the aceA (AH-3::aceA) 1,349-bp DNA fragment containing the aceA gene was amplified by PCR from A. hydrophila AH-3 genomic DNA using the oligonucleotides aceAF1 (5’-CGACAAGTGCATCTCAATC-3’) and aceAR1 (5’-GATTGAAAGGAGAC-3’), ligated into the vector pGEM-Teasy (Promega), and transformed into E. coli XL1-Blue. The Tn5-derived kanamycin resistance cassette (nptII) from pUC4-KIXX was inserted, using the endonuclease BamHI, in the gene according to its orientation. The KmR cassette contains an outward-reading promoter that ensures the expression of downstream genes when in-frame deletion mutant.

To obtain the aceA (AH-3::aceA) gene was amplified by PCR from A. hydrophila AH-3 genomic DNA using the oligonucleotides aceAF1 (5’-CGACAAGTGCATCTCAATC-3’) and aceAR1 (5’-GATTGAAAGGAGAC-3’), ligated into the vector pGEM-Teasy (Promega), and transformed into E. coli XL1-Blue.

### TABLE 1. Bacterial strains and plasmids used in this study

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<th>Strain/plasmid</th>
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| Promoterless gfp gene fusions' construction. The putative promoter regions of the genes aopN and aceT (GenBank accession no. AY528667 and EF442031, respectively) were amplified from A. hydrophila AH-3 genomic DNA using the 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. The ligation product was transformed into E. coli DH5α strain AH-405 by triparental mating using the mobilizing strain HB101/pRK2073. To complete the allelic exchange, the integrated suicide vector pDM4 (34), electrotransferred into E. coli MC1061 (Kmr), and plated on LB with Cm at 30°C. The plasmid with the mutated aceA gene was transferred into the A. hydrophila rifampin-resistant (RifR) strain AH-405 by triparental mating using E. coli MC1061 (Kmr) 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 chromosome.

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Mutants, selecting for RifR and Kmr (35). Briefly, an internal fragment of the selected gene was amplified by PCR using pFS100 (37).
oligonucleotides PassNF1 (5′-AATTGGAAATAGGCCTTCGTCAGTGC-3′) and BaapNRs3 (5′-CCGGATCTTTGTTGGGATTTCTTG-3′) and BaapNRs5 (5′-CGGCGATCCCGGCTGATGTCACAT-3′) and aexTF1 (5′-GGTTAGTGCCATCGTGTTGG-3′) and aexTR10 (5′-CGGAGTGGTACATCGTCTCC-3′), respectively. The BamHI site is underlined, and the PsiI 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-T easy and transformed into E. coli DH5α and E. coli XL1-Blue. The DNA in inserts were recovered by BamHII/PsiI 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 (AmpR). Recombinants containing gfp fusions were identified 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 phosphorylated-treated pCM100 (46) plasmid vector. The ligation products were transformed into E. coli MC1061 (pir) and selected for CmR. 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-μl cultures of the different strains (A. hydrophila) gfp fusion constructs were grown overnight under various environmental conditions, harvested by centrifugation, rinsed in PBS (1×) and centrifuged again, and resuspended in PBS (1×) to a cell density in the range of 105 cells/ml, and aliquots were added to a 96-well plate (200 μl/well). Fluorescence was assayed in an FL600 microplate fluorochrome reader (BioTek). Spectrofluorometer analysis and settings were as follows: 475-nm excitation, 505-nm emission, and data recorded in relative fluorescent units. All strains were analyzed in duplicate in at least three independent experiments. The fluorescence was calculated as the quotient of relative fluorescent units (average of repetitions) and the optical density at 600 nm (OD600), being then designated specific relative fluorescent units (relative fluorescent 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 × 106 CFU/ml) were collected and mixed with RNAprotect bacterial 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 μg random primers and 1 μM oligonucleotides PascNF1 (5′-CGGGATCCCTGTGGTTGCATCGTCTCC-3′) and PascNF2 (5′-CGGAGTGGTACATCGTCTCC-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-aopNp, and pCM-aexTp, respectively. Both were independently transferred by mating into strain AH-405 (AH-3 RifR [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 the respective strains 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 ± 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
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⁺ 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 (μ = 0.588 ± 0.02 h⁻¹). The AH-3::*aceA* mutant showed a reduction in its growth rate that was com-
pensated 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 MgCl2 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 (noninducing 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 flaA, flgL, and lafK mutant strains (AH-3::flaA, AH-2325, and AH-5503, respectively) and the fluorescence under inducing conditions assessed. The flaA 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 flaA 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 flaA 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 flaA 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).

**α54 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 waaL.
mutants (AH-3::wzy and AH-3::waaL, respectively). The \textit{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 \textit{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 \textit{wzy} and \textit{waaL} mutants, respectively, in comparison to the wild-type strain. In agreement with these results, qRT-PCR analysis showed reductions of 10 to 20% and 70 to 80% in the mRNA levels of \textit{aopN} and \textit{aexT}, respectively, in the aforementioned mutants (Fig. 4B).

\textbf{PhoPQ two-component system.} We also introduced, independently, the two \textit{gfp} fusions into a \textit{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\textsuperscript{2+}-limiting environments and transcriptionally regulates numerous virulence determinants in different bacteria.

In both cases, a slight repetitive increase (approximately
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 \textit{aopN} and \textit{aexT}, respectively (Fig. 4B).

**A**

**Inducing conditions**

**Non inducing conditions**

![Graph A]

**B**

**Inducing conditions**

![Graph B]

**C**

**Inducing conditions**

(semisolid)

![Graph C]

**FIG. 4.** \textit{aopN} (white bars) or \textit{aexT} (black bars) gene expression relative to that of 16S rRNA in different \textit{A. hydrophila} AH-3 mutant strains. The \textit{aopN} expression level was set to 100%. AH-3-1114 (\textit{aopN}) and AH-3::\textit{axsA} (A), AH-3::\textit{rpoN}, AH-3::\textit{flrA}, AH-3::\textit{flgL}, AH-3::\textit{wzy}, AH-3::\textit{waaL}, AH-3::\textit{phoP}, AH-3::\textit{ahyI}, AH-3::\textit{ahyR}, and AH-3::\textit{aceA} (B), or AH-3::\textit{lafK} (C) is analyzed. The values represent the means ± 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.

\textbf{AhyIR quorum sensing system.} We assessed whether mutations in the \textit{A. hydrophila} AH-3 quorum sensing system may affect type III gene expression. The \textit{ahyI} and \textit{ahyR} mutants are devoid of the unique quorum sensing system found in \textit{Aeromonas} (AH-3 sequences unpublished). The \textit{ahyI} and \textit{ahyR}
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 deshydrogenase (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 of 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, YscN, YscB, and YscE, 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 fltA and flgL 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 ΔaopN and ΔexsB 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 ΔaopN and ΔexsD 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 (Dulbecco’s modified Eagle medium) in a humidified atmosphere of 5% CO₂. Furthermore, deletion of exsA in either the ΔaopN and ΔexsD 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 exsA 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 rpoD and fltA mutants could be explained by the roles of the FlrA and RpoN proteins. FlrA is the master regulator of polar flagellum. RpoN, as a σ54 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-1. 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 system
circuits, LuxM/LuxN and LuxS-LuxPO 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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