Regulation of Motility in *Erwinia carotovora* subsp. *carotovora*: Quorum-Sensing Signal Controls FlhDC, the Global Regulator of Flagellar and Exoprotein Genes, by Modulating the Production of RsmA, an RNA-Binding Protein

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Erwinia carotovora subsp. *carotovora* causes soft-rotting (tissue-macerating) disease in many plants and plant organs. Although pectinases are the primary determinants of virulence, several ancillary factors that augment bacterial virulence have also been identified. One such factor is bacterial motility. Flagellum formation and bacterial movement are regulated in many enterobacteria, including *E. carotovora* subsp. *carotovora*, by FlhDC, the master regulator of flagellar genes and FliA, a flagellum-specific σ factor. We document here that motility of *E. carotovora* subsp. *carotovora* is positively regulated by the quorum-sensing signal, *N*-acylhomoserine lactone (AHL), and negatively regulated by RsmA, a post-transcriptional regulator. RsmA, an RNA-binding protein, causes translational repression and promotes RNA decay. Our data show that RsmA negatively regulates *flhDC* and *fliA* expression. Moreover, the chemical stabilities of transcripts of these genes are greater in an RsmA⁻ mutant than in RsmA⁺ bacteria. These observations contrast with positive regulation of *flhDC* and motility by CsrA (= RsmA) in *Escherichia coli*. In the absence of AHL, the AHL receptors ExpR1/ExpR2 (= AhlR) in *Erwinia carotovora* subsp. *carotovora* negatively regulate motility and expression of *flhDC* and *fliA* by activating RsmA production. In the presence of AHL, regulatory effects of ExpR1/ExpR2 are neutralized, resulting in reduced levels of *rsmA* expression and enhanced motility.

Flagellar motility contributes to virulence of several animal pathogens and plant-pathogenic bacteria, including *Erwinia carotovora* (Josenhans and Suerbaum 2002; Matsumoto et al. 2003; Hossain et al. 2005; Gauger et al. 2007). Evidence for this in *E. carotovora* subsp. *carotovora* comes from *AfliC* and *AmotA* mutants of *E. carotovora* subsp. *carotovora* EC1 (Hossain et al. 2005). These nonmotile mutants were reduced in their ability to cause soft-rotting disease in Chinese cabbage. Although the flagellar regulon of *E. carotovora* subsp. *carotovora* has not been fully characterized, extensive studies in *Escherichia coli* and *Salmonella typhimurium* serve as a good guide in predicting the structural and regulatory features. The flagellar regulon in *E. coli* and *Salmonella* spp. consists of

approximately 50 genes organized into at least 14 operons and in a three-tier hierarchy: class I, class II, and class III genes (Aizawa and Kubori 1998; Claret and Hughes 2000; Soutourina and Bertin 2003; McCarter 2006). *flhD* and *flhC* are the class I genes, whose products are known to form an FlhD₄C₂ hexamer complex (Wang et al. 2006). Both FlhD and FlhC subunits are essential for effective transcription. The FlhDC complex binds promoter regions of the class II genes and activates their transcription (Liu and Matsumura 1994). Within the class II flagellar regulon are genes for proteins for the basal body and the hook of the flagellum as well as two regulators, FliA and FlgM. FliA is an alternative σ factor (σ^{28}) specific for the flagellar regulon and FlgM acts as an anti- σ factor that inhibits FliA-dependent transcription by stripping FliA from the core of RNA polymerase as well as by preventing the σ -core interaction (Chadsey and Hughes 2001; Aldridge et al. 2006). FliA is required for the expression of several class II genes and all class III genes. The class III genes specify components for assembly of the flagellar filament, chemotaxis proteins, and motor activity (Aizawa and Kubori 1998; Claret and Hughes 2000; Aldridge et al. 2006; McCarter 2006).

In addition to the flagellar genes for swimming motility, the flagellar master operon FlhDC in *E. carotovora* subsp. *carotovora* controls an array of traits, including virulence, type III secretion system, and extracellular enzyme production (Cui et al. 2008). Matsumoto and associates (2003) showed that the FlhC and FlhD deficiencies in *E. carotovora* subsp. *carotovora* EC1-N resulted in reduced levels of transcripts of *fliC* and *fliA*, flagellum synthesis, and virulence in Chinese cabbage and potato. We subsequently documented that this pleiotropic effect of FlhDC deficiency results from reduced expression of *gacA*, *rsmC*, and *fliA* and increased expression of *hexA*: FliA is responsible for the expression of flagellar genes and bacterial movement (Cui et al. 2008); HexA, an LrhA homolog, is a LysR-like regulator and negatively regulates FlhDC, *GacA*, and *rsmB* RNA (A. Chatterjee, *unpublished*) (Mukherjee et al. 2000); *GacA*, a response regulator of a two-component system, positively regulates *rsmB* (Cui et al. 2001); and RsmC specifies an FlhD-binding protein and behaves as an anti-FlhD4FlhC2 factor (Chatterjee et al. 2009). There are reports of FlhDC-mediated control of other traits in several enterobacteria (Givskov et al. 1995; Liu et al. 2000; Blevels et al. 2002; Kapatral et al. 2004). Thus, it is safe to conclude that, in many enterobacteria, including *E. carotovora* subsp. *carotovora*, FlhDC behaves as a global positive regulator.

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Expression of *flhDC* genes is controlled by environmental cues and several regulators (Aizawa and Kubori 1998; Wei et al. 2001; Lehnen et al. 2002; Sperandio et al. 2002; Soutourina and Bertin 2003; McCarter 2006). One such regulator in *Escherichia coli* is CsrA (Wei et al. 2001). It is an RNA-binding protein, generally responsible for translational repression and RNA decay. Surprisingly, it was shown to positively regulate *flhDC* expression (Wei et al. 2001), although the molecular basis for this regulation remains unclear. In *Erwinia carotovora* subsp. *carotovora*, this post-transcriptional regulator, designated RsmA (= CsrA), plays a central role in the production of *N*-acylhomoserine lactone (AHL) and extracellular enzymes or proteins and virulence. In a series of studies, we have determined that RsmA, thus far without any exception, negatively controls gene expression (Chatterjee et al. 1995; Cui et al. 1995; Mukherjee et al. 1996). Thus, the observation in *Escherichia coli* regarding the positive regulation of *flhDC* by CsrA (RsmA) was unexpected. This apparent anomaly prompted us to examine the role of RsmA on FlhDC expression in *Erwinia carotovora* subsp. *carotovora*. The data presented here show that, contrary to the positive effect of CsrA in *Escherichia coli*, RsmA negatively regulates FlhDC in *Erwinia carotovora* subsp. *carotovora*. We also document that AHL positively controls motility, and this effect is mediated by the negative effect of AHL on *rsmA* expression.

RESULTS AND DISCUSSION

RsmA differentially affects *flhDC* expression and motility in *E. carotovora* subsp. *carotovora* and *Escherichia coli*.

Wei and associates (2001) reported that expression of *flhDC* in *Escherichia coli* is positively regulated by CsrA, the homolog of RsmA. This result was unexpected because RsmA and its homologs have been found to be responsible for translational repression and RNA decay. Consistent with these modes of action, RsmA and RsmA-like proteins are known to negatively control many genes in different bacterial species (Chatterjee et al. 1995; Cui et al. 1995; Mukherjee et al. 1996; Romeo et al. 1998; Babitzke and Romeo 2007; Lapouge et al. 2008). Therefore, it was of interest to determine whether RsmA positively regulated *flhDC* in *Erwinia carotovora* subsp. *carotovora* as well. We compared the levels of *flhDC* transcripts of *E. carotovora* subsp. *carotovora* Ecc71 and its RsmA⁻ mutant, AC5071. The levels of *flhDC* transcript in strain RsmA⁻ were higher than that in the parent strain (Fig. 1A). By contrast, the levels of FlhDC transcripts were higher with the CsrA⁺ *Escherichia coli* BW3414 compared with the levels in its CsrA⁻ derivative TR1-5 (Fig. 1B), as previously reported by Wei and associates (2001).

Wei and associates (2001) had also observed a positive effect of CsrA on motility in *Escherichia coli*. To our surprise, preliminary motility tests revealed that Ecc71, the wild-type strain we

had used in studies of various regulatory systems, exhibits little or no motility. Because it expresses *fliA*, the gene for flagellum-specific σ factor, we presumed that the absence of motility may have resulted from a defect in other class II or class III genes. A comparison of Ecc71 and Ecc7N2, a motile strain, revealed that expressions of *fliA* and *fliE*, the gene for flagellar basal body protein are comparable in the two strains (Fig. 2). However, a remarkable difference was seen in the expression of *fliC*, the gene for flagellin. Whereas Ecc7N2 produces very high levels of *fliC* RNA, the levels of *fliC* transcript are low in Ecc71. Thus, it seems plausible that reduced levels of flagellin production may account for the nonmotile state of Ecc71 and its derivatives.

To test the effects of RsmA deficiency on motility in *Erwinia carotovora* subsp. *carotovora*, we first constructed an RsmA⁻ mutant (AC5152) of a motile *E. carotovora* subsp. *carotovora* Ecc7N2. Similar to strain Ecc71, deficiency of RsmA in Ecc7N2 causes hyperproduction of exoproteins and hypervirulence (Fig. 3A and B). Motility test results in tryptone swarm agar (Fig. 3C) revealed that the RsmA deficiency of Ecc7N2 leads to hypermotility. Northern blot analysis (Fig. 3D) demonstrated that the transcript levels of *flhDC*, *fliA*, *fliE*, and *fliC* were higher in the RsmA⁻ mutant AC5152 than in Ecc7N2. These results revealed that, contrary to positive regulation of expression of *flhDC* and motility by CsrA in *Escherichia coli*, in *Erwinia carotovora* subsp. *carotovora*, RsmA negatively controls the transcript levels of *flhDC* and its downstream genes *fliA*, *fliE*, and *fliC* as well as motility.

To further examine whether the differential effects of CsrA and RsmA on expression of *flhDC* and motility could be attributed to an intrinsic property of RsmA, we transferred the cloning vector pCL1920 and the Ecc71 *rsmA*⁺ DNA pAKC880 into the CsrA⁻ mutant TR1-5. Northern blot analysis results (Fig. 4A) revealed that TR1-5 carrying pAKC880 produced higher levels of *flhDC* and *fliA* transcript compared with TR1-5 carrying the vector. As would be expected, a motility test in tryptone swarm agar showed that pAKC880 restored motility in the CsrA⁻ mutant (Fig. 4B).

Stabilities of *flhDC*, *fliA*, *fliE*, and *fliC* mRNAs in RsmA⁺ and RsmA⁻ *E. carotovora* subsp. *carotovora* strains.

Our previous data (Cui et al. 1995) had demonstrated that the high levels of transcripts of exoenzyme genes and *hrpL*, the σ factor required for expression of type III effector genes, were due to the greater stability of mRNAs of those genes in the RsmA⁻ strain than in the RsmA⁺ strain. Extrapolating upon

Fig. 1. Effects of RsmA/CsrA on transcript levels of *flhDC* in *Erwinia carotovora* subsp. *carotovora* and *Escherichia coli*. **A**, Northern blot analysis of *flhDC* of 1, *Erwinia carotovora* subsp. *carotovora* Ecc71 and 2, its RsmA⁻ mutant AC5071; **B**, 1, *Escherichia coli* BW3414 and 2, its CsrA⁻ mutant TR1-5. Bacterial cells were grown in King's B medium to a Klett value of approximately 220 for total RNA extraction. Each lane contained 15 μ g of total RNA.

Fig. 2. Transcript levels of *fliA*, *fliE*, and *fliC* in 1, *Erwinia carotovora* subsp. *carotovora* Ecc71 and 2, *E. carotovora* subsp. *carotovora* Ecc7N2. Bacterial cells were grown in minimal salts plus sucrose medium to a Klett value of approximately 200 for total RNA extraction. Each lane contained 10 μ g of total RNA. For *fliA* and *fliE*, X-ray films were exposed 24 h at -80°C and, for *fliC*, X-ray film was exposed for 1 h.

Fig. 3. Characterization of an RsmA⁻ mutant of *Erwinia carotovora* subsp. *carotovora* Ecc7N2. 1, Ecc7N2 and 2, its RsmA⁻ mutant AC5152. **A**, Agarose plate assays of pectate lyase (Pel), polygalacturonase (Peh), protease (Prt), and cellulase (Cel) activities. **B**, Soft-rot disease symptoms in celery petiole. **C**, Motility in tryptone swarm agar. The inoculated plates were incubated at 28°C for 18 h. **D**, Northern blot analysis of *flhDC*, *fliA*, *fliE*, and *fliC*. Bacterial cells were grown in King's B medium to a Klett value of approximately 220 for total RNA extraction. Each lane contained 15 µg of total RNA. For *flhDC*, *fliA*, and *fliE*, X-ray films were exposed 24 h at -80°C and, for *fliC*, X-ray film was exposed for 1 h.

those observations, we predicted that the higher levels of *flhDC*, *fliA*, *fliC*, and *fliE* transcripts in RsmA⁻ bacteria also were due to greater stability of those transcripts in the RsmA⁻ strain compared with the parent. Indeed, the results in Figure 5 clearly demonstrate that the mRNAs of those genes were more stable in RsmA⁻ strain AC5152 than in the RsmA⁺ parent Ecc7N2. These results contrast with those of Wei and associates (2001) in that the *flhDC* transcripts were approximately threefold more stable in CsrA⁺ wild-type *Escherichia coli* versus the CsrA⁻ mutant cells. Of the several *fli* transcripts, *fliC* RNA of EccN2 was least susceptible to degradation or processing, and this pattern remained unchanged in the absence of RsmA. The basis for this remarkable stability of *fliC* transcripts awaits clarification.

AHL positively controls motility in *Erwinia carotovora* subsp. *carotovora*.

The effects of AHL on motility have been determined in several bacterial species and the results vary depending on the bacterial species. For example, null mutation of AHL biosynthesis has no effect on swimming motility in *Pseudomonas aeruginosa* (Reimann et al. 2002), whereas AHL deficiency in *Yersinia enterocolitica* results in decreased swimming and swarming motility (Atkinson et al. 2006). Recently, Hussain and associates (2008) reported that null mutation of AHL increased swimming and swarming motility of *E. chrysanthemi* pv. *zeae*. To examine the effects of AHL on motility in *E. carotovora* subsp. *carotovora*, we made an AhlI⁻ mutant of the motile *E. carotovora* subsp. *carotovora* Ecc7N2. This AhlI⁻ strain, AC5151, was then tested for motility in tryptone swarm agar. The results (Fig. 6A) demonstrate that AHL deficiency of this *E. carotovora* subsp. *carotovora* resulted in reduced motility. Northern blot analysis revealed that levels of transcript of *flhDC*, *fliA*, and *fliC* were lower in the AhlI⁻ mutant than in the parent (Fig. 6B). It should be noted that, in contrast to the levels of transcripts of *flhDC* and *fli* genes, the level of *rsmA* RNA was higher in the AhlI⁻ mutant of Ecc7N2 (Fig 6B). The latter finding was reminiscent of the effects of AHL deficiency in Ecc71 and raised the possibility that the AHL effect on motility may be operating via RsmA.

The AHL receptors ExpR1 and ExpR2 negatively regulate expression of *flhDC*, *fliA*, and *fliC* in the absence of AHL.

Having established a positive regulatory role of AHL on motility of *E. carotovora* subsp. *carotovora*, we focused on the

Fig. 4. RsmA of *Erwinia carotovora* subsp. *carotovora* Ecc71 restores the transcript levels of *flhDC* and *fliA* as well as motility of *Escherichia coli* CsrA⁻ mutant TR1-5. 1, TR1-5 carrying the cloning vector pCL1920 and 2, TR1-5 carrying the *rsmA*_{Ecc}⁺ plasmid pAKC880. **A**, Northern blot analysis of *flhDC* and *fliA*. Bacterial cells were grown in King's B medium supplemented with spectinomycin to a Klett value of approximately 220 for total RNA extraction. Each lane contained 15 µg of total RNA. **B**, Motility in tryptone swarm agar plate supplemented with spectinomycin. The inoculated plate was incubated at 37°C for 48 h.

underlying mechanism. We should recall that the seminal contributions of G. Salmond and T. Palva have established that extracellular enzyme production and virulence are controlled by AHL (Jones et al. 1993; Pirhonen et al. 1993). Subsequent work in our laboratory and elsewhere has clarified the modes of AHL action (Cui et al. 2005, 2006; Sjöblom et al. 2006). In *E. carotovora* subsp. *carotovora*, there are two distinct regulatory pathways involving target-specific receptors (Barnard et

Fig. 5. Stabilities of *flhDC*, *fliA*, *fliC*, and *fliE* transcripts in *Erwinia carotovora* subsp. *carotovora* Ecc7N2 (lanes 1 through 8) and its RsmA⁻ mutant AC5152 (lanes 9 through 16). Samples were collected at 0, 1, 2, 4, 6, 8, 10, and 15 min after the addition of rifampin. Lanes 1 through 8, each lane contained 15 µg of total RNA and lanes 9 through 16, each lane contained 10 µg of total RNA.

Fig. 6. Effects of AhlI deficiency on motility and expression of *flhDC*, *fliA*, *fliC*, and *rsmA* in *Erwinia carotovora* subsp. *carotovora* Ecc7N2 (Ecc7N2). 1, Ecc7N2 and 2, its AhlI⁻ mutant AC5151. **A**, Motility in tryptone swarm agar. The inoculated plate was incubated at 28°C for 22 h. **B**, Northern blot analysis of *flhDC*, *fliA*, *fliC*, and *rsmA*. Bacterial cells were grown in King's B medium to a Klett value of approximately 220 for total RNA extraction. Each lane contained 15 µg of total RNA. For *flhDC*, *fliA*, and *rsmA*, X-ray films were exposed 24 h at -80°C and, for *fliC*, X-ray film was exposed for 1 h.

al. 2007). In a subsets of *E. carotovora* subsp. *carotovora* strains, the regulation of antibiotic production follows the LUX paradigm in that CarR, the AHL receptor, binds AHL and this complex activates the antibiotic (carbapenem = Car) biosynthetic (*car*) genes (Whitehead et al. 2002). To the best of our knowledge, the CarR-AHL complex does not control any other phenotype, including AHL production. The other AHL receptors, ExpR1 and ExpR2 (= VirR) (Sjöblom et al. 2006), activate *rsmA* transcription in the absence of AHL; this activator function is abrogated upon AHL binding (Cui et al. 2005, 2006). Thus, AHL modulates the levels of RsmA through its effects on ExpR action and, thereby, controls multiple phenotypes of *E. carotovora* subsp. *carotovora*. In light of the global

Fig. 7. Effects of ExpR1/ExpR2 on expression of *flhDC*, *fliA*, *fliC*, and *rsmA* in the absence of *N*-acylhomoserine lactone (AHL). Northern blot analysis of (1) AC5091 (AhlI⁻ExpR1⁺ExpR2⁺); 2, AC5099 (AhlI⁻ExpR1⁻ExpR2⁺); 3, AC5117 (AhlI⁻ExpR1⁺ExpR2⁻); and 4, AC5118 (AhlI⁻ExpR1⁻ExpR2⁻). Bacterial cells were grown in King's B medium to a Klett value of approximately 220 for total RNA extraction. Each lane contained 15 µg of total RNA. For *flhDC*, *fliA*, and *rsmA*, X-ray films were exposed 24 h at -80°C and, for *fliC*, X-ray film was exposed for 3 h.

effects of RsmA, including its role in motility (described above), we were led to examine the responses of ExpR (i.e., activators of *rsmA* transcription) in the absence of AHL. To study the effects of ExpR1 and ExpR2 on expression of *flhDC*, *fliA*, and *fliC*, we compared the transcript levels of those genes in several mutants of Ecc71: AhlI⁻ExpR1⁺ExpR2⁺ (AC5091), AhlI⁻ExpR1⁻ExpR2⁺ (AC5099), AhlI⁻ExpR1⁺ExpR2⁻ (AC5117), and AhlI⁻ExpR1⁻ExpR2⁻ (AC5118). Aside from the availability of well-characterized mutants, the rationale for using Ecc71 derivatives for this analysis was our finding that the expressions of *flhDC* and *fliA* are comparable in both Ecc71 and Ecc7N2 (described above; Fig. 2). Therefore, we predicted that the responses we see with Ecc71 should also apply for Ecc7N2. The results (Fig. 7) revealed that i) the levels of *flhDC*, *fliA*, and *fliC* transcripts were slightly higher in the AhlI⁻ExpR1⁻ExpR2⁺ strain (column 2) than that of AhlI⁻ExpR1⁺ExpR2⁺ strain (column 1), ii) the levels of those transcripts were much higher in AhlI⁻ExpR1⁺ExpR2⁻ (column 3) and AhlI⁻ExpR1⁻ExpR2⁻ (column 4) than that of AhlI⁻ExpR1⁺ExpR2⁺ (column 1), and iii) the levels of *flhDC*, *fliA*, and *fliC* transcripts correlated with the levels of *rsmA* tran-

script in those strains. These observations indicate that both ExpR1 and ExpR2 negatively regulate expression of those genes via stimulation of the expression of *rsmA*. However, ExpR2 compared with ExpR1 is more effective in controlling expression of those genes.

The AHL effect is channeled via RsmA.

To test whether the AHL effect is channeled via the RsmA pathway, we compared the levels of transcripts of flagellar genes and the degree of motility in RsmA⁻ strains of Ecc7N2 that were either AhlI⁺ (AC5152) or AhlI⁻ (AC5153). The motility assay results (Fig. 8A) revealed that both of those strains are hypermotile compared with the AhlI⁻RsmA⁺ strain AC5151. Moreover, transcript levels of *flhDC*, *fliA*, and *fliC* were comparable in the AhlI⁻RsmA⁻ and AhlI⁺RsmA⁻ strains (Fig. 8B). We should note that these two strains produced similar levels of exoenzymes (Fig. 8C) as well as transcripts of several exoenzyme genes: *pel-1*, *peh-1*, and *celV* (Fig. 8D). We have previously documented that the levels of exoenzymes in RsmA⁻AhlI⁺ and RsmA⁻AhlI⁻ mutants of *E. carotovora* subsp. *carotovora* Ecc71 are similar (Chatterjee et al. 1995). Thus, in the absence of RsmA, AHL has no effect on motility and exoprotein production, proving that the AHL effect is channeled via RsmA.

Fig. 8. Effect of *N*-acylhomoserine lactone (AHL) deficiency on motility, exoenzyme production, and expression of *flhDC*, *fliA*, *fliC*, *pel-1*, *peh-1*, and *celV* in the absence of RsmA. 1, AC5151 (AhlI⁻RsmA⁺); 2, AC5152 (AhlI⁺RsmA⁻); and 3, AC5153 (AhlI⁻RsmA⁻). **A**, Motility in tryptone swarm agar. The inoculated plate was incubated at 28°C for 22 h. **B** and **D**, Northern blot analysis of *flhDC*, *fliA*, *fliC*, *pel-1*, *peh-1*, and *celV*. Bacterial cells were grown in King's B medium to a Klett value of approximately 220 for total RNA extraction for *flhDC*, *fliA*, and *fliC*. For *pel-1*, *peh-1*, and *celV*, RNAs were isolated from bacteria grown in minimal-salts plus sucrose (0.5%, wt/vol) medium to a Klett value of approximately 220. Each lane contained 15 µg of total RNA. For *flhDC*, *fliA*, *pel-1*, *peh-1*, and *celV*, X-ray films were exposed for 24 h at -80°C and, for *fliC*, X-ray film was exposed for 1 h. **C**, Agarose plate assays of pectate lyase (Pel), polygalacturonase (Peh), protease (Prt), and cellulase (Cel) activities. Culture supernatants from bacteria grown in minimal-salts plus sucrose medium were used for the plate assays.

Conclusions.

The findings reported here allow three major conclusions (Fig. 9): motility and flagellar genes are negatively regulated by RsmA and positively regulated by AHL, and the AHL effect is mediated through RsmA involving the actions of the AHL receptors ExpR1 and ExpR2. The RsmA effect manifests itself through modulation of chemical stabilities of transcripts of flagellar genes affecting the levels of RNA species. This effect is consistent with RsmA actions on genes for various exoproteins and their regulators, as previously reported. The evidence presented here also shows that ExpR1/ExpR2, in the absence of AHL, stimulates transcription of *rsmA*. In the presence of AHL, this activity is abolished and, in this manner, AHL modulates the pool of RsmA. In *Escherichia coli*, RsmA (= CsrA) positively regulates *flhDC* expression and motility. Our observations are essentially the same as those previously reported by Wei and associates (2001). They found greater stability of *flhDC* RNA and increased transcription of *flhDC-lacZ* transcriptional fusion in CsrA⁺ than in CsrA⁻ *Escherichia coli* strains. The molecular basis for this response is not clear. One possibility is that CsrA binding retards the decay of *flhDC* RNA, unlike the destabilizing action of RsmA on most other RNA species. The other possibility is that CsrA promotes a

Fig. 9. Model showing motility and flagellar genes are negatively regulated by RsmA and positively regulated by *N*-acylhomoserine lactone (AHL) in *Erwinia carotovora* subsp. *carotovora* Ecc7N2. The AHL effect is mediated through RsmA involving the actions of the AHL receptors, ExpR1 and ExpR2. ExpR1 and ExpR2 bind upstream DNA of *rsmA* and stimulate *rsmA* expression. The activator function of ExpR1 and ExpR2 is abrogated upon AHL binding. Thus, ExpR1, ExpR2, the ExpR1-AHL (3-oxo-C6-HL) complex, and the ExpR2-AHL (3-oxo-C6-HL and 3-oxo-C8-HL) complex modulate the levels of RsmA. In addition, RpoS, the stress response σ factor, possibly regulates *rsmA* expression. The RsmA effect manifests itself through modulation of chemical stabilities of transcripts of flagellar genes affecting the levels of RNA species which, in turn, control motility. Motility is also regulated by several positive and negative regulators. Positive regulators are *rsmB*, an untranslated RNA that binds RsmA and neutralizes its negative effect (Rsm = regulator of secondary metabolism); GacA, the response regulator of a widely prevalent two-component system and a positive regulator of *rsmB*; and FlhDC, the master regulator of flagellar genes, which controls exoprotein production and motility by positively controlling *gacA*, *fliA*, and *rsmC* and negatively controlling *hexA* (FliA, a flagellum-specific σ factor; RsmC, an anti-FlhDC factor, and HexA, a LysR-like protein and a homolog of *Escherichia coli* LrhA). HexA negatively regulates *flhDC*, *gacA*, and *rsmB* expression (A. Chatterjee, unpublished data), and these repressions are presumed to account for its remarkable effects on quorum sensing-signal and exoprotein production and motility (A. Chatterjee, unpublished data).

rapid decay of a negative regulator of flagellar genes and this, in turn, stimulates the expression of FlhDC and other flagellar genes. Despite these uncertainties, it is clear that RsmA effects on *flhDC* in *Escherichia coli* and *E. carotovora* subsp. *carotovora* are fundamentally different.

In *E. carotovora* subsp. *carotovora*, motility, a component of the virulence arsenal, is coregulated by RsmA and AHL with extracellular enzymes or proteins, the primary determinants of virulence. This coregulation may be significant in enhancing the disease potential of the pathogen. The actions of secreted enzymes or proteins would cause plant cell death, releasing nutrients for bacterial sustenance. The depletion of nutrients by feeding of the pathogenic bacterium and commensals present at the infection site would necessitate movement of bacteria to a suitable niche to inflict additional damage and nutrient release. This progressive degradation of plant tissue and movement may be critical in disease severity. The pool size of free RsmA and its differential affinity for various RNA species may determine the temporal sequence in which the genes for motility and exoproteins are expressed during the course of disease development.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media.

Bacterial strains and plasmids are described in Table 1. *E. carotovora* subsp. *carotovora* and *Escherichia coli* strains were maintained on Luria-Bertani (LB) agar. The strains carrying antibiotic markers were maintained on LB agar containing appropriate antibiotics.

The compositions of LB, King's B (KB), and minimal-salts (MM) media have been described in previous publications (Chatterjee et al. 1995, 2003; Murata et al. 1991). When required, antibiotics were supplemented as follows: gentamicin at 10 µg/ml, kanamycin at 50 µg/ml, spectinomycin at 50 µg/ml, and tetracycline at 10 µg/ml. Media were solidified using 1.5% (wt/vol) agar.

The composition of media for agarose plate assays for enzymatic activities was described by Chatterjee and associates (1995).

Motility assay.

Cells of Ecc7N2, AC5151, AC5152, and AC5153 were stab inoculated into tryptone swarm agar (Harris et al. 1998) and plates were supplemented with spectinomycin for inoculation of bacterial cells harboring pCL1920 or pAKC880. Inoculated plates were incubated at 28°C for 22 h as indicated in the figure legends. Motility of the bacteria was visually examined.

Extracellular enzyme assays.

Bacterial cells were grown in MM supplemented with sucrose (0.5%, wt/vol) to a Klett value of approximately 250 and the cultural supernatants were used for assays. The semiquantitative pectate lyase (Pel), polygalacturonase (Peh), protease (Prt), and cellulase (Cel) agarose plate assays were performed according to previously published procedures (Chatterjee et al. 1995). Briefly, wells were made in agarose media with a no. 2 cork borer and the bottoms were sealed with molten agarose (0.8% [wt/vol]). Samples were applied to the wells and the plates were incubated at 28°C. After 16 to 18 h, Pel and Peh assay plates were developed with 4 N HCl, and the Cel assay plates were developed with 0.1% (wt/vol) Congo red and 1 M NaCl solutions. Halos around the wells due to protease activity became visible in Prt assay plates within 24 to 36 h without any further treatment.

Plant tissue maceration.

The celery petiole assay was performed as described by Murata and associates (1991). Bacterial cells (2×10^7) were inoculated into each site. The extent of tissue maceration was estimated visually. For each assay, five different petioles were used and the experiments were repeated at least three to four times. In addition, the positions of the inoculation sites for the parent and the mutant were exchanged to minimize regional differences, if any.

Table 1. Bacterial strains and plasmids used in this study

Strains, plasmids	Relevant characteristics	Source, reference
Strains		
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>		
Ecc71	Wild type	Zink et al. 1984
AC5006	Lac ⁻ derivative of Ecc71	Murata et al. 1991
AC5071	Km ^r , RsmA ⁻ derivative of Ecc71	Chatterjee et al. 1995
AC5091	Sp ^r , AhlI ⁻ ExpR1 ⁺ ExpR2 ⁺ derivative of AC5006	Chatterjee et al. 1995
AC5099	Km ^r , Sp ^r , AhlI ⁻ ExpR1 ⁺ ExpR2 ⁺ derivative of AC5006	Cui et al. 2005
AC5117	Km ^r , Gm ^r , AhlI ⁻ ExpR1 ⁺ ExpR2 ⁻ derivative of AC5006	Cui et al. 2006
AC5118	Km ^r , Sp ^r , Gm ^r , AhlI ⁻ ExpR1 ⁺ ExpR2 ⁻ derivative of AC5006	Cui et al. 2006
Ecc7N2	Wild type	Lab. collection
AC5151	Km ^r , AhlI ⁻ derivative of Ecc7N2	This study
AC5152	Km ^r , RsmA ⁻ derivative of Ecc7N2	This study
AC5153	Km ^r , Sp ^r , AhlI ⁻ RsmA ⁻ derivative of Ecc7N2 of Ecc7N2	This study
<i>Escherichia coli</i>		
DH5α	φ80 <i>lacZ</i> AM15 Δ(<i>lacZYA-argF</i>), U169 <i>hsdR17 recA1 endA1 thi-1</i>	Gibco BRL
BW3414	Δ <i>lacU169 rpoS</i> (Am)	T. Romeo
TR1-5	CsrA ⁻ mutant of BW3414	T. Romeo
Plasmids		
pCL1920	Sp ^r , Sm ^r	Lerner and Inouye 1990
pAKC781	Ap ^r , <i>peh-1</i> DNA in pBluescriptSK(+)	Liu et al. 1994
pAKC783	Ap ^r , <i>pel-1</i> DNA in pBluescriptSK(+)	Liu et al. 1994
pAKC854	Km ^r , Tc ^r , inactivated <i>ahII</i> DNA in pRK415	Chatterjee et al. 1995
pAKC855	Sp ^r , Tc ^r , inactivated <i>ahII</i> DNA in pRK415	Chatterjee et al. 1995
pAKC870	Km ^r , Tc ^r , inactivated <i>rsmA</i> DNA in pLARF5	Chatterjee et al. 1995
pAKC880	Sp ^r , <i>rsmA</i> of Ecc71 in pCL1920	Cui et al. 1995
pAKC882	Ap ^r , <i>rsmA</i> coding region in pT7-7	Mukherjee et al. 1997
pAKC1034	Ap ^r , 200-bp <i>celV</i> fragment in pGEM-T Easy	Liu et al. 1999
pAKC1246	Sp ^r , <i>plac-flhA</i> in pCL1920	Cui et al. 2008

^a Km^r, Sp^r, Gm^r, Sm^r, Ap^r, and Tc^r indicate resistance to kanamycin, spectinomycin, gentamicin, streptomycin, ampicillin, and tetracycline, respectively.

DNA techniques.

Standard procedures were used in the isolation of plasmid DNAs, gel electrophoresis, polymerase chain reactions (PCR), DNA ligation, transformation, and electroporation (Sambrook et al. 1989). Restriction and modification enzymes were obtained from Promega Biotec (Madison, WI, U.S.A.). Prime-a-Gene DNA labeling system (Promega Biotec) was used for labeling DNA probes.

Construction of AhlI⁻, RsmA⁻ and AhlI⁻RsmA⁻ mutants of *E. carotovora* subsp. *carotovora* Ecc7N2.

The AhlI⁻ mutant AC5151 was constructed by marker exchange of Ecc7N2 with plasmid pAKC854 which contains inactivated *ahlI* with flanking DNAs. The RsmA⁻ mutant AC5152 was obtained by marker exchange of Ecc7N2 with inactivated *rsmA* plasmid pAKC870 with flanking DNAs. For making the AhlI⁻RsmA⁻ double mutant AC5153, inactivated *ahlI* plasmid pAKC855 was used for marker exchange with Ecc7N2. This AhlI⁻ mutant was then marker exchanged with pAKC870 to yield AC5153. The procedures for marker exchange have been described in Chatterjee and associates (1995). Inactivation of target genes in mutants was confirmed by Northern blot analysis.

Northern blot analysis.

Bacterial cultures were grown at 28°C in KB or MM supplemented with sucrose (0.5% wt/vol). Cells in late exponential growth phase were harvested as the cultures reached a Klett value of approximately 200 in MM + sucrose medium or 220 in KB medium. RNA extractions were done from at least two independently grown cultures. RNA isolation and Northern blot analysis were performed as described by Liu and associates (1994). To determine the stability of *flhDC*, *fliA*, *fliC*, and *fliE* of Ecc7N2 and its RsmA⁻ mutant AC5152, the bacteria were grown in KB medium to a Klett value of approximately 200, at which point rifampin (0.2 mg/ml, wt/vol) was added to block further initiation. Culture samples (8 ml each) were then withdrawn at various time points into tubes containing 5 ml of ice-cold water, and total RNA was extracted. The probes used were the 183-bp *NdeI*-*SalI* fragment of *rsmA* from pAKC882, 314-bp *EcoRV*-*KpnI* fragment of *pel-1* from pAKC783, 743-bp *HindIII* fragment of *peh-1* from pAKC781, 200-bp *EcoRI* fragment of *celV* from pAKC1034, and 811-bp *BamHI*-*HindIII* fragment of *fliA* from pAKC1246. The *flhDC*, *fliC*, and *fliE* of *E. carotovora* subsp. *carotovora* as well as *flhDC* and *fliA* of *Escherichia coli* probes were PCR amplified using primers described by Cui and associates (2008) and Chatterjee and associates (2009). Equal loading of RNA was checked by hybridization of the blot with a probe corresponding to 16S ribosomal RNA (16S rDNA).

Extracellular enzyme assays, Northern blot, and pathogenicity tests were performed at least twice and the results were reproducible.

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