Two-Component Regulators Involved in the Global Control of Virulence in *Erwinia carotovora* subsp. *carotovora*

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Production of extracellular, plant cell wall degrading enzymes, the main virulence determinants of the plant pathogen *Erwinia carotovora* subsp. *carotovora*, is coordinately controlled by a complex regulatory network. Insertion mutants in the *exp* (extracellular enzyme production) loci exhibit pleiotropic defects in virulence and the growth-phase-dependent transcriptional activation of genes encoding extracellular enzymes. Two new *exp* mutations, designated *expA* and *expS*, were characterized. Introduction of the corresponding wild-type alleles to the mutants complemented both the lack of virulence and the impaired production of plant cell wall degrading enzymes. The *expA* gene was shown to encode a 24-kDa polypeptide that is structurally and functionally related to the *uvrY* gene product of *Escherichia coli* and the GacA response regulator of *Pseudomonas fluorescens*. Functional similarity of *expA* and *uvrY* was demonstrated by genetic complementation. The *expA* gene is organized in an operon together with a *uvrC*-like gene, identical to the organization of *uvrY* and *uvrC* in *E. coli*. The unlinked *expS* gene encodes a putative sensor kinase that shows 92% identity to the recently described *rpfA* gene product from another *E. carotovora* subsp. *carotovora* strain. Our data suggest that ExpS and ExpA are members of two-component sensor kinase and response regulator families, respectively. These two proteins might interact in controlling virulence gene expression in *E. carotovora* subsp. *carotovora*.

The plant-pathogenic enterobacterium *Erwinia carotovora* subsp. *carotovora* and related *Erwinia* spp. are capable of infecting a number of different plant species, including several economically important crops (Pérombelon and Kelman

1980). The soft rot disease caused by these broad-host-range pathogens is characterized by extensive maceration of the affected tissue. The maceration ability is dependent on the production of a number of plant cell wall degrading enzymes. The importance of these extracellular enzymes is reflected by the impressive arsenal of different types of enzymes produced by *E. carotovora* subsp. *carotovora*, including isoforms of pectate lyase (Pel), pectin lyase (Pnl), polygalacturonase (Peh), cellulase (Cel), and protease (Prt) (Collmer and Keen 1986).

The production and secretion of these enzymes appear to be vital for virulence. This is demonstrated by isolation of avirulent mutants of *E. carotovora* subsp. *carotovora* that are pleiotropically affected in either extracellular enzyme production (*exp*, *aep*, and *rex* mutants) (Jones et al. 1993; Murata et al. 1991; Pirhonen et al. 1991, 1993) or secretion of the enzymes (*out* mutants) (Andro et al. 1984; Ji et al. 1987; Pirhonen et al. 1991; Thurn and Chatterjee 1985). The synthesis of these enzymes appears to be a tightly controlled process involving complex coordinate regulation by both quorum sensing and other global regulatory factors. The *expI* (*rexI*) genes of *E. carotovora* subsp. *carotovora* have been shown to direct the synthesis of a freely diffusible signal molecule, the *Erwinia* autoinducer (EAI), that is required for coordinate activation of extracellular enzyme encoding genes and hence virulence (Jones et al. 1993; Pirhonen et al. 1993). Other positive regulators include AepA and AepH, originally described in the *E. carotovora* subsp. *carotovora* strain 71 (Liu et al. 1993; Murata et al. 1991, 1994). The complex regulatory network controlling virulence in *E. carotovora* subsp. *carotovora* also includes negative regulation. Recently, one such negative regulator, *rsmA*, (repressor of secondary metabolites) was characterized. Inactivation of *rsmA* results in overproduction of extracellular enzymes and hypervirulence. In addition, *rsmA* mutants seem to exhibit EAI-independent extracellular enzyme production (Chatterjee et al. 1995; Cui et al. 1995, 1996; Mukherjee et al. 1996).

In many bacteria, two-component systems composed of a membrane-bound sensor kinase and a cytoplasmic response regulator seem to be a common way by which the bacteria sense and respond to their environment. These regulatory systems have been shown to regulate virulence gene expression in both plant and animal pathogens, including the phyto-

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pathogens *Agrobacterium tumefaciens* and *Erwinia amylovora*, as well as the animal pathogens *Vibrio cholerae*, *Shigella flexneri*, and *Yersinia* spp. (for a review, see Dziejann and Mekalanos 1996). Surprisingly, no two-component regulatory system involved in controlling virulence has been described in *E. carotovora* subsp. *carotovora*. Recently, however, Frederick et al. (1997) described a gene, *rpfA*, encoding a putative sensor kinase involved in regulation of virulence in *E. carotovora* subsp. *carotovora*.

The apparent lack of two-component regulators together with the complexity of virulence regulation in *E. carotovora* subsp. *carotovora* prompted us to characterize new regulatory genes. We have used transposon mutagenesis and virulence screening on axenic tobacco seedlings as a strategy to isolate avirulent mutants and identify genes involved in virulence (Pirhonen et al. 1991). Many of the avirulent mutants isolated showed a pleiotropic phenotype with reduced production of several extracellular enzymes, including Peh, Pel, and Cel. These *exp* mutants could be divided into two broad categories. Mutations in *expI* were deficient in enzyme gene expression due to a lack of EAI production (Pirhonen et al. 1993). However, most of the other avirulent mutants did produce apparently normal amounts of EAI but were still impaired in production of several extracellular enzymes, suggesting that the corresponding mutations could define novel virulence regulators (Pirhonen et al. 1991, 1993).

In this work, we describe the genetic analysis and molecular characterization of two new *exp* genes that are required for production of extracellular enzymes and virulence in *E. carotovora* subsp. *carotovora*. We show that the genes are likely to encode polypeptides belonging to the two-component regulator category of proteins. One of these proteins, ExpA, appears both structurally and functionally similar to response regulators UvrY of *Escherichia coli* and GacA of *Pseudomonas fluorescens*. The other characterized gene, *expS*, encodes a putative sensor kinase highly similar to the *rpfA* gene product (Frederick et al. 1997). ExpS and ExpA might constitute a two-component system that regulates virulence gene expression in *E. carotovora* subsp. *carotovora*.

RESULTS

Isolation of pleiotropic *exp* mutants affected in virulence.

We previously isolated a number of *E. carotovora* subsp. *carotovora* (strain SCC3193) transposon mutants showing a pleiotropic phenotype: reduced virulence on axenic tobacco seedlings and impaired production of extracellular enzymes (Pirhonen et al. 1991, 1993). Besides *expI* mutants defective in quorum sensing, we obtained 15 other *exp* mutants that appeared normal in EAI production. Southern analysis of these strains indicated that the transposon insertions were at two unlinked (at least several kb apart) loci, suggesting that two separate *exp* genes (named *expA* and *expS*) were affected. The two mutant classes also appeared phenotypically distinct: *expA* mutants were essentially avirulent while those in *expS* showed a reduced virulence phenotype. This was evident when virulence (maceration capacity) of representative strains of each category was tested on potato tubers (Fig. 1). The virulence of one of the *expA* mutant strains, SCC3060, and one of the *expS* mutants, SCC3009, was compared with that of the wild-type parent, SCC3193 (Fig. 1). The results show

that the *expA* mutant has a markedly reduced capacity to macerate potato tuber tissue while the *expS* strain still retains about 70% of its maceration capacity.

Avirulent *expA* mutants show impaired production of several extracellular enzymes.

To define the molecular basis of the avirulent phenotype in *expA* mutants we first characterized the growth and production of extracellular enzymes of in vitro propagated SCC3193 and SCC3060 (Fig. 2A). The mutant did not show any growth defect, but the production of extracellular plant cell wall degrading enzymes Pel, Peh, and Cel was drastically reduced.

To verify the results of the enzyme assays and to characterize the effect of the *expA* mutation on the expression of genes encoding extracellular enzymes at the transcriptional level, we performed a Northern (RNA) blot analysis. Transcript accumulation was followed for the *pelC*, *pehA*, and *celVI* genes (Fig. 2B). In the wild type, the accumulation of mRNAs for these genes was induced in a growth-phase-dependent manner, but in the *expA* mutant the accumulation of these mRNAs was reduced (Fig. 2B). A similar reduction in transcript accumulation was observed for all three mRNA species, *pehA*, *pelC*, and *celVI* in the mutant strain. Interestingly, the residual expression of these genes was still responsive to the growth phase regulation. Taken together, these data demonstrate that the *expA* gene is required for activation of several different categories of virulence genes in *E. carotovora* subsp. *carotovora*, and suggest that ExpA may exert its effect on virulence by controlling expression of these genes.

Cloning and characterization of the *expA* wild-type allele.

To characterize the *expA* gene and to get insight in its function, the gene was first isolated. We started this by isolation of the *expA* mutant allele from the strain SCC3060. Partial sequencing of the DNA flanking the transposon was employed to design oligonucleotide primers. These primers were used to amplify, by polymerase chain reaction (PCR), the wild-type allele of *expA* from the *E. carotovora* subsp. *carotovora* strain SCC3193.

Fig. 1. *Erwinia carotovora* subsp. *carotovora* *exp* mutants exhibit reduced maceration capacity on potato tubers. Maceration by *expA* (SCC3060) and *expS* (SCC3009) mutants was compared with that of the wild-type strain (SCC3193). Tubers were inoculated with 10^6 CFU and macerated tissue was weighed after 48 h of incubation at 28°C and 100% humidity. Each value is a mean of 16 inoculations.

To confirm that the cloned genomic fragment coded for a functional gene product the clone was used for complementation studies with the *expA* mutant SCC3060. Complementation was assessed by assaying Pel activity and testing maceration capacity on potato tubers (Fig. 3). Comparison of *expA* mutant strains carrying either the wild-type (pRA903) or the mutant (pRA903-1) allele of the *expA* gene on a plasmid showed that the wild-type allele restores both the extracellular enzyme production in vitro (Fig. 3A) and the ability to macerate potato tubers (Fig. 3B). The SCC3060 mutant harboring the plasmid-borne *expA* gene even produced more extracellular enzymes in vitro and had a higher capacity to macerate potato tuber tissue than the wild-type strain SCC3193. Additional virulence tests were done on axenic tobacco seedlings and on potato stems, with similar results (data not shown).

Fig. 2. A, ExpA is required for extracellular enzyme production in *Erwinia carotovora* subsp. *carotovora*. Accumulation of polygalacturonase (Peh), pectate lyase (Pel), and cellulase (Cel) was followed for 24 h in SCC3193 (wild type, black square) and SCC3060 (*expA* mutant, open circle). **B**, ExpA is necessary for transcriptional activation of genes encoding extracellular enzymes. Northern (RNA) blot analysis of SCC3193 (wild type) and SCC3060 (*expA* mutant) with 5 µg of total RNA analyzed at each time point. Filters were probed with specific DNA fragments of *pehA* (Saarilahti et al. 1990), *celV1* (Mäe et al. 1995), and *pelC*.

Fig. 3. A, The *expA* and the *Escherichia coli uvrY* clones restore pectate lyase activity to the *expA* mutant (SCC3060) in vitro. SCC3193 (wild type, black box), SCC3060/pRA903 (*expA* mutant harboring the *expA* gene, black triangle) and SCC3060/pRA903-1 (*expA* mutant harboring a plasmid-borne mutant allele of the *expA* gene, open circle). SCC3060/pCA9505 (*expA* mutant harboring the *E. coli uvrY* gene, open triangle) **B**, Cloned *expA* and *E. coli uvrY* genes restore macerating capacity to the *expA* mutant. Macerating capacity of SCC3060/pRA903 (*expA* mutant carrying the *expA* wild-type allele) and SCC3060/pRA903-1 (*expA* mutant harboring a plasmid-borne mutant allele of the *expA* gene), SCC3060/pCA9505 (*expA* mutant harboring the *E. coli uvrY* gene) and of SCC3193 (wild type) was examined on potato tubers. Tubers were inoculated with 10^6 CFU. Macerated tissue was collected after 48 h of incubation at 28°C and 100% humidity and weighed. Each value is a mean of 16 inoculations.

These experiments clearly show that the cloned genomic fragment carrying *expA* can complement the avirulent and enzyme-deficient phenotype of SCC3060.

ExpA is structurally similar to two-component response regulators UvrY and GacA.

We determined the nucleotide sequence of the genomic fragment in pRA903-1 that was shown to contain three open reading frames (ORFs), two complete and one partial (Fig. 4A). The first ORF, corresponding to the *expA* gene, consists of 654 nucleotides. The deduced amino acid sequence of *expA* results in a polypeptide with a calculated molecular mass of 24,032 Da. The assignment of the TTG start codon was based on the potential ribosome binding site and by sequence comparisons with corresponding genes in other bacteria.

To characterize the product of the *expA* gene we used the maxicell method (Sancar et al. 1979). *E. coli* DH5 α cells harboring the plasmid pRA903 (carrying the *expA* wild-type al-

lele) were converted into maxicells and the plasmid-encoded proteins analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 5). A 24-kDa polypeptide was produced in the pRA903 carrying strain, in addition to the 29-kDa band corresponding to the β -lactamase encoded by the vector. The apparent molecular mass of this putative ExpA protein correlates well with the deduced size of the ExpA polypeptide.

Search of protein data bases with the deduced amino acid sequence of the ExpA polypeptide showed significant similarity of ExpA to the UvrY and GacA proteins of *E. coli* and *Pseudomonas fluorescens*, respectively (Gaffney et al. 1994; Laville et al. 1992; Moolenaar et al. 1987; Rich et al. 1994; Sancar et al. 1984; Sharma et al. 1986; van Sluis et al. 1983). Both UvrY and GacA are two-component response regulators (transcriptional activators) belonging to the FixJ family. The alignment between these polypeptides (Fig. 4B) shows that they are structurally related to each other and share identical amino acids throughout the whole protein sequence. The predicted sequence of ExpA is 82.6% identical to UvrY and 57.3% identical to GacA. Both the N-terminal receiver module containing the phosphorylated aspartate residue and the C-terminal DNA binding domain appear to be conserved (Fig. 4B).

A second ORF was found downstream of *expA*, apparently organized in the same operon. The deduced partial polypeptide sequence shows similarity to the *uvrC* gene products from both *E. coli* and *P. fluorescens*. (Laville et al. 1992; Sancar et al. 1984; van Sluis et al. 1983). The *uvrC* gene, which is found in the same relative position in these three bacterial species, encodes one of the components of the UV repair system. The operon structure of these genes suggests that polar mutations in the upstream gene might result in a UV-sensitive phenotype, as was shown to be the case with insertions in the *gacA* gene in *P. fluorescens*. We tested the UV sensitivity of the *E. carotovora* subsp. *carotovora* *expA* transposon mutant

Fig. 4. Genomic organization of the *expA-uvrC* operon and amino acid alignment of ExpA. **A**, Organization of the *expA* locus from *Erwinia carotovora* subsp. *carotovora* strain SCC3193. The clone complementing the *expA* mutant strain SCC3060 is indicated. Genes are boxed, direction of transcription is indicated, and position of transposon insertion is marked with a circle. **B**, Alignment of ExpA from *E. carotovora* subsp. *carotovora* (SCC3193) with UvrY from *Escherichia coli* (Sharma et al. 1986) and GacA from *Pseudomonas fluorescens* (Laville et al. 1992). Highly conserved residues in the response regulator family are boxed. P indicates a potential phosphorylation site. The helix-turn-helix DNA binding motif is overlined.

Fig. 5. Characterization of the *expA* gene product by maxicells. *Escherichia coli* DH5 α cells harboring pBluescript and pRA903 (pBluescript with the *expA* gene), respectively, were converted to maxicells (Sancar et al. 1979). After being labeled with [³⁵S]methionine, the proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the labeled protein products were visualized by autoradiography of the stained and dried gel. Protein size marker is indicated in the left margin in kDa. Gene products are indicated at the right: Bla, β -lactamase; ExpA, the *expA* gene product.

(SCC3060) in a plate assay and observed a distinct UV-sensitive phenotype (data not shown). Introduction of the wild-type *expA* allele (pRA903) or the pRA903-1 plasmid containing the mutated *expA* allele did not complement the increased sensitivity to UV light in the *expA* mutant. These results suggest that the *expA* gene is not directly involved in the UV repair but the altered UV phenotype is due to a polar *expA* mutation in the *expA-uvrC* operon.

Further downstream of the *expA* and *uvrC* genes, we found the 5' part of a housekeeping gene, *pgsA* (phosphatidylglycerophosphate synthase).

UvrY of *E. coli* is a functional analog of ExpA.

The similar organization and structural homology of the *expA-uvrC* and *uvrY-uvrC* operons suggested that the corresponding proteins might be also functionally related. To test whether UvrY from *E. coli* is a functional analog of the ExpA protein in *E. carotovora* subsp. *carotovora*, we introduced the *E. coli* *uvrY* gene into the *expA* mutant SCC3060. These strains were assayed for Pel activity (Fig. 3A) as well as tested for their maceration capacity on potato tubers (Fig. 3B). Our results indicate that the plasmid-borne *uvrY* gene from *E. coli* is able to restore the production of extracellular enzymes to the *expA* mutant SCC3060. Similarly, *uvrY* restored the maceration capacity on potato tubers (Fig. 3B) as well as virulence on axenic tobacco seedlings and potato stems (data not shown). Consequently, the UvrY polypeptide of *E. coli* is not only structurally but also functionally similar to the ExpA protein of *E. carotovora* subsp. *carotovora*.

A functional *expS* gene is required for high-level expression of virulence genes.

Mutations in *expS* resulted in a reduced virulence phenotype, and as discussed (Fig. 1) these strains still retained most of their capacity to macerate potato tubers. To elucidate the reason for this reduction in virulence we characterized the effect of the mutation on production of extracellular enzymes Cel, Pel, and Peh (Fig. 6A). Comparison of enzyme activities of the *expS* mutant, SCC3009, with the wild-type parent demonstrated that the mutant still produced substantial amounts of these enzymes in vitro in a growth-phase-dependent manner. In contrast to the wild type, however, the enzyme levels were reduced and the production delayed (Fig. 6A). The effect of *expS* was at the transcriptional level, as shown by characterization of mRNA accumulation by Northern analysis (Fig. 6B). A clear reduction in transcripts corresponding to several virulence genes was evident in the *expS* mutant background. The discrepancy between the Pel enzyme activity and the *pelC* Northern can be explained by the fact that several different pectate lyases are produced by SCC3193 (Heikinheimo et al. 1995; and H. Saarilahti, M. Pirhonen, and E. T. Palva, *unpublished results*). These results show that a functional *expS* allele is required for high-level expression of several types of virulence genes and suggest that the reduced expression of these genes in the mutants results in reduced virulence.

The *expS* gene encodes a putative sensor kinase.

To elucidate the function of the *expS* gene it was structurally characterized. We first cloned the mutant allele, which was partially sequenced. The obtained sequence information was used to generate primers for PCR amplification of the

corresponding wild-type allele from SCC3193. The PCR fragment was cloned into pBluescriptII to generate pRA904. This fragment was found to contain a single ORF, designated *expS*, encoding a polypeptide of 928 amino acids. The transposon insertion in the mutant was by sequencing mapped 1,160 bp downstream of the translation start codon.

Search of protein data bases with the deduced amino acid sequence of *expS* revealed that it was similar to a class of sensor kinases with linked transmitter and receiver domains, including LemA of *Pseudomonas syringae* (Hrabak and Willis 1992), RepA of *P. viridiflava* (Liao et al. 1994), BarA of *E. coli* (Nagasawa et al. 1992), BvgS of *Bordetella pertussis* (Aricò et al. 1989), and RpfA of *E. carotovora* subsp. *carotovora* (Frederick et al. 1997). Alignment of the protein sequences (Fig. 7) demonstrated that the highest similarity (92.2% amino acid identity) was to the product of the *rpfA*

Fig. 6. ExpS is required for high level production of extracellular enzymes. **A**, Characterization of the extracellular enzyme activity of SCC3193 (wild type, black box) and SCC3009 (*expS* mutant, open circle). Activity of the extracellular enzymes polygalacturonase (Peh), pectate lyase (Pel), and cellulase (Cel) was followed during 24 h of growth. **B**, Transcriptional activation of genes encoding extracellular enzymes was followed by Northern (RNA) blot analysis in SCC3193 (wild type) and SCC3009 (*expS* mutant). Each lane was loaded with 5 µg of total RNA. Filters were probed with specific fragments of *pehA* (Saarilahti et al. 1990), *celVI* (Mäe et al. 1995), and *pelC*.

gene that was recently isolated from another *E. carotovora* subsp. *carotovora* strain, AH2 (Frederick et al. 1997). In one short region (amino acids 51 to 57), however, ExpS and RpfA differ completely, which could be due to a frame shift in one of the gene sequences. Like ExpS, RpfA is involved in extracellular enzyme production as well as pathogenicity in *E. carotovora* subsp. *carotovora*.

To determine the functionality of the cloned *expS* gene we tested the complementation of the *expS* mutation in SCC3009 with the plasmid-borne wild-type allele of *expS* (pRA904). This plasmid was electroporated into the mutant strain and the strains assessed for virulence on axenic tobacco and for the production of cellulase by plate assays. We found that pRA904 almost fully restored the virulence as well as the cellulase production of SCC3009 (data not shown). These results indicate that the cloned *expS* gene in pRA904 is functional and is able to complement the mutation in SCC3009.

We also tested the effect of a multicopy *expA* gene (pRA903) in the *expS* mutant SCC3009 background. The results show that pRA903 suppresses the reduced virulence phenotype of *expS* and even restores cellulase production in this mutant (data not shown).

DISCUSSION

Production of virulence factors in pathogenic bacteria is normally a strictly controlled process that responds to a number of environmental cues. Accordingly, in the plant pathogen *E. carotovora* subsp. *carotovora*, expression of virulence genes encoding extracellular plant cell wall degrading enzymes is controlled by a complex regulatory network involving both positive and negative global regulators. We have employed a genetic approach for identification of new genes controlling virulence and pathogenicity in *E. carotovora* subsp. *carotovora* (Pirhonen et al. 1991, 1993). In this paper we show that virulence of *E. carotovora* subsp. *carotovora* depends on *expA*, which encodes a putative transcriptional activator of a two-component response regulator family, and on *expS*, which encodes a putative two-component sensor kinase with linked transmitter and receiver domains.

We demonstrate that insertion mutations in either of these genes result in an avirulent or reduced virulent phenotype. These mutants are impaired in production of several plant cell wall degrading enzymes, including Pel, Peh, and Cel, and exhibit a reduced accumulation of the corresponding transcripts

ExpS	MTKYSRLRMLILILAPTLMIGLLSSFFVIHRYNQLQTLQADSGTRIE	50	ExpS	ISFQSQLNGSTFWFHITLPLNPHTPVIEPVYTMQKGLHAYVEYHPIAA	546
RpfA	MTKYSRLRMLILILAPTLMIGLLSSFFVIHRYNQLQTLQADSGASIID	50	RpfA	ISFQSELSKSGSTFWFHITLPLNPHAITPEPAYTMLQKGLHAYVEYHPIAA	548
BarA	MTNYSRLRMLILILAPTLMIGLLSSFFVIHRYNQLQTLQADSGASIE	50	BarA	ISFHSQPNRGSTFWFHINLDLNPNIIEGFPSTQCLAGKRLAYVEPNASAA	549
LemA	M-----LLTILPASLMAAMLGYYFTWMQLSELQSQLQRGEMIAQ	41	LemA	IGVDSTPGESEFWISLKLK-KAREDKESLNIPLGGLRAAVLEHHDLAR	530
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ExpS	PLATISAYATHRQMDTIPITLNTLHRRHSAIIRTISVFDARNQLLATN	100	ExpS	QATLDILSQTPLMVSYSPTFEQL-----PEGEFDILLAI PAQYRN	587
RpfA	AAGNHQRYATHRQMDTIPALINTLHRRHSAIIRTISVFDARNQLLATK	100	RpfA	QATLDILSQTPLIVSYSPTFEQL-----PEGEFDILLAI PAQYRN	589
BarA	PLAVSTEYGMISLQNRISGLISLVLRHSDIVRAISVDENNRLFTVN	100	BarA	QCTLDILSETPLEVVSPTFSAL-----PPAHYDMLLGIATVFRE	590
LemA	DLAPLAANALGRKDKVLSRIAT--QTLEQTDVRAVSFLDRTDTVLA--	86	LemA	QALEHQLEDCGLQTVFNNLNLNGVTAAHETPAADLAVLVGTA----	576
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ExpS	VRNNVTLPQLSSDALSYNKLHLHHTNDALILHMPIVNDSFMPGG--VQP	148	ExpS	TLLDYTPRLRDI CRAP---CVILALPSLAQMDAEQLKAFGVHACL-SKP	633
RpfA	LRNKRTLQCLSNVLAHNKLHLHHTNDALILHMPIVNDSFMPAGTTPVLP	150	RpfA	TLLDYTPRLRDI CRSP---CVILALPSLAQMDAEQLKTFGVHACL-SKP	635
BarA	FHLDPSSMQLGSNVFPFRLQTLVTRDGMILRTPIISES--YSPDESPPSD	149	BarA	PLTMQHERLAKAVSMTD---FLMLALPCHAQVNAEKLKQDGIGACL-LKP	636
LemA	-HAGPTMISPSPIGSGSLSSSTGTATRYL-LPVFGSRQRLT--SPIIP	132	LemA	LEISPERLRQHIWDLNLNCKVMVLCPTTEHALFQLAVHDVYTLQAKP	625
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ExpS	GTATP--LGYLVIELDTSTIRLQYQEIFIAALLLSLGAIVFAYRLM	196	ExpS	LAASRLPLQLNSTFLQSLFSLPDDTSHQGTVRHRLPLSVMAVDNPA	683
RpfA	GTATP--LGYLVIELDTSTIRLQYQEIFIAALLLSLGAIVFAYRLM	198	RpfA	LAASRLPLQLNSTFLQSLFSLPDDTSHQSAVRHRLPLSVMAVDNPA	685
BarA	AKNSQNMGLYIALELDLKSVRLLQYKEIFISSVMMLFCIGIALIFGWRM	199	BarA	LTPTRLPALTE-----FCHHKQNTLLPVTDKSLAMTMAVDNPA	678
LemA	A-EADTLLGWVEISHNGLTLRGVRSFLASLLILTLGAFTATVLA--	181	LemA	ACTRLQKALSE-----LIAPRAVADIGPSSRAP-RVLCVDNPA	667
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ExpS	RDVTTPIRNMVDTVDRIIRGQLDSRVEGYMLGELDMKNGINSMAMSLTA	246	ExpS	NLKLIGTLLLEQVDITILCESGTEAIAQAKMHQIDIIILMDIOMPGMDGIC	733
RpfA	RDVTTPIRNMVDTVDRIIRGQLDSRVEGYMLGELDMKNGINSMAMSLTA	248	RpfA	NLKLIGTLLLEQVDITILCESGQDAIAQELNQCDIILMDIOMPGMDGIC	735
BarA	RDVTTPIRNMVDTVDRIIRGQLDSRVEGYMLGELDMKNGINSMAMSLAA	249	BarA	NLKLIGALLEDMVQHVLCDSGHQAVRAKMPFDLILMDIOMPGMDGIR	728
LemA	RTINGPMSQIKQAVSQKLDGNLETRPLPLGSRELDLDELASGINMAATLQ	231	LemA	NLLVQTLLEDMDGAEEVAVEGGYAAVNAVQQAEDFLVMDIOMPGMDGRQ	717
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ExpS	YHEEMQONIDQATYDLRETLQMEIQNVLDLAKKRAQEAARIKSEFLAN	296	ExpS	ASELIHQ---IPHHATTPIIAVTAQMTGEREHLRSGMDDYLAKPIDEQ	780
RpfA	YHEEMQONIDQATYDLRETLQMEIQNVLDLAKKRAQEAARIKSEFLAN	298	RpfA	ASELIHQ---IPHHATTPIIAVTAQMTGEREHLRSGMDDYLAKPIDEQ	782
BarA	YHEEMQONIDQATYDLRETLQMEIQNVLDLAKKRAQEAARIKSEFLAN	299	BarA	ACELIHQ---LPHQQQTPVIAVTAHAMAGQEKLLGAGMSDYLAKEIEE	775
LemA	AQEELQSLIDQATEDVRQNLDTIEIQNVLDLAKKRAQEAARIKSEFLAN	281	LemA	ATEAIRAWEAERNQSSLPVIALTAHAMANEKRSLLQSGMDDYLTTPISER	767
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ExpS	MSHELRTPLNGVIGFTRQLTKTSINTTQTDYLLTIERSANNLLNI INDVL	346	ExpS	MLKSVLTHARKDPLKRDADRMVGLLSEHDDSQLSLDWALAQQAANKPE	830
RpfA	MSHELRTPLNGVIGFTRQLTKTSINTTQTDYLLTIERSANNLLNI INDVL	348	RpfA	MLKSVLTHARKDPLKRNRGEMTGLLNEHDDSQLSLDWALAQQAANKPE	832
BarA	MSHELRTPLNGVIGFTRQLTKTSINTTQTDYLLTIERSANNLLAI INDVL	349	BarA	RLHNNLLRYKPGSGIS-SRVVTPVNEIVVFNATLQWLQALRQAAGKTD	824
LemA	MSHELRTPLNGVIGFTRQLTKTSINTTQTDYLLTIERSANNLLSI INEIL	331	LemA	LQAQVVLKWTGLALRNPAAPERQNEALEVHVGP-LVL DHEEGLRLAAGKAD	816
	* * * * *			* * * * *	
ExpS	DFSKLEAGKLVLEDIPFSLHNTLDDVMLLAHTAHEKGLLETLISQNDVP	396	ExpS	LARDLLQMLLDLFLPEVQRKIENVLNQTDDDDIIELVHKLHGSCSYSGVPR	880
RpfA	DFSKLEAGKLVLEDIPFSLHNTLDDVMLLAHTAHEKGLLETLISQNDVP	398	RpfA	LGRDQLQMLLDLFLPEVQRKIENVLNQTDDDDIIELVHKLHGSCSYSGVP-	881
BarA	DFSKLEAGKLVLEDIPFSLHNTLDDVMLLAHTAHEKGLLETLISQNDVP	399	BarA	LARDMLQMLLDLFLPEVRNKVEEQLGVENFEGVLVLIHKLHGSCSYSGVPR	874
LemA	DFSKIEAGKLVLEDIPFSLHNTLDDVMLLAHTAHEKGLLETLISQNDVP	381	LemA	LMADMLAMLLASLDADREARVRANQDVHAIERIRLHGATRYCGVQ	866
	* * * * *			* * * * *	
ExpS	EQYVGDPRLRQIITNLLGNAIKFTEQGNIDIRVEKRRQEHQVQLEVI	446	ExpS	LKRICRYLEQQLRKGVDHLEPEWLELLDEIDNVNNAQPHINRPLP	928
RpfA	EQYVGDPRLRQIITNLLGNAIKFTEQGNIDIRVEKRRQELHQLVLEVI	448	RpfA	LQPICRYLEQQLRKGVDHLEPEWLELLDEIDNVNNAQPHINRPLP	929
BarA	DNVIGDPLRLQIITNLLGNAIKFTEQGNIDIRVEKRRQELHQLVLEVI	449	BarA	MKNLQQLIEQLRSGTKEEDLEPELLELLEDMONVAREA----SKILG	918
LemA	LALSGLDPLRLRQIITNLLGNAIKFTEQGNIDIRVEKRRQELHQLVLEVI	431	LemA	LRASQQRATLLKQNAHPTE-----EALNLDKAIIRLEAE-ARVMA	907
	* * * * *			* * * * *	
ExpS	CDTGIGIAELQSQQLFQAFQADTSISRRHGGTGLGLVITQRLVKEMGGD	496			
RpfA	CDTGIGIAELQSQQLFQAFQADTSISRRHGGTGLGLVITQRLVKEMGGD	498			
BarA	CDTGIGIPERDQSLFQAFQADTSISRRHGGTGLGLVITQRLVKEMGGD	499			
LemA	QDTGIGLSSQDVRALFQAFQADTSISRRHGGTGLGLVITQRLVKEMGGD	481			
	* * * * *				

Fig. 7. Alignment of ExpS with RpfA from *Erwinia carotovora* subsp. *carotovora* AH2 (Frederick et al. 1997), BarA from *Escherichia coli* (Nagasawa et al. 1992), and LemA from *Pseudomonas syringae* (Hrabak and Willis 1992). Identical amino acids are indicated with asterisks and similar amino acids with dots under the sequences. The potentially phosphorylated residues are boxed.

(*pelC*, *pehA*, and *celVI*). Both phenotypes (virulence and enzyme production) of the mutants could be complemented by the cloned wild-type alleles of *expA* and *expS*, respectively. In addition, the phenotype of the *expS* mutant was also suppressed by the cloned wild-type allele of the unlinked *expA* gene.

The *expA* gene encodes a 24-kDa polypeptide highly similar to several two-component response regulators of the FixJ family. The deduced amino acid sequence of ExpA shows 82.6% amino acid identity to UvrY from *E. coli* (Sharma et al. 1986), 57.3% identity to GacA from *P. fluorescens* (Laville et al. 1992), and 82.6% identity to SirA from *Salmonella typhimurium* (Johnston et al. 1996). These proteins include both the carboxy-terminal helix-turn-helix DNA binding domain and the amino terminal receiver module characteristic of the FixJ family of proteins (Kahn and Ditta 1991). The structural features of these proteins together with mutant and overexpression phenotypes of the corresponding genes suggest that they encode transcriptional activators.

Many of these *expA* homologs appear to control cellular functions related to pathogenicity. SirA is required for transcriptional activation of invasion genes in *S. typhimurium* (Johnston et al. 1996), and GacA has been shown to be important for pathogenicity in *Pseudomonas aeruginosa* (Rahme et al. 1995) and *P. syringae* (Rich et al. 1994) and for synthesis of antifungal factors in *P. fluorescens* (Laville et al. 1992). Similar regulators have also been characterized from *Pseudomonas viridiflava* and *P. marginalis*, where they control production of pectate lyases and pathogenicity (Liao et al. 1996;1997). In contrast, the function of *uvrY* in *E. coli* remains to be elucidated. Interestingly, we could demonstrate the functional similarity of UvrY and ExpA by complementation experiments, showing that UvrY can mediate transcriptional activation of virulence genes in *E. carotovora* subsp. *carotovora*. It is possible that UvrY might also be a regulator of virulence in *E. coli*.

The *expA* gene appears to be part of an operon involved in UV repair. Transposon insertions in *expA* exhibit a UV-sensitive phenotype. This is likely due to a polar effect on a downstream gene homologous to *uvrC*. In *E. coli*, *P. fluorescens*, and *S. typhimurium*, the *uvrC* gene, required for the repair of UV-induced DNA damage, is similarly located downstream of the *uvrY/gacA/sirA* gene (Johnston et al. 1996; Laville et al. 1992; Rich et al. 1994; Sharma et al. 1986). Our results show that this genomic organization is also conserved in *E. carotovora* subsp. *carotovora*. This raises the question of possible relevance of this conservation for bacterial pathogenesis, both in animal and plant pathogens. Part of the host defense against invading bacteria could involve production of DNA-damaging substances. In this case it would make sense for the bacteria to have genes of the DNA repair system linked to genes controlling virulence.

The *expS* gene encodes a putative sensor kinase related to a number of unorthodox two-component sensor kinases with linked transmitter and receiver modules, including LemA, BarA, and BvgS. The highest similarity is, however, to a recently described gene product, RpfA, characterized from a related *E. carotovora* subsp. *carotovora* strain, AH2 (Friederick et al. 1997). Both proteins are involved in the regulation of virulence and extracellular enzyme production, thus appearing to have the corresponding function in the two *E.*

carotovora subsp. *carotovora* strains. However, there are some differences in the phenotype of the *expS* mutant when compared with the *rpfA* mutant. First, the *rpfA* insertion mutant is affected in the production of cellulase but not in the production of pectate lyase and polygalacturonase, while the *expS* mutant shows reduced production of all these enzymes. Second, the *rpfA* mutant has less than 10% of the macerating capacity of the corresponding wild type on potato tubers, in contrast to the 70% residual capacity in the *expS* mutant. Both mutants are due to insertions that should inactivate the respective genes. The differences between the phenotypes could be partly explained by the location of the insertion, which in the *rpfA* mutant is about 600 bp downstream of that in the *expS* mutant. It is possible that a truncated version of the protein is produced in one or both of the mutants and that this polypeptide still retains some function. Alternatively, the two strains might have somewhat different enzyme arsenals, resulting in different mutant phenotypes.

Are ExpS and ExpA part of the same two-component system? In *P. syringae*, GacA is thought to activate the transcription of target genes after phosphorylation by the sensory kinase LemA (Rich et al. 1994). Based on the structural similarity of ExpA and GacA, it is likely that *E. carotovora* subsp. *carotovora* also contains a sensory protein related to LemA that senses environmental signals and activates ExpA by phosphorylation. We propose that ExpS is the cognate sensor of ExpA. This is based on the structural similarity of the ExpS and LemA as well as ExpA and GacA proteins, respectively. Furthermore, ExpS and ExpA appear to regulate the same target genes encoding extracellular enzymes. The mutant phenotypes are similar although the enzyme production in the *expS* mutant is less affected in vitro. That the sensor mutant still retains some expression of the regulated genes is known from other two-component systems, e.g., the EnvZ/OmpR system of *E. coli* and the VirA/VirG system of *Agrobacterium tumefaciens* (for a review, see Stock et al. 1989). We found also that overexpression of *expA* in SCC3009 suppressed the avirulent phenotype of this mutant. Similar results have been obtained in other systems, where overexpression of the response regulator renders the cells independent of the corresponding sensor (Stock et al. 1989; Uhl and Miller 1996). This could be due to the fact that alternative kinases phosphorylate the response regulator, or that the response regulator, when present in high concentrations, is able to activate transcription also in the absence of phosphorylation. We cannot, however, rule out the possibility that ExpS in addition to/instead of phosphorylation of ExpA phosphorylates other response regulators that are needed during plant infection.

The types of signals sensed by this putative two-component system remain to be elucidated. These might include (i) signals derived from the host plant, e.g., cell wall fragments that have been released from wounded plant cells or by the action of extracellular pectinolytic enzymes of *E. carotovora* subsp. *carotovora*, (ii) molecules leaking from the plant cell cytoplasm, or (iii) signals produced by the bacteria themselves.

Whether the different global sensory and regulatory systems controlling virulence in *E. carotovora* subsp. *carotovora* act in a hierarchical manner or in parallel remains to be elucidated. We are currently studying the interaction between the different virulence regulators of this pathogen.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The *E. coli* strain DH5 α (Hanahan 1983) was used for plasmid maintenance and DNA preparations. The *E. carotovora* subsp. *carotovora* wild-type strain SCC3193 (Pirhonen et al. 1988), and the *expA* mutant strain SCC3060 have been described earlier (Pirhonen et al. 1993). SCC3009 is a mutant of SCC3193 with a transposon insertion in the *expS* gene (Pirhonen et al. 1991; this study). The plasmid vector used for cloning was pBluescript II (Stratagene, La Jolla, CA). The plasmid pCA9505 carrying the *E. coli uvrY* gene has been described earlier (Moolenaar et al. 1987). The plasmids pRA903, pRA903-1, and pRA904 were constructed in this study. Transfer of the plasmids was done by standard transformation technique (*E. coli*) or by electroporation (*E. carotovora* subsp. *carotovora*) with a Bio-Rad gene pulser (Bio-Rad Laboratories, Hercules, CA). Plasmid preparations were performed with Qiagen plasmid kits (Qiagen, Hilden, Germany).

Culture conditions and chemicals.

E. coli strains were grown in L medium (Miller 1972) at 37°C and *E. carotovora* subsp. *carotovora* strains were grown in the same medium at 28°C. For Northern analysis and enzyme assays *E. carotovora* subsp. *carotovora* was grown in L medium supplemented with 0.4% polygalacturonic acid (PGA; P-1879 from Sigma Chemical Sweden, Stockholm) to induce extracellular enzyme production. When appropriate, antibiotics were added to the media at the following concentrations: ampicillin, 150 μ g/ml; and kanamycin, 25 μ g/ml. Restriction enzymes, T4 ligase, multiprime DNA labeling kit, and radiolabeled ddATP were from Amersham International (Buckinghamshire, UK). The DNA sequencing kit was from United States Biochemicals (Cleveland, OH). Synthetic oligonucleotides were purchased from Kebo Lab (Kebo, Stockholm) and Pharmacia Biotech (Uppsala, Sweden).

Cloning techniques and DNA sequencing.

The mutated *expA* gene was cloned by cutting chromosomal DNA from the *expA* transposon mutant SCC3060 to completion with *Pst*I, ligating this DNA to *Pst*I-digested pBluescript II, followed by transformation into the *E. coli* strain DH5 α . The relevant clone was obtained by direct selection for the kanamycin resistance gene of the transposon. The obtained plasmid was called pRA903-1. The DNA flanking the transposon was then partially sequenced with the Sanger dideoxy-chain termination method (Sanger et al. 1977) with oligonucleotides annealing to the vector DNA and synthetic oligonucleotides designed from the obtained sequences as primers. PCR primers were then designed from these sequences in order to clone the corresponding wild-type allele with chromosomal DNA from SCC3193 as a template. The resulting 991-bp PCR fragment containing the *expA* gene was ligated to *Eco*RV-digested pBluescript II to create plasmid pRA903. The mutated *expS* gene was cloned by a similar approach. Chromosomal DNA from SCC3009 was digested with *Pst*I followed by ligation to pBluescriptII and transformation to DH5 α . By selecting for the kanamycin resistance gene of the transposon we isolated the mutant allele. The wild-type allele of *expS* was cloned from SCC3193 by PCR with primers designed from the DNA sequence flanking the transposon. The

complete sequence of both strands was determined. All PCR reactions were performed with the proofreading Vent DNA polymerase (New England Biolabs, Beverly, MA).

Analysis of plasmid encoded proteins.

L-[³⁵S]methionine-labeled, plasmid-encoded proteins were produced by converting DH5 α cells harboring pRA903 to maxicells (Sancar et al. 1979). Proteins were separated by SDS-PAGE according to Laemmli (1970). Labeled proteins were visualized by autoradiography.

UV sensitivity tests.

The bacterial strains to be tested were streaked on L-agar plates and the plates immediately exposed to UV irradiation with a short wave, hand-held lamp. The lamp was kept at 10 cm distance from the bacterial streaks, which were irradiated for 0, 5, 10, 20, and 30 s. The growth of the bacteria was recorded after incubation for 18 h at 28°C.

Isolation of total RNA and northern blot analysis.

Total RNA was isolated with the Qiagen RNA isolation kit (Qiagen, Hilden, Germany). Five micrograms of RNA was denatured in formaldehyde and formamide and separated by formaldehyde gel electrophoresis. The gels were blotted on nylon membranes (Hybond-N, Amersham International, Buckinghamshire, UK) as described (Sambrook et al. 1989) and hybridized overnight at 65°C in 5 $\times$ Denhardt's, 5 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaPO₄, and 1 mM EDTA [pH 7.7]), 0.2% SDS, and 500 μ g of denatured herring sperm DNA per ml. A 1,103-bp *Bst*XI fragment of *celVI* from pAMV1 (Mäe et al. 1995) was used as a *celVI*-specific probe; for a specific *pehA* probe, a 563-bp PCR fragment was used, and for *pelC* the plasmid pHSK17 (Hannu Saarilahti and E. Tapio Palva, *unpublished*) was used. Probes were labeled with [α -³²P]dATP by random priming (Amersham International, Buckinghamshire, UK). After hybridization, membranes were washed in standard washes (6 $\times$ SSC [1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% SDS; 3 $\times$ SSC, 0.5% SDS; 1 $\times$ SSC, 0.5% SDS; at 65°C for 30 min), and exposed to X-ray film. The mRNA hybridization was also quantified by PhosphorImager and the data were processed with the Image Quant program (Molecular Dynamics, Sunnyvale, CA).

Enzyme assays.

The activities of polygalacturonase (Peh), pectate lyase (Pel), and cellulase (Cel) were assayed as described previously (Pirhonen et al. 1991).

Virulence tests.

The virulence of *E. carotovora* subsp. *carotovora* strains was assayed on axenic tobacco seedlings (*Nicotiana tabacum* cv. Samsun) cultivated essentially as described (Pirhonen et al. 1991). Bacterial inoculations and recording of symptom development were done as described (Pirhonen et al. 1993). Complementation of the *expS* mutant was performed essentially as described in Pirhonen et al. (1993). Virulence assays performed on greenhouse grown potato (*Solanum tuberosum* cv. Bintje) stems were essentially done as described by McMillan et al. (1993) and development of the symptoms was followed for 48 h. Maceration of potato tubers from *S. tuberosum* cv. Bintje was tested by injection of 10⁶ CFU from an

overnight bacterial culture into the tubers. The inoculated tubers were incubated at 28°C and 100% humidity, and the development of symptoms was evaluated after 48 h by measuring the weight of the macerated tissue.

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