Global Regulators ExpA (GacA) and KdgR Modulate Extracellular Enzyme Gene Expression Through the RsmA–*rsmB* System in *Erwinia carotovora* subsp. *carotovora*

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The production of the main virulence determinants, the extracellular plant cell wall-degrading enzymes, and hence virulence of *Erwinia carotovora* subsp. *carotovora* is controlled by a complex regulatory network. One of the global regulators, the response regulator ExpA, a GacA homolog, is required for transcriptional activation of the extracellular enzyme genes of this soft-rot pathogen. To elucidate the mechanism of ExpA control as well as interactions with other regulatory systems, we isolated second-site transposon mutants that would suppress the enzyme-negative phenotype of an *expA* (*gacA*) mutant. Inactivation of *kdgR* resulted in partial restoration of extracellular enzyme production and virulence to the *expA* mutant, suggesting an interaction between the two regulatory pathways. This interaction was mediated by the RsmA–*rsmB* system. Northern analysis was used to show that the regulatory *rsmB* RNA was under positive control of ExpA. Conversely, the expression of *rsmA* encoding a global repressor was under negative control of ExpA and positive control of KdgR. This study indicates a central role for the RsmA–*rsmB* regulatory system during pathogenesis, integrating signals from the ExpA (GacA) and KdgR global regulators of extracellular enzyme production in *E. carotovora* subsp. *carotovora*.

Additional keywords: regulation of virulence, transposon mutagenesis.

The pathogenicity of *Erwinia carotovora* subsp. *carotovora* is the result mainly of the production of a large arsenal of extracellular enzymes such as pectate lyases (Pel), polygalacturonase (Peh), cellulases (Cel), and proteases (Prt). These enzymes degrade the plant cell wall and thus allow the bacteria to macerate the plant tissue. Regulation of enzyme production and, hence, virulence is a complex and very tightly controlled process involving negative and positive factors. One of the central controls of virulence is quorum sensing, which allows coordinate expression of the virulence genes when sufficient cell density is reached (Andersson et al. 2000; Jones

et al. 1993; Pirhonen et al. 1991; Pirhonen et al. 1993; Swift et al. 1993). In addition to quorum sensing, a number of global (AepA, ExpS–ExpA, ExpM, RpfA, *rsmB* RNA, RexZ) and specific (PehS–PehR) positive regulators of extracellular enzyme gene expression have been characterized (Andersson et al. 1999b; Eriksson et al. 1998; Flego et al. 2000; Frederick et al. 1997; Liu et al. 1993; Liu et al. 1998; Murata et al. 1991; Murata et al. 1994; Thomson et al. 1999). The extracellular enzyme genes also are controlled by several negative regulators, including HexA, KdgR, RpoS, RsmA, and RsmC (Andersson et al. 1999a; Chatterjee et al. 1995; Cui et al. 1995; Cui et al. 1996; Cui et al. 1999; Harris et al. 1998; Liu et al. 1999; Mukherjee et al. 1996; Mukherjee et al. 1998; Thomson et al. 1999).

ExpA is one of the central regulators of virulence in *E. carotovora* subsp. *carotovora*, and mutations in *expA* result in an avirulent phenotype (Eriksson et al. 1998). ExpA, together with the putative sensor kinase ExpS (RpfA), appear to form a two-component signal transduction system that is required for the production of plant cell wall-degrading enzymes and virulence. Homologs (GacS–GacA) of the ExpS–ExpA signal transduction system have been characterized from many gram-negative bacteria, in particular the *Pseudomonas* species, where this system controls the production of a variety of virulence factors and secondary metabolites (Hrabak and Willis 1992; Laville et al. 1992; Liao et al. 1994; Rich et al. 1994). In *Pseudomonas syringae*, this conserved two-component signal transduction system is necessary for the production of protease and the toxin syringomycin as well as for lesion formation on bean (Hrabak and Willis 1992; Kitten et al. 1998). The signal activating this conserved two-component system is still unknown. The ExpA (GacA) protein has a helix-turn-helix DNA binding motif, but the direct binding of ExpA (GacA) to the putative target gene promoter remains to be demonstrated.

The virulence genes of *E. carotovora* subsp. *carotovora* also are subject to posttranscriptional control (Liu et al. 1998). This control is exerted by the RsmA–*rsmB* system, a homolog of the CsrA–*csrB* system in *Escherichia coli*, where it controls carbon metabolism on a wide scale (Liu et al. 1997; Sabnis et al. 1995; Yang et al. 1996). RsmA is a RNA binding protein that promotes decay of the enzyme gene transcripts (Chatterjee et al. 1995; Cui et al. 1995). The activity of RsmA

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is in turn controlled by the *rsmB* RNA (formerly *aepH*). Binding of *rsmB* RNA to RsmA neutralizes the effect of RsmA on transcript stability, allowing increased expression of virulence genes. *rsmB* and *rsmA* are modulated by RsmC, which negatively controls virulence in *E. carotovora* subsp. *carotovora* (Cui et al. 1999). The *rsmB* also is controlled negatively by the KdgR repressor (Liu et al. 1999). This global repressor of virulence genes has been characterized extensively from *Erwinia chrysanthemi* (Hugouvieux-Cotte-Pattat et al. 1996) but also exists in other *Erwinia* species as well as in *E. coli* (Negre et al. 1991; Thomson et al. 1999). In *E. carotovora* subsp. *carotovora*, KdgR was suggested to directly control extracellular enzyme gene expression as well as prevent expression of *rsmB* (Liu et al. 1999).

The interactions and cross talk between the different regulators that form the complex regulatory network controlling virulence gene expression in *E. carotovora* subsp. *carotovora* are largely unknown. To elucidate some of these interactions, we isolated second-site mutants that could suppress the extracellular enzyme-negative and avirulent phenotype of an *expA* mutant. This resulted in insertions in the *kdgR* gene. Characterization of the effect of ExpA and KdgR on the RsmA-*rsmB* regulatory system demonstrated that expression of *rsmA* and *rsmB* are modulated by ExpA and KdgR. We suggest that ExpA (GacA) and KdgR interact by controlling virulence gene expression through the RsmA-*rsmB* system.

RESULTS

Insertional inactivation of *kdgR* suppresses the protease-negative phenotype of an *expA* mutant.

To obtain second-site mutants where the extracellular enzyme-negative phenotype of an *expA* mutant would be restored, we employed transposon mutagenesis by random insertion of the mini-Tn5Cm^R::*gusA* transposon into the *expA* mutant background (SCC3060, *expA*::Km^R). The Km^RCm^R colonies were screened on protease indicator plates for enhanced protease production. Out of 10,000 mutants tested, one, SCC500, showed clear restoration of the protease activity and was selected for further studies. To identify the locus with the transposon insertion, the mutant allele was isolated by arbitrary polymerase chain reaction (PCR) and sequenced. The sequence of the flanking region from this mutant was most homologous to the *kdgR* gene of *E. carotovora* subsp. *carotovora* strains (Hinton and Salmond 1987; Zink et al. 1984), and the insertion site was located 240 bp upstream of the translational stop codon in *kdgR*. The wild-type *kdgR* allele was amplified by PCR from SCC3193, cloned to pBluescript II to yield pHJH23, and sequenced. The nucleotide sequence of the *kdgR* gene in pHJH23 showed 93.7% homology to that of *E. carotovora* subsp. *carotovora* strain SCRI193 (Hinton and Salmond 1987). The identity of the corresponding deduced amino acid sequences was 97.7%. This extensive similarity suggests that the transposon insertion was indeed in the *kdgR* gene. The disruption of *expA* and *kdgR* genes in the mutant SCC500 was confirmed by Southern blot analysis (data not shown).

kdgR inactivation results in partial restoration of extracellular enzyme production and virulence.

To elucidate the phenotypic differences between the *expA*-*kdgR* double mutant SCC500 and the corresponding *expA* and

kdgR single mutants, the *kdgR* mutant allele was transferred to the wild-type background by phage T4GT7 transduction. Chloramphenicol (Cm)-resistant transductants were obtained, and one of these, SCC510, was used for additional studies. Strain SCC510 was confirmed by Southern analysis to contain the wild-type allele of the *expA* gene and a mutated *kdgR* gene.

In order to characterize the ability of *kdgR* to suppress the *expA* mutant phenotype, we compared the extracellular enzyme production and virulence of the *expA*-*kdgR* double mutant to those in the *expA* or *kdgR* single mutants and to those in the wild-type strain. Pectate lyase activity in the culture supernatant of the *expA*-*kdgR* double mutant SCC500 was restored to 40% of wild-type level, whereas the Pel activity of the parental *expA* mutant strain SCC3060 was essentially zero (Fig. 1B). In contrast, the Pel activity of the *kdgR* mutant SCC510 was approximately 30% higher than the corresponding activity of wild-type strain SCC3193 (Fig. 1B). Similar characterization of cellulase, polygalacturonase, and protease on indicator plates showed that the production of these extracellular enzymes was restored partially in the *expA*-*kdgR* double mutant SCC500 compared with the *expA* mutant strain SCC3060 (Fig. 1C). The *kdgR* mutant SCC510 showed enhanced production of, particularly, protease compared with wild-type strain SCC3193. In conclusion, inactivation of *kdgR* resulted in the partial restoration of extracellular enzyme production in the *expA* mutant background.

To determine whether *kdgR* inactivation also would restore virulence to the *expA* mutant strain, we used a potato leaf assay to compare the virulence of the strains (Fig. 2). The result was in accordance with the extracellular enzyme production profile. The maceration capacity of the *expA*-*kdgR* double mutant SCC500 was restored partially, whereas the avirulent *expA* mutant SCC3060 did not macerate plant tissue. The *kdgR* mutant SCC510 was as virulent as wild-type strain SCC3193. Thus the inactivation of *kdgR* was sufficient to partially suppress the avirulent phenotype of an *expA* mutant.

ExpA appears to control the transcript levels of extracellular enzyme genes (Eriksson et al. 1998). Northern blot analysis was used to determine whether the observed restoration of enzyme production and virulence was the result of enhanced transcription of these genes in the *expA*-*kdgR* mutant SCC500 (Fig. 3A). A clear increase in enzyme gene transcripts was evident when *kdgR* was inactivated in the *expA* mutant background. The levels of *celVI*, *pehA*, and *priW* transcripts were substantially higher in the *expA*-*kdgR* mutant SCC500 than in the parental *expA* mutant SCC3060, although the amounts did not reach the levels observed in the wild-type strain SCC3193. In the *kdgR* mutant SCC510, the levels of *celVI*, *pehA*, and *priW* transcripts were similar or slightly increased when compared with those in wild-type strain SCC3193. Complementation of the *expA* and *kdgR* mutants in *trans* with respective plasmid clones confirmed that the phenotypes were indeed caused by the respective mutations (Fig. 3B). Introduction of the *kdgR* gene repressed enzyme gene expression, except in the case of residual *pehA* activity in the *expA* mutant SCC3060 (Fig. 3B). When the *expA* gene was introduced on a multicopy plasmid (pRA903) into the *expA* mutant SCC3060 or the *expA*-*kdgR* double mutant SCC500, it complemented the *expA* mutant phenotype. Furthermore, the introduction of *expA* also increased the

Fig. 1. Suppression of the extracellular enzyme-negative phenotype of an *expA* mutant by *kdgR* in *Erwinia carotovora* subsp. *carotovora*. Strains used were the SCC3193 wild type, SCC3060 *expA* mutant, SCC500 *expA-kdgR* mutant, and SCC510 *kdgR* mutant strain. **A**, Growth rate of SCC3193 (◆), SCC3060 (■), SCC500 (▲), and SCC510 (○), grown in L medium at 28°C degrees. **B**, Pectate lyase (Pel) activity in culture supernatants collected at stationary phase (24 h). Each value represents the mean of five experiments. Bar indicates standard deviation. **C**, Indicator plate assays showing Peh, Cel, and Prt activities. Strains were grown for 24 h in L medium, and 8.0×10^6 CFU were used for each assay.

levels of *celVI*, *pehA*, and *prtW* transcripts in the *kdgR*⁻ and wild-type backgrounds (Fig. 3B).

Expression of *rsmA* and *rsmB* is controlled by ExpA and KdgR.

Do ExpA and KdgR interact to control extracellular enzyme gene expression? Previous work by Liu et al. (1999) demonstrated that KdgR repression of the extracellular enzyme genes (*peh-1* and *pel-1*) is executed through direct control of target genes and through the *rsmB* gene in *E. carotovora* subsp. *carotovora*. To explore whether the suppression of the *expA* mutant phenotype by inactivation of *kdgR* involved the RsmA-*rsmB* system, we characterized the expression of *rsmA* and *rsmB* in the wild-type and mutant backgrounds by Northern analysis (Fig. 3A and B). The results indicate that ExpA positively controls *rsmB* expression. The primary and processed *rsmB* RNA were reduced substantially in the *expA* mutant SCC3060 (Fig. 3A). This negative effect of the *expA* mutation was complemented partially by the introduction of the wild-type *expA* allele in *trans*. This introduction of *expA* on a multicopy plasmid resulted in increased levels of *rsmB* RNA in all strains tested (Fig. 3B). Inactivation of *kdgR* in the *expA* mutant background (SCC500) suppressed the negative effect of the *expA* mutation on *rsmB* expression (Fig. 3A). The *rsmB*

Fig. 2. Effect of *kdgR* mutation on virulence of the *expA* mutant strain. Virulence was tested on potato leaves with local inoculations of *Erwinia carotovora* subsp. *carotovora* strains. Strains used were SCC3193 (wild type, 1), SCC3060 (*expA* mutant, 2), SCC500 (*expA-kdgR* mutant, 3), and SCC510 (*kdgR* mutant, 4). Strains were grown for 24 h in L medium, and cells were pelleted and washed once with 0.9% NaCl prior to inoculation. Each inoculation was adjusted to contain 2×10^7 CFU.

RNA level in the *expA*–*kdgR* double mutant SCC500 was almost as high as in the wild-type strain SCC3193. Introduction of the *kdgR* wild-type allele in *trans* prevented this suppression in the *expA*–*kdgR* mutant SCC500 as well as repressed *rsmB* expression in the wild-type and *kdgR* mutant SCC510 (Fig. 3B).

Expression of *rsmA* also is under ExpA control (Fig. 3A and B). Transcription of the *rsmA* gene was up regulated in the *expA* mutant strain SCC3060 during logarithmic growth (Fig. 3A). This negative control of ExpA on *rsmA* expression was confirmed by the introduction of the wild-type allele of *expA* in *trans*. Such introduction of the plasmid construct pRA903 (*expA*⁺) resulted in a marked reduction of *rsmA* transcript levels in all backgrounds tested, except in the *kdgR* mutant strain SCC510 (Fig. 3B). The up regulation of *rsmA* seen in the

expA mutant background was abolished in the *expA*–*kdgR* double mutant SCC500 (Fig. 3A). Expression of *rsmA* was reduced in the *kdgR* mutant strain SCC510 compared with the wild-type control, suggesting that *rsmA* expression requires functional KdgR. This was supported by the introduction of the *kdgR* wild-type allele in *trans* on plasmid construct pHJH23 (*kdgR*⁺) (Fig. 3B). The presence of this *kdgR* plasmid increased *rsmA* transcript levels in other backgrounds, except in the *expA* mutant strain SCC3060. Thus, the global regulators ExpA and KdgR appear to modulate the RsmA–*rsmB* system at the transcript level.

DISCUSSION

The complex regulatory network that controls production of virulence factors has been the subject of intensive studies in

Fig. 3. Effect of *kdgR* and *expA* on the expression of extracellular enzyme genes and *rsmA* and *rsmB* regulatory genes. **A**, Northern blot analysis of total RNA from *Erwinia carotovora* subsp. *carotovora* wild-type and mutant derivatives. The strains were grown in L medium at 28°C and total RNAs were extracted at optical density (OD) 600 nm of 1.3 and 1.5. SCC3193 (wild type, lanes 1 and 2), SCC3060 (*expA* mutant, lanes 3 and 4), SCC500 (*expA* *kdgR* mutant, lanes 5 and 6), and SCC510 (*kdgR* mutant, lanes 7 and 8). Each lane contained 5 µg of total RNA, except the blot hybridized with a *rsmB* probe, which contained 2 µg of total RNA. rRNA indicates ethidium bromide-stained formaldehyde gel, used as a loading control, showing 23S (approximately 3,000 nucleotides) and 16S (approximately 1,500 nucleotides) rRNAs. **B**, Northern blot analysis of total RNA from *E. carotovora* subsp. *carotovora* wild-type and mutant derivatives containing plasmids pRA903 (*expA*⁺) and pHJH23 (*kdgR*⁺). c indicates vector control pBluescript II. Strains were grown in L medium at 28°C, and total RNAs were extracted at OD₆₀₀ of 1.2. SCC3193 (wild type, lanes 1–3), SCC3060 (*expA* mutant, lanes 4–6), SCC500 (*expA*–*kdgR* mutant, lanes 7–9), and SCC510 (*kdgR* mutant, lanes 10–12). Each lane contained 5 µg of total RNA, except the blot hybridized with the *rsmB* probe contained 2 µg of total RNA. rRNA indicates ethidium bromide-stained formaldehyde gel, used as a loading control.

many gram-negative pathogens during the last decade, especially in the *Pseudomonas*, *Salmonella*, and *Erwinia* species. Although many of the regulatory proteins share a great degree of similarity among different pathogenic bacteria, the interactions and the hierarchical arrangement of the characterized positive and negative regulators remain to be elucidated. In this study, we characterized the genetic interaction of two central regulators, ExpA (GacA) and KdgR, in the plant pathogen *E. carotovora* subsp. *carotovora*. We first demonstrate that inactivation of the *kdgR* gene is sufficient to suppress the extracellular enzyme-negative phenotype of an *expA* (*gacA*) mutant strain and partially restores production of extracellular enzymes and virulence. The production of these enzymes, cellulases, pectate lyases, polygalacturonase, and protease was restored to approximately 40% of the wild-type level in the *expA-kdgR* double mutant. Similarly, virulence on potato leaves was partially restored in this mutant. *expA* (Eriksson et al. 1998) and *kdgR* (Liu et al. 1999) appear to control production of extracellular enzymes by activating or repressing, respectively, expression of the corresponding genes. Northern analysis was used to confirm that the enhanced production of extracellular enzymes in the *expA-kdgR* double mutant was the result of an increased expression of the *celVI*, *pehA*, and *prrW* genes. A similar increase in *celVI*, *pehA*, and *prrW* transcripts was noticed when the *expA* was introduced in *trans*. Conversely, reduction of these same transcripts was seen when the *kdgR*⁺ plasmid was introduced in *trans*. In contrast to the apparent ExpA requirement for *celVI* and *prrW* expression, *pehA* expression was less affected in the *expA* mutant. The relatively high residual levels of *pehA* transcripts and polygalacturonase activity in the *expA* mutant could be explained by the specific control of *pehA* expression by the PehR–PehS two-component system (Flego et al. 2000).

How are the two global regulators ExpA (GacA) and KdgR arranged in the regulatory network controlling extracellular enzyme gene expression? Is the suppression of the *expA* (*gacA*) phenotype by inactivation of *kdgR* caused by independent but opposite controls of the extracellular enzyme genes or do the two signal pathways interact to execute this control? ExpA does not appear to negatively regulate *kdgR* because there was no reduction in the level of *kdgR* transcripts in the *expA* mutant strain SCC3060 compared with the wild-type strain SCC3193 (data not shown). Earlier work by Liu et al. (1999) suggests that KdgR control of enzyme genes is partly through direct control and partly through control of *rsmB* transcription. Our results are best explained by a model where ExpA and KdgR modulate expression of *rsmA* and *rsmB*, which in turn controls the stability of enzyme gene transcripts (Fig. 4). The model does not rule out direct interactions of ExpA and KdgR with the enzyme genes, part of the control could indeed be direct, as suggested for KdgR by Liu et al. (1999). According to the model, ExpA and KdgR have opposite effects on *rsmA* and *rsmB* expression. Our results as well as those of Liu et al. (1999) demonstrate that KdgR represses *rsmB* transcription, which in turn could increase the free RsmA pool and lead to decreased stability of enzyme gene transcripts. Furthermore, our results show that KdgR positively controls *rsmA* transcription. Whether this control is direct or mediated by some other regulator remains to be demonstrated. One possibility is that this control could proceed through *rsmB* RNA, which prevents *rsmA* gene transcrip-

tion, as suggested by Liu et al. (1998). In contrast, ExpA appears to have a negative effect on *rsmA* gene expression, in particular during logarithmic growth, which in turn could decrease the RsmA levels and lead to an increased stability of the enzyme gene transcripts. This negative effect on RsmA levels would be further enhanced by the positive control, which ExpA has on *rsmB* expression (Fig. 4). The opposite effects of KdgR and ExpA on *rsmA* and *rsmB* expression are evidenced by several types of data. These include the obvious decrease of *rsmB* and the increase of *rsmA* transcripts in the *expA* mutant background as well as partial restoration of the wild-type levels in the *expA-kdgR* double mutant. Additional evidence comes from complementation and overexpression studies where the presence of plasmidborne *expA* reduced *rsmA* but enhanced *rsmB* expression. The converse was true for the introduction of a plasmidborne copy of *kdgR*. Our results indicate, therefore, that ExpA (GacA) and KdgR interact in the control of extracellular enzyme gene expression and that this interaction is mediated by the RsmA–*rsmB* system (Fig. 4). Whether ExpA represses *rsmA* transcription and activates *rsmB* transcription and/or processing directly or indirectly via some other regulator remains to be demonstrated. The initial gel-shift binding assays with purified ExpA protein and a *rsmB* DNA fragment did not show any complex formation (data not shown) supporting the indirect route.

Two recent studies support the model presented (Fig. 4). The ExpA homolog GacA controls the synthesis of two different exoproducts, hydrogen cyanide and extracellular protease, by a posttranscriptional mechanism in *Pseudomonas fluorescens* (Blumer et al. 1999). Overexpression of *rsmA* mimicked the partial loss of GacA function, suggesting that the GacA control could be mediated through *rsmA* and that RsmA is a downstream regulatory element of the GacA control cascade. Blumer et al. (1999) also speculate that the GacS–GacA system could directly affect *rsmA* expression. This is in accordance with our data showing that *rsmA* expression is negatively controlled by ExpA (GacA). Furthermore, Blumer et al. (1999) hypothesize that a *rsmB*-like RNA molecule that is up regulated by GacA could antagonize the action of RsmA repressor in *P. fluorescens*. Recently, Aarons et al. (2000) identified a structural homolog of *rsmB* RNA, *prrB* RNA, from another strain, F113 of *P. fluorescens*. This RNA is involved in the positive regulation of secondary metabolites and exoprotease mediated by the GacS–GacA system. The *prrB* transcripts were not detectable in a *gacA* mutant strain. Aarons et al. (2000) located consensus recognition sequences for KdgR within the *prrB* gene and suggested that, as in *E. carotovora* subsp. *carotovora*, a KdgR-like protein in *P. fluorescens* could negatively regulate *prrB* expression.

This study confirmed the central role of the ExpA (GacA) response regulator during the pathogenesis in *E. carotovora* subsp. *carotovora*. Furthermore, the work has started to unravel the molecular mechanism of this regulation and indicates the central role of the RsmA–*rsmB* system in mediating this control as well as mediating the cross talk between different regulators acting on the same downstream target genes.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The *E. coli* strain HB101 was used for plasmid maintenance and DNA preparation (Boyer and Roulland-Dussoix 1969).

The *E. coli* strain S17-1 λ -pir containing a suicide vector pPRG (pUT-mini-Tn5Cm^R::*gusA*) was used for transposon mutagenesis (De Lorenzo et al. 1990; Marits et al. 1999). The bacteriophage T4GT7 (Wilson et al. 1979) was propagated in the *E. coli* *supE* host strain TG1 (Carter et al. 1985). The *E. carotovora* subsp. *carotovora* wild-type strain SCC3193 (Pirhonen and Palva 1988) and the *expA* mutant strain SCC3060 (Eriksson et al. 1998; Pirhonen et al. 1991; Pirhonen et al. 1993) have been described. The *E. carotovora* subsp. *carotovora* mutant strains SCC500 and SCC510 were constructed in this study. The plasmid pRA903 containing the *expA* gene has been described by Eriksson et al. (1998), and the plasmid pHJH23 containing *kdgR* gene was constructed in this study. Transfer of plasmids was performed by standard transformation technique or electroporation with a Bio-Rad gene pulser (Richmond, CA, U.S.A.).

Media, culture conditions, and chemicals.

E. coli was propagated in L medium (Miller 1972) at 37°C and *E. carotovora* subsp. *carotovora* at 28°C. Cm and kanamycin (Km) were added to media at 50 μ g per ml, and 150 μ g of ampicillin (Amp) per ml was added, when required.

Cloning techniques and DNA sequencing.

For the generation of double mutants of *E. carotovora* subsp. *carotovora*, SCC3060 was used as a recipient. The donor strain *E. coli* S17-1 λ -pir containing a suicide vector pUT-mini-Tn5Cm^R::*gusA* was used in biparental matings, as described by

De Lorenzo et al. (1990) and Marits et al. (1999). Transconjugants were screened on L agar plates containing 5% skim milk, Cm, and Km. With additional propagation, the loss of the vector marker, Amp, was confirmed and the SCC500 mutant was characterized by arbitrary PCR (Caetano-Annoles, G. 1993; O'Toole and Kolter, 1998). In this method, the DNA flanking insertion site is enriched in two rounds of amplification with primers specific to the ends of the Tn5 element and primers specific to the random sequence, which can anneal to chromosomal sequences flanking the transposon. Additionally, Southern analysis with *expA*, *kdgR*, Cm, and Km gene-specific probes was employed. The *kdgR* mutant SCC510 was constructed as follows. The T4GT7 phage lysate was prepared from the SCC500 mutant strain, as described in Pirhonen et al. (1991), and used to infect the recipient *E. carotovora* subsp. *carotovora* strain SCC3193. The transductants were plated on L agar plates containing Cm. The further characterization was made by Southern analysis with the same probes as above. Plasmid pHJH23, which contains the *kdgR* gene in pBluescript, was constructed by PCR amplification with primers 5'-CCTTTACGCCGATAATAACAAG-3' and 5'-TCAGAAAGGATAATCGTGGTAACC-3' and chromosomal DNA from SCC3193 as a template. PCR was performed with the proofreading polymerase *Pfu* (Stratagene, La Jolla, CA, U.S.A.). All of the sequencing reactions were performed by an automatic laser fluorescent sequencer (Pharmacia, Uppsala, Sweden) at the Institute of Biotechnology (University of Helsinki).

Isolation of chromosomal DNA and total RNA and Southern and Northern blot analyses.

The chromosomal DNA from *E. carotovora* subsp. *carotovora* strains was isolated with a DNA isolation kit (Promega, Madison, WI, U.S.A.), and total RNA was isolated as described by Sambrook et al. (1989). For Southern analysis, 5 μ g of chromosomal DNA was digested with restriction enzymes (Promega) and run on an 0.8% agarose gel. Northern analysis was performed with 5 μ g of total RNA separated in 1.2% formaldehyde gel. The Northern analysis with a *rsmB*-specific probe was performed with 2 μ g of total RNA separated in 1.5% formaldehyde gel. Filters were probed with specific DNA fragments of *celV1* (Mäe et al. 1995), *pehA* (Saarilahti et al. 1990), *prtW* (Marits et al. 1999), *rsmA* (Cui et al. 1995), and *rsmB* (Murata et al. 1994). The blotting, hybridization, and digoxigenin (DIG) detection were performed with the DIG System (Boehringer Mannheim, Mannheim, Germany), according to the manufacturer's instructions.

Enzyme assays.

E. carotovora subsp. *carotovora* strains were grown overnight in L medium and subcultured 1:100 into a 50-ml culture. After 24 h, samples were taken and estimated to contain 4×10^9 CFU/ml. The extracellular enzyme indicator plates (Peh and Cel) were developed as in Pirhonen et al. (1993), and protease activity (Prt) was detected as in Marits et al. (1999). Pectate lyase activity was measured by the thiobarbituric acid method (Sherwood 1966).

Virulence tests.

Potato plants (*Solanum tuberosum* cv. Bintje) were propagated axenically in MS medium (Murashige and Skoog 1962) and grown in a controlled environment (22°C; 14 h light, 80 to

Fig. 4. Model for RsmA-*rsmB*-mediated cross talk in ExpA (GacA)- and KdgR-controlled expression of extracellular enzyme genes in *Erwinia carotovora* subsp. *carotovora*. ExpA and KdgR control expression of the *rsmB* and *rsmA* genes. ExpA activates *rsmB* RNA expression, which in turn decreases the pool of free RsmA repressor, leading to enhanced expression of extracellular enzyme genes. The effect is accentuated by negative control of the *rsmA* expression by ExpA. This could be direct or proceeding through *rsmB* RNA, where after the RsmA-*rsmB* complex formation, processed *rsmB* negatively affects *rsmA* transcription. KdgR has an opposite effect on *rsmA* and *rsmB* expression, activating *rsmA* and repressing *rsmB*. For the repression of *rsmB* gene expression by KdgR and the regulation of the *rsmB* RNA molecule and RsmA protein, see models by Liu et al. (1998; 1999).

100 $\mu\text{mol per s m}^{-2}$) for 4 to 5 weeks before inoculation. Local bacterial inoculations were performed by gently pressing the leaf surface with the tip of an automatic pipette and leaving a 5- μl drop, containing 2 to 3 $\times 10^7$ CFU of bacterial suspension, on top. After the inoculations, plants were kept under the previously indicated plant growth conditions and the progress of the infection was evaluated by the development of disease symptoms and documented photographically.

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