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Global regulation in *Erwinia* species by *Erwinia carotovora rsmA*, a homologue of *Escherichia coli csrA*: repression of secondary metabolites, pathogenicity and hypersensitive reaction

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Our previous studies revealed that *rsmA* of *Erwinia carotovora* subsp. *carotovora* strain 71 suppressed the synthesis of the cell density (quorum) sensing signal *N*-(3-oxohexanoyl)-L-homoserine lactone, the production of extracellular enzymes and tissue macerating ability in soft-rotting *Erwinia* species and that homologues of this negative regulator gene were present in other *Erwinia* species. Northern blot data presented here demonstrate that *rsmA* and *rsmA*-like genes are also expressed in soft-rotting and non-soft-rotting *Erwinia* spp. such as *E. amylovora*, *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, *E. carotovora* subsp. *carotovora*, *E. chrysanthemi*, *E. herbicola* and *E. stewartii*. A low-copy plasmid carrying *rsmA* of *E. carotovora* subsp. *carotovora* strain 71 caused suppression of antibiotic production in *E. carotovora* subsp. *betavasculorum*, flagellum formation in *E. carotovora* subsp. *carotovora*, carotenoid production in *E. herbicola* and *E. stewartii*, and indigoidine production in *E. chrysanthemi*. In *E. amylovora*, *rsmA* of *E. carotovora* subsp. *carotovora* suppressed the elicitation of the hypersensitive reaction in tobacco leaves and the production of disease symptoms in apple shoots, in addition to repressing motility and extracellular polysaccharide production. We conclude that *rsmA* homologues function as global regulators of secondary metabolic pathways as well as factors controlling host interaction of *Erwinia* species.

Keywords: *Erwinia*, motility, pathogenicity factors, global regulation, hypersensitive reaction

INTRODUCTION

The enterobacterial genus *Erwinia* contains plant-pathogenic and plant-associated bacteria that are capable of producing extracellular enzymes, polysaccharides, antibiotics or pigments (Chatterjee & Vidaver, 1986; Starr, 1983; Vanneste, 1995). A number of regulatory genes have been identified in *Erwinia* that specifically control the production of extracellular enzymes (Barras *et al.*, 1994; Collmer & Keen, 1986; Kotoujansky, 1987), polysaccharides (Leigh & Coplin, 1992; Roberts & Coleman, 1991) and antibiotics (McGowan *et al.*, 1995). Such specific regulatory systems are generally activated during late-exponential growth or early-stationary phase when bacteria reach high cell density, when experiencing nutrient limitation (starvation) or both. This growth-phase dependence suggests that production of extracellular enzymes and secondary metabolites such as antibiotics, pigments and polysaccharides may respond to a common regulator. Evidence supports the hypothesis that the cell density (quorum) sensing signal *N*-(3-oxohexanoyl)-L-homoserine lactone (HSL), or its structural analogues could be a component of such a regulatory system. It has been discovered that in *Erwinia carotovora* subsp. *carotovora*, HSL is required for the production of extracellular enzymes and an antibiotic as well as for pathogenicity (Bainton *et al.*, 1992; Jones *et al.*, 1993; Pirhonen *et al.*, 1993). Also, many soft-rotting and non-soft-rotting *Erwinia* species are now known to produce HSL (Bainton *et al.*, 1992; Beck von Bodman & Farrand, 1995; Chatterjee *et al.*, 1995; Cui *et al.*, 1995; Jones *et al.*, 1993;

Abbreviations: EPS, extracellular polysaccharide; HR, hypersensitive reaction; HSL, *N*-(3-oxohexanoyl)-L-homoserine lactone.

Pirhonen *et al.*, 1993; Salmond *et al.*, 1995; Williams *et al.*, 1992; A. K. Chatterjee, unpublished results).

We recently identified a negative regulator gene, *rsmA* of *E. carotovora* subsp. *carotovora* strain 71 (Chatterjee *et al.*, 1995; Cui *et al.*, 1995), which suppressed the synthesis of HSL, the production of extracellular pectate lyase (Pel), polygalacturonase (Peh), cellulases (Cel) and proteases (Prt), and pathogenicity in soft-rotting *Erwinia* species. This pleiotropic effect of RsmA, as well as extensive similarity between *rsmA* and *csrA* of *Escherichia coli*, and the occurrence of *rsmA* homologues in all other enterobacterial species tested, led us to consider the possibility that *rsmA* or its homologues may function as global regulators in *Erwinia*. In this communication, we demonstrate that *rsmA* homologues are normally expressed in *Erwinia* species, and that *rsmA* of *E. carotovora* suppresses such diverse traits as the production of an antibiotic, pigments and extracellular polysaccharides (EPS), flagellum formation and motility, pathogenicity and elicitation of the hypersensitive reaction (HR).

METHODS

Bacterial strains, plasmids and media. Bacterial strains and plasmids used in this study are listed in Table 1. The strains carrying drug markers were maintained on LB agar containing appropriate antibiotics. The wild-type strains of *Erwinia* were maintained on yeast extract/glucose/calcium carbonate (YGC) agar.

The compositions of KB medium, LB medium, minimal medium, nutrient gelatin agar, and YGC agar have been previously described (Barras *et al.*, 1987; Chatterjee, 1980; Chatterjee *et al.*, 1995; Murata *et al.*, 1990, 1991). Nutrient agar glycerol (NAG) medium was prepared according to Costa & Loper (1994). For the detection of EPS production, nutrient agar supplemented with 1% (w/v) galactose was used. When required, ampicillin and spectinomycin were supplemented at 50 μg ml^{-1}. Media were solidified by the addition of 1·5% (w/v) agar, except soft KB, which contained 0·4% (w/v) agar.

Antibiotic assay. The procedure described by Costa & Loper (1994) was utilized for the detection of antibiotic production. Briefly, cells of *E. carotovora* subsp. *betavasculorum* strain Ecb11129 harbouring the $RsmA^+$ plasmid pAKC880 or the cloning vector pCL1920 were patched on NAG medium supplemented with spectinomycin and incubated at 28 °C for 48 h. A culture of the test strain *E. carotovora* subsp. *carotovora* 193 harbouring pCL1920, grown to a Klett value of approximately 30 (OD_{600} 0·24) in LB medium plus spectinomycin, was then sprayed onto the surface of seeded agar plates. The plates were incubated at 28 °C for an additional 18 h. Antibiotic production was indicated by zones of clearing around the growth of Ecb11129(pCL1920).

Pigment production. Bacteria harbouring pAKC880 or

Table 1. Bacterial strains and plasmids

Bacteria and plasmids	Relevant characteristics	Reference or source
Strain		
E. carotovora* subsp. *carotovora		
71	Wild-type	Zink *et al.* (1984)
193	Wild-type	Zink *et al.* (1984)
AC5070	$RsmA^-$ Km^r	Chatterjee *et al.* (1995)
SCC3193	Wild-type	Pirhonen *et al.* (1993)
SCRI193	Wild-type	Salmond *et al.* (1986)
E. carotovora* subsp. *atroseptica		
Eca12	Wild-type	Zink *et al.* (1984)
E. carotovora* subsp. *betavasculorum		
Ecb11129	Wild-type	J. E. Loper, Agricultural Research Service, USDA, OR, USA
E. chrysanthemi		
EC183	Wild-type	Chatterjee & Brown (1981)
CU156	Wild-type	Laboratory collection
E. herbicola		
EH105	Wild-type	Laboratory collection
E. amylovora		
E9	Wild-type	Politis & Goodman (1980)
E. stewartii		
DC283	Wild-type	D. L. Coplin, Ohio State University, OH, USA
Plasmid		
pCL1920	Spc^r Sm^r	Lerner & Inouye (1990)
pAKC880	$RsmA^+$ Spc^r	Cui *et al.* (1995)
pAKC882	pT7-*rsmA*, Ap^r	PCR product of pAKC880 cloned into pT7-7 (Tabor & Richardson, 1985)
pAKC783	*pel-1*$^+$ Ap^r	Liu *et al.* (1994)

pCL1920 were patched on appropriate media supplemented with spectinomycin and incubated at 28 °C. Indigoidine production by *E. chrysanthemi* (CU156) was assayed on YGC agar (Chatterjee & Brown, 1981) and carotenoid production by *E. herbicola* and *E. stewartii* was assayed on LB agar. Pigment production was examined visually after 24 h incubation.

Motility assay and detection of flagella. Bacterial cells harbouring pAKC880 or pCL1920 were stab-inoculated into soft KB agar supplemented with spectinomycin and incubated at 28 °C. Motility of the bacteria was visually examined.

For detection of flagella, electron microscopy techniques were employed in which cells were negatively stained using 1 % (v/v) phosphotungstic acid and mounted on carbon-coated 200-mesh copper grids. Samples were visualized using transmission electron microscope model JEOL JEM 100B.

RNA analysis. Bacterial cultures were grown to a value of approximately 200 Klett units (OD_{600} 1·6) at 28 °C in LB medium. The procedures for RNA isolation and Northern blot analysis described by Chatterjee *et al.* (1991) and Liu *et al.* (1993) were followed. Blots containing total RNA samples from *Erwinia* strains were hybridized using the 138 bp *Nde*I–*Bgl*II *rsmA* fragment of pAKC882 or the 314 bp *Eco*RV–*Kpn*I *pel-1* fragment of pAKC783 as a probe.

Test for EPS production. Bacteria harbouring pCL1920 or pAKC880 were patched on appropriate media supplemented with spectinomycin and incubated at 28 °C for 24–48 h. Cells of *E. amylovora* E9 and *E. herbicola* EH105 constructs were inoculated on nutrient agar supplemented with galactose (1 %, w/v) and *E. stewartii* DC283 constructs were inoculated on minimal agar medium containing 1 % (w/v) sucrose. Production of EPS was indicated by a slimy and mucoid growth.

Elicitation of HR. Bacteria were grown overnight at 28 °C on LB agar supplemented with spectinomycin or on KB agar. Cells were suspended in water or spectinomycin solution (50 μg ml^{-1}) to a concentration of approximately 2×10^8 c.f.u. ml^{-1}. Young fully expanded third and fourth leaves of 6-week-old plants of *Nicotiana tobacum* L. cv. Samsun were infiltrated with bacterial cell suspensions. Inoculated plants were incubated at 28 °C for 24 h in a growth chamber with a 14/10 h daylight regime and visually monitored for reactions.

Pathogenicity assays on apple shoots. One-year-old branched Jonathan apple plants were transplanted into pots and maintained in a growth chamber at 28 °C with a 14/10 h daylight regime. Four-week-old transplants with four to five side branches were used for the assay. The laminae of the first three to five fully expanded leaves were cut at the junction of the petioles. Cells of *E. amylovora* strain E9 harbouring pAKC880 or pCL1920, grown overnight on LB agar supplemented with spectinomycin, were suspended in spectinomycin solution (50 μg ml^{-1}) to a concentration of approximately 2×10^8 c.f.u. ml^{-1}. Aliquots (5 μl) of cell suspensions were added to the cut surfaces and allowed to penetrate. This process was repeated until each petiole had absorbed a total of 30 μl cell suspension. Inoculated plants were incubated for 24–48 h under the environmental conditions described above.

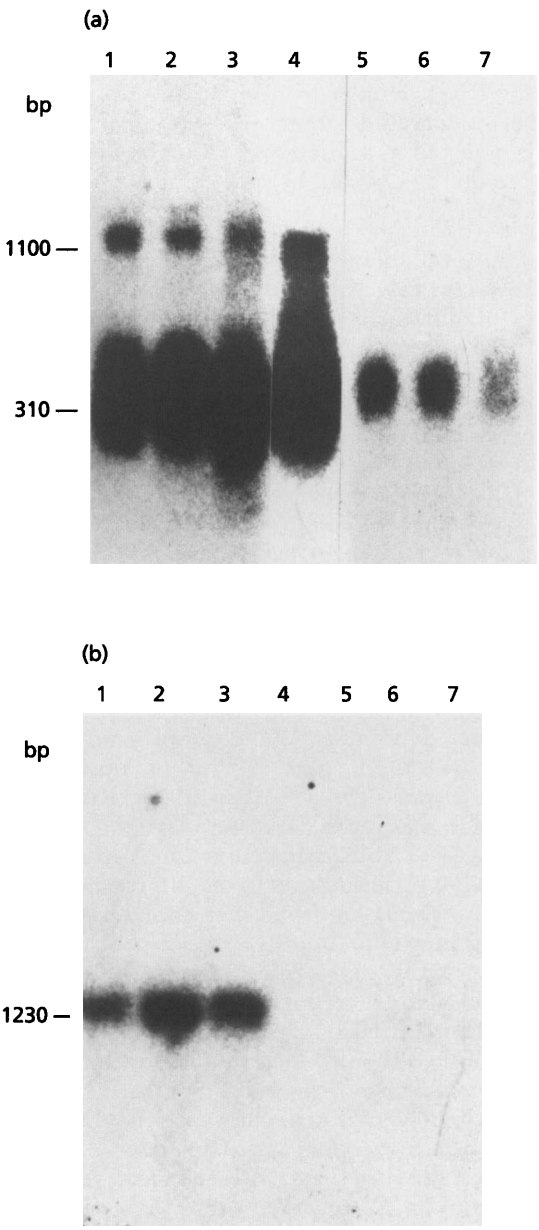

Fig. 1. **Northern blot analysis of total RNA from *Erwinia* species using *rsmA* DNA (a) and *pel-1* DNA (b) of *E. carotovora* subsp. *carotovora* 71 as probes. Lanes: 1, *E. carotovora* subsp. *carotovora* 71; 2, *E. carotovora* subsp. *atroseptica* Eca12; 3, *E. carotovora* subsp. *betavasculorum* Ecb11129; 4, *E. chrysanthemi* EC183; 5, *E. stewartii* DC283; 6, *E. amylovora* E9; 7, *E. herbicola* EH105. Lanes 1–4 contained 10 μg total RNA each; lanes 5–7 contained 20 μg total RNA each.**

RESULTS AND DISCUSSION

Occurrence of *rsmA* transcripts in *Erwinia* species

rsmA homologues have been detected in many enterobacteria, including *Erwinia* species (Cui *et al.*, 1995). To determine if *rsmA*-like genes are normally expressed in these bacteria we conducted Northern analysis using the *rsmA* DNA of *E. carotovora* subsp. *carotovora* as the probe. The data presented in Fig. 1(a) show that *rsmA* transcripts were present in the wild-type strains of *E. amylovora*, *E. carotovora* subsp. *atroseptica*, *E. carotovora* subsp. *betavasculorum*, *E. carotovora* subsp. *carotovora*, *E. chrysanthemi*, *E. herbicola* and *E. stewartii*. A duplicate blot was hybridized under similar conditions with *pel-1* DNA of *E.*

carotovora subsp. *carotovora* strain 71 (Liu *et al.*, 1994). In the latter case, we detected signals with the RNA samples from strains belonging to *E. carotovora* subspecies but not with the RNA samples from other *Erwinia* species (Fig. 1b). This observation strengthens our conclusion that the signals detected with the *rsmA* DNA as the probe (Fig. 1a) were due to specific hybridization with the cognate mRNA species.

Transcripts of about 310 bp hybridizing with the *E. carotovora rsmA* were detected in all bacterial species tested. In addition, another larger transcript hybridized with the *rsmA* probe in soft-rotting *Erwinia* species, but not in *E. herbicola* and *E. stewartii* (Fig. 1a). We should note that the larger transcript was observed with *E. amylovora* samples only upon longer exposure (i.e. 7 d instead of 2 d) of the X-ray film (data not shown). The low intensity of the signal of this transcript could be due to the use of a heterologous probe. We are currently testing this hypothesis by using *E. amylovora rsmA* DNA as the probe.

These findings suggest that *rsmA* may be organized as a multicistronic operon. Recent observations in our laboratory with *E. carotovora* subsp. *carotovora* strain 71 support this hypothesis. While the plasmid carrying the 183 bp *rsmA* ORF plus 245 bp of upstream DNA produced a transcript of 310 bp in the $RsmA^-$ mutant AC5070 (Cui *et al.*, 1995), a plasmid carrying this ORF and additional (> 2 kb) downstream DNA yielded a transcript of 1100 bp in addition to the 310 bp transcript (data not shown). The latter profile was similar to that found in the wild-type strains of *E. carotovora* subspecies and *E. chrysanthemi* (Fig. 1a). The presence of a mini-Tn*5* transposon within the upstream DNA sequences of the *rsmA* ORF in AC5070 (Chatterjee *et al.*, 1995; Cui *et al.*, 1995) abolished the production of both the 310 and the 1100 bp transcripts. Analysis by Southern hybridization of appropriately digested chromosomal DNA and an $RsmA^+$ cosmid carrying approximately 22 kb chromosomal DNA revealed that *rsmA* sequences were present in a single copy in *E. carotovora* subsp. *carotovora* strain 71. Since there is no evidence of gene duplication, the existence of two species of *rsmA* transcripts might be due to the utilization of different start and termination sites. Additional studies are clearly needed to understand the mechanism, as well as the physiological significance, of the production of these transcripts.

Effect of *rsmA* on antibiotic production in *E. carotovora* subsp. *betavasculorum*

E. carotovora subsp. *betavasculorum* strains are known to produce antibiotics that inhibit *E. carotovora* subsp. *carotovora* (Axelrood *et al.*, 1988; Costa & Loper, 1994). The suppression of HSL synthesis and extracellular enzyme production in *E. carotovora* subsp. *betavasculorum* by *rsmA* of *E. carotovora* subsp. *carotovora* strain 71 (Cui *et al.*, 1995) prompted us to determine if *rsmA* also had an effect on antibiotic production. In preliminary trials we noted that *E. carotovora* subsp. *betavasculorum* strain Ecb11129 produced an antibiotic(s) which inhibited the growth of *E. carotovora* subsp. *carotovora* strains 71, 193, SCRI193 and SCC3193. To test the effect of *rsmA*, cells of Ecb11129 harbouring pAKC880 or pCL1920 were grown on NAG agar plus spectinomycin for 48 h and then oversprayed with cells of *E. carotovora* subsp. *carotovora* strain 193 harbouring pCL1920. After 18 h incubation, a zone of clearing was observed around the growth of Ecb11129(pCL1920). By contrast, no clear zone was detected around the growth of Ecb11129(pAKC880), indicating a severe repression of antibiotic production. By extrapolating from our previous observation that multiple copies of *rsmA* suppressed HSL production in Ecb11129 (Cui *et al.*, 1995), we suggest that the pleiotropic effect of *rsmA* on the production of antibiotic and extracellular enzymes, as well as on pathogenicity, is due to the repression of the cell density (quorum) sensing signal. In this context, we should note that the biosynthesis of a β-lactam antibiotic, 1-carbapen-2-em-3-carboxylic acid, is controlled by HSL in *E. carotovora* subsp. *carotovora* (Bainton *et al.*, 1992; Salmond *et al.*, 1995), a bacterium closely related to *E. carotovora* subsp. *betavasculorum*.

Effect of *rsmA* on pigment production

The *rsmA* plasmid pAKC880 suppressed the production of carotenoid pigment in *E. herbicola* strain EH105 and in *E. stewartii* strain DC283 and indigoidine pigment in *E. chrysanthemi* strain CU156 (data not shown). The genes that regulate the production of these pigments have not yet been identified, with the exception of indigoidine. In that case, a negative regulator gene, *pecS*, was found to control pectinases as well as the pigment in *E. chrysanthemi* strain 3937 (Reverchon *et al.*, 1994). Since *rsmA* and *pecS* genes do not share sequence homology and *rsmA* homologues have been detected in *E. chrysanthemi* (Cui *et al.*, 1995), we postulate that these genes control indigoidine production by different mechanisms.

rsmA suppresses EPS production

The data of Cui *et al.* (1995) revealed that *rsmA* suppressed glycogen accumulation in *E. coli* B, suggesting that it was also responsible for repression of EPS production in *Erwinia* species. To test this possibility, pCL1920 or pAKC880 was transferred into *E. amylovora* strain E9, *E. herbicola* strain EH105, and *E. stewartii* strain DC283, after which the drug-resistant transformants were scored for EPS production on agar media. *E. amylovora* E9(pCL1920) produced copious amounts of polysaccharide as indicated by glistening, extremely mucoid growth. By contrast, the growth of bacteria harbouring pAKC880 mostly remained non-mucoid. Similar results were found with other bacterial strains. Since EPS production in *E. amylovora* and *E. stewartii* is controlled by the *rcs* genes (Coleman *et al.*, 1990; Roberts & Coleman, 1991; Leigh & Coplin, 1992; Bernhard *et al.*, 1990; Vanneste, 1995), it would be of interest to determine if *rsmA* affects polysaccharide production by modulating the expression of these genes.

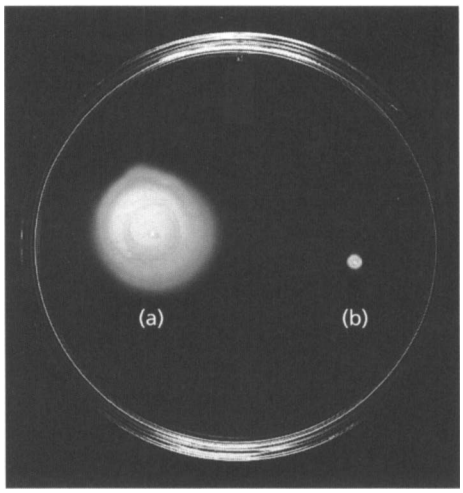

Fig. 2. Inhibition of motility of *E. carotovora* subsp. *carotovora* SCC3193 by *rsmA*. SCC3193(pCL1920) (a) and SCC3193(pAKC880) (b) were stab-inoculated into soft KB agar supplemented with spectinomycin using a straight needle and incubated at 28 °C for 24 h.

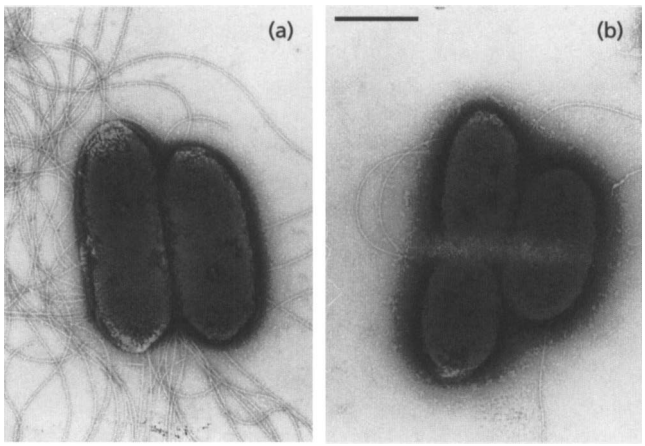

Fig. 3. Electron microscopic examination of flagella of *E. carotovora* subsp. *carotovora* SCC3193 harbouring pCL1920 (a) and pAKC880 (b). Bacterial cells were harvested from soft KB agar, stained with 1 % (v/v) phosphotungstic acid, and mounted on 200-mesh carbon-coated grid. Samples were examined using a transmission electron microscope model JEOL JEM 100B. Bar, 1 μm.

Inhibition of motility by *rsmA*

Most *Erwinia* species and other enterobacteria are motile, as indicated by the radial diffusion of growth beyond the point of inoculation on soft agar. The occurrence of twitching type motility has been documented for a number of enterobacterial species (see Harshey, 1994, for a review). Indeed, *E. carotovora* subsp. *carotovora* strain SCC3193 swarms extensively, covering the entire 10 cm agar surface of a standard Petri plate upon incubation for 36–48 h at 28 °C (data not shown). As shown in Fig. 2, while cells of SCC3193 harbouring pCL1920 were motile, those harbouring pAKC880 were non-motile. Similarly, motility was suppressed by pAKC880 in *E. amylovora*, *E. rhapontici* and other *Erwinia* species (A. K. Chatterjee, unpublished results).

To ascertain if the suppression of motility by *rsmA* was due to the inhibition of flagella formation, we examined negatively stained bacterial cells by transmission electron microscopy. As shown in Fig. 3, while cells of strain SCC3193(pCL1920) possessed numerous flagella, those of SCC3193(pAKC880) either were non-flagellated or at most contained one to two flagella. Thus, in addition to suppressing various secondary metabolic systems (see above), *rsmA* also causes a marked reduction in flagellum formation. Since *rsmA* suppressed motility in most *Erwinia* species in addition to *E. carotovora* subsp. *carotovora*, it appears that regulation of flagellum formation is a generalized function of *rsmA*.

Effect of *rsmA* on the elicitation of HR

When Gram-negative phytopathogenic bacteria are infiltrated into non-host plants, they elicit rapid cell death followed by a drop in the viability of bacterial cells. The necrogenic reaction, also generally known as HR (Willis *et al.*, 1991; Goodman & Novacky, 1994), remains limited to the area infiltrated with the bacterial suspension. *E. amylovora* strains are known to elicit HR in tobacco leaves (Klement & Goodman, 1966), and the genes that elicit HR, the *hrp* genes, as well as an elicitor of HR have been isolated (Beer *et al.*, 1991; Wei *et al.*, 1992a, b; Baker *et al.*, 1993). To test if *rsmA* affected the ability to elicit HR, we compared the responses with *E. amylovora* strain E9 harbouring pCL1920 or pAKC880. The results clearly demonstrated that E9(pCL1920) elicited typical HR in tobacco leaves, whereas E9(pAKC880) failed to elicit any discernible response under similar conditions (data not shown). The molecular basis for this differential response is not yet known. A plausible explanation is that the *rsmA* product suppresses the expression of one or more *hrp* genes.

Attenuation of pathogenicity in *E. amylovora* by *rsmA*

Several studies have established that an EPS, also known as amylovoran, is required for pathogenicity of *E. amylovora* (Bugert & Geider, 1995, and references cited therein). The suppression of polysaccharide production in *E. amylovora* strain E9 by *E. carotovora* subsp. *carotovora rsmA* (see above) raised the possibility that virulence of strain E9 could also be affected by multiple copies of this gene. The results of an apple shoot assay (data not shown) showed that E9(pCL1920) caused tip bending and wilting of the apple shoot, typical responses elicited by a virulent fire-blight pathogen (Van der Zwet & Beer, 1992). By contrast, these symptoms were not elicited by E9(pAKC880). Whether this repression was due to the lack of polysaccharide production or repression of other disease-specific genes or the *hrp* cluster awaits clarification.

In summary, we have shown that: (i) *rsmA* homologues occur in all *Erwinia* strains tested (Cui *et al.*, 1995); (ii) *rsmA* or *rsmA*-like genes are normally expressed in

Erwinia species; (iii) the *E. carotovora rsmA* locus suppresses an array of secondary metabolic systems in several *Erwinia* species; and (iv) multiple copies of *rsmA* affect the induction of HR by *E. amylovora* and pathogenicity of various *Erwinia* species (Cui *et al.*, 1995; and this report). Moreover, *rsmA* suppresses the levels of HSL in most *Erwinia* species tested and substantially reduces the levels of transcripts of *hslI*, a homologue of *carI* (Swift *et al.*, 1993) and *expI* (Pirhonen *et al.*, 1993), required for HSL production in *E. carotovora* subsp. *carotovora* strain 71 (Chatterjee *et al.*, 1995; Cui *et al.*, 1995).

The mechanism by which *rsmA* regulates these responses in so many different bacteria is not yet understood. However, in a recent report, Liu *et al.* (1995) have proposed that the product of the negative regulator gene *csrA* of *E. coli* binds to mRNA of the target gene, *glgC*, accelerating the degradation of the transcripts. Thus, in this model CsrA is proposed to regulate expression of the target gene by shortening the half-life of its transcript. As stated elsewhere (Cui *et al.*, 1995), RsmA and CsrA sequences are highly similar and both proteins possess the putative RNA-binding motif. Therefore, we suggest that RsmA, like CsrA, controls gene expression by causing a rapid decay of mRNA. It remains to be determined if RsmA acts directly on the transcripts of *pel*, *peh*, *cel*, *prt*, *fla* and an array of other genes or if it affects the expression of these genes indirectly by accelerating the decay of transcripts of one or more global transcriptional factors. Examples of such putative factors may include specialized sigma factors (Givskov *et al.*, 1995; Hengge-Aronis, 1993; Kolter *et al.*, 1993; Macnab, 1992; Xiao *et al.*, 1994), the products of *aep* genes (Liu *et al.*, 1993; Murata *et al.*, 1991, 1994) and HSL or its structural analogues and other components of the cell density sensing signalling system (Fuqua *et al.*, 1994; McGowan *et al.*, 1995; Salmond *et al.*, 1995; Swift *et al.*, 1994), now known to control various secondary metabolites.

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