A Two-Component Regulatory System, *pehR-pehS*, Controls Endopolygalacturonase Production and Virulence in the Plant Pathogen *Erwinia carotovora* subsp. *carotovora*

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Genes coding for the main virulence determinants of the plant pathogen *Erwinia carotovora* subsp. *carotovora*, the plant cell wall-degrading enzymes, are under the coordinate control of global regulator systems including both positive and negative factors. In addition to this global control, some virulence determinants are subject to specific regulation. We have previously shown that mutations in the *pehR* locus result in reduced virulence and impaired production of one of these enzymes, an endopolygalacturonase (PehA). In contrast, these *pehR* strains produce essentially wild-type levels of other extracellular enzymes including pectate lyases and cellulases. In this work, we characterized the *pehR* locus and showed that the DNA sequence is composed of two genes, designated *pehR* and *pehS*, present in an operon. Mutations in either *pehR* or *pehS* caused a Peh-negative phenotype and resulted in reduced virulence on tobacco seedlings. Complementation experiments indicated that both genes are required for transcriptional activation of the endopolygalacturonase gene, *pehA*, as well as restoration of virulence. Structural characterization of the *pehR-pehS* operon demonstrated that the corresponding polypeptides are highly similar to the two-component transcriptional regulators PhoP-PhoQ of both *Escherichia coli* and *Salmonella typhimurium*. Functional similarity of PehR-PehS with PhoP-PhoQ of *E. coli* and *S. typhimurium* was demonstrated by genetic complementation.

The plant-pathogenic enterobacterium *Erwinia carotovora* subsp. *carotovora* belongs to the soft-rot group of erwinias

and has the ability to infect a number of plant species, including several economically important crops. These bacteria secrete a large arsenal of plant cell wall-degrading enzymes, causing maceration of different plant organs and tissues (Pérombelon and Kelman 1980; Collmer and Keen 1986; Barras et al. 1994). The crucial role of these extracellular enzymes in virulence is demonstrated by isolation of avirulent mutants that are defective either in enzyme production (*exp* mutants; Pirhonen et al. 1991, 1993) (*aep* mutants; Murata et al. 1991, 1994; Liu et al. 1993) (*rex* mutants; Jones et al. 1993) or in secretion of the enzymes (*out* mutants) (Andro et al. 1984; Ji et al. 1987; Pirhonen et al. 1991; Thurn and Chatterjee 1985).

The production of these extracellular enzymes is coordinately controlled by a number of global regulators. A central control in this regulatory network is exerted by the cell density-dependent accumulation of a diffusible signal molecule, N-(3-oxohexanoyl)-L-homoserine lactone (OHHL), the *E. carotovora* autoinducer (Jones et al. 1993; Pirhonen et al. 1993). In addition to the *expI* gene directing the synthesis of OHHL, other global regulatory genes required for extracellular enzyme production and virulence have been characterized. These include *expA* and *expS* encoding proteins of the two-component regulator family (Pirhonen et al. 1993; Eriksson et al. 1998), as well as *aepA* and *aepH* (*rsmB*) (Liu et al. 1993; Murata et al. 1994; Liu et al. 1998). However, not only is the synthesis of virulence determinants controlled by the positive factors discussed above, but also a negative global regulator (*RsmA*) has been described (Chatterjee et al. 1995; Cui et al. 1995; Mukherjee et al. 1996; Liu et al. 1998).

In addition to global regulators, specific regulators, which can modify the expression of one gene or a subset of genes, are also involved in controlling the production of extracellular enzymes. In a related pathogen, *Erwinia chrysanthemi*, differential expression of genes for pectate lyases (Pels) has been observed in different hosts (Beaulieu et al. 1993) and even in different tissues of the same host (Lojowska et al. 1993). Some Pel isoforms are produced only during infection in planta (Kelemu and Collmer 1993).

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We have previously characterized mutants of *E. carotovora* subsp. *carotovora* that are impaired in the production of an endopolygalacturonase (PehA) but that produced and secreted Pels and cellulases (Cels) at the wild-type level (Saarilahti et al. 1992). The mutation was shown to affect transcriptional activation of the endopolygalacturonase gene, *pehA*. This suggested that the mutated locus, designated *pehR*, could encode a specific regulator of PehA. Mutations in *pehR*, as well as in the structural gene, *pehA*, exhibited a similar reduction in virulence on in vitro grown tobacco seedlings (Saarilahti et al. 1992). In this work we show that the *pehR* locus contains an operon of two genes, designated *pehR* and *pehS*, both of which are required for *pehA* expression and virulence. We also show that PehR-PehS is both structurally and functionally related to the two-component regulatory system PhoP-PhoQ that controls several pathogenicity properties of *Salmonella typhimurium* and *Escherichia coli*.

RESULTS

The *pehR* locus contains an operon of two genes.

For molecular characterization of the *pehR* locus we first cloned the transposon mutant allele present in strain SCC1935 of *E. carotovora* subsp. *carotovora*. The relevant clone was obtained by selecting for the kanamycin resistance provided by the transposon. Partial sequencing of the clone, carrying a 3.2-kb *Sall*-*EcoRI* fragment of chromosomal origin, was established and used to design oligonucleotide primers for polymerase chain reaction (PCR) amplification of the wild-type allele. Structural characterization of the *pehR* locus revealed two complete open reading frames (ORFs), apparently present as an operon (Fig. 1; GenBank accession number AF022772). The first 684-bp ORF, identified as *pehR*, is located between nucleotides 253 and 936. This ORF contained the original transposon insertion at 88 nucleotides (nt) downstream from the start codon (Fig. 1). The second 1,455-bp ORF, designated *pehS*, is located between nucleotides 933 and 2387. Both *pehR* and *pehS* coding regions begin with an ATG codon and end with a TGA codon. The start codon of *pehS* overlaps the stop codon of *pehR* by 2 nt, suggesting translational coupling commonly seen with two-component regulators (Govantes et al. 1998). A Shine-Dalgarno sequence, GAGGA, precedes the ATG codon of *pehR* by 7 nt; the *pehS* gene has a sequence AGG 6 nt upstream of the start codon. A putative -10 sequence, GATAAT, is found at nucleotides 125 to 130. The -35 region is replaced by a similar direct hexanucleotide repeat sequence GTTTA(T or C) as originally characterized in the phoP promoter (Soncini et al. 1995). Sequences that may form stem-loop structures and function as transcriptional terminators can be found 134 nt upstream of *pehR* and 82 nt downstream of the termination codon of *pehS*.

Both *pehR* and *pehS* mutants are affected in the production of endopolygalacturonase and exhibit reduced virulence.

We first wanted to assess the role of the individual genes at the *pehR* locus, *pehR* and *pehS*, on virulence and production of extracellular enzymes. To characterize the contribution of the *pehS* gene, we constructed a *pehS* insertion mutant (SCC4194) by marker exchange. Comparison of the extracel-

lular enzyme phenotypes of SCC1935 and SCC4194 showed that they were rather similar. Neither of the mutants produced measurable amounts of polygalacturonase (Fig. 2) but secreted both Pels and Cels close to the wild-type levels (not shown). Northern (RNA) blot analysis of *pehA* expression demonstrated that both mutants failed to accumulate detectable amounts of *pehA* mRNA (Fig. 2).

Virulence tests with in vitro grown tobacco seedlings showed that both mutants exhibit reduced virulence (Table 1). The reduced virulence phenotype of the *pehR* mutant SCC1935 was similar to that of a *pehA* mutant (SCC4080), but the *pehS* mutant SCC4194 appeared to be even less virulent.

For complementation analysis, the genomic fragment harboring the wild-type alleles of both *pehR* and *pehS* was first cloned into pBluescript to obtain plasmid pREM100 (Fig. 1). To facilitate the reliability of the complementation, the 2.5-kb fragment carrying *pehR-pehS* was incorporated into the low copy number vector pMW118. The resulting plasmid, pREM200, and its mutant derivatives, pREM201 (*pehR* mutant-*pehS*⁺) and pREM202 (*pehR*⁺-*pehS* mutant), were used for the actual complementation experiments. The mutation introduced into *pehR* (in pREM201) was a 2-bp deletion at the *NdeI* cleavage site resulting in a frameshift. The *pehS* mutant allele (in pREM202) harbored an 859-bp deletion (from *MluI* to *SphI*). Introduction of pREM200 to the mutants restored the Peh⁺ phenotype to both SCC1935 and SCC4194. In contrast, the mutant plasmids pREM201 and pREM202 only comple-

Fig. 1. The organization of the *Sall*-*EcoRI* chromosomal fragment containing the *pehR-pehS* operon and plasmid constructs derived from this region. Location of transposon insertion in the *pehR* gene of SCC1935 and location of interposon Ω_{Cm} in the *pehS* gene of SCC4194 are indicated by T and Ω , respectively. The *pehR* and *pehS* open reading frames, determined by sequencing, are shown by an open bar and filled bar, respectively. $\Delta NdeI$ shows position of the 2-bp deletion in the *pehR* gene on pREM201. Restriction enzymes *MluI* and *SphI* were used to delete a 859-bp fragment (thin line) from the *pehS* gene on pREM202. Complementation of the *pehR* mutation in SCC1935 and the *pehS* mutation in SCC4194, determined by Northern (RNA) hybridization and assay of Peh activity, is indicated by + or -, respectively. nd, not determined. The measured Peh activity is shown in parentheses. Nucleotide sequence of the *pehR-pehS* operon of *Erwinia carotovora* subsp. *carotovora* will appear in the GenBank data base under accession number AF022772.

mented the *pehS* and *pehR* mutants, respectively (Fig. 1). The plasmid pREM201, carrying a frameshift-mutated *pehR* gene but an intact *pehS* gene, could not complement the *pehR* mutant SCC1935, but restored the Peh⁺ phenotype to the *pehS* mutant SCC4194. The plasmid pREM202, carrying an intact *pehR* gene and a deletion of the *pehS* gene, failed to restore the Peh⁺ phenotype to the *pehS* mutant SCC4194 as expected but complemented the *pehR* mutant SCC1935 (Fig. 1). This was unexpected since the transposon insertion in *pehR* should be polar.

A more extensive complementation analysis by Northern hybridization and enzyme assays showed that pREM200 restores *pehA* expression and Peh activity to both *pehR* (SCC1935) and *pehS* (SCC4194) mutants. The enzyme activity obtained in both backgrounds by the complementing clone was about 35% of the wild-type level (Fig. 2). Furthermore, we assessed the effect of this plasmid on virulence. The results (Table 1) showed that while pREM200 was able to restore the virulence of SCC1935 to wild-type level, the virulence of SCC4194 was only partially restored.

Taken together, these results indicate that both *pehR* and *pehS* are required for *pehA* expression and that the *pehR-pehS* operon controls polygalacturonase production and virulence in *E. carotovora* subsp. *carotovora*.

Fig. 2. Effect of the *pehR* and *pehS* mutations on *pehA* expression and production of polygalacturonase (Peh) and complementation of the mutants by a *pehR-pehS* clone. **A**, Northern (RNA) blot analysis of *pehA* expression in *Erwinia carotovora* subsp. *carotovora* wild-type SCC3193, *pehR* mutant SCC1935, *pehS* mutant SCC4194, and mutants carrying the complementing plasmid pREM200 (*pehR*⁺-*pehS*⁺). Accumulation of RNA was followed during growth and samples were collected at indicated time points. Each lane contains 5 µg of total RNA. The *pehA* probe was prepared from an 1.2-kb internal *EcoRV* fragment from the *pehA* gene in pHSK24. **B**, Peh activity in the same strains, including the wild-type strain SCC3193 carrying the vector plasmid pMW118. The strains are indicated as follows: SCC3193 (■), SCC3193(pMW118) (□), SCC1935 (▲), SCC1935(pREM200) (Δ), SCC4194 (●), SCC4194(pREM200) (○). **C**, Corresponding growth curves; strains were grown in L⁻medium supplemented with appropriate antibiotics at 28°C.

PehR and PehS are structurally similar to the two-component regulators PhoP and PhoQ of *E. coli* and *S. typhimurium*.

The ORFs of *pehR* and *pehS* code for polypeptides of 227 and 484 amino acids with calculated molecular masses of 26 and 55 kDa, respectively. When protein data bases were searched with the deduced amino acid sequences of the PehR and PehS polypeptides, they were found to be similar to the two-component regulatory system PhoP-PhoQ of *E. coli* (Kasahara et al. 1992) and *S. typhimurium* (Miller et al. 1989). The putative response regulators PehR of *E. carotovora* subsp. *carotovora* and PhoP of *E. coli* and *S. typhimurium* share identity throughout the whole amino acid sequence. The amino acid identity of PehR with PhoP of *E. coli* is 76.2% and with PhoP of *S. typhimurium* is 74.6%. The crucial regions for phosphorylation as well as the putative DNA binding region are very well conserved (Fig. 3). The PehS protein is highly similar to the PhoQ proteins of *E. coli* and *S. typhimurium*, which belong to the histidine kinase family. The amino acid identity between PehS and the PhoQ sensor kinase of *E. coli* and *S. typhimurium* is 58.6 and 57.4%, respectively (Fig. 3B). In PehS, the two hydrophobic transmembrane segments are very well conserved (amino acids 16 to 42 and 188 to 216). In contrast, the sequence between them, which by similarity would correspond to the periplasmic loop (Miller et al. 1989) is the part of the polypeptide that differs most. An additional feature that relates PehS to the sensor kinase family is a sequence (amino acids 269 to 277) and a histidine residue (amino acid 275) positioned similarly to those involved in CheA (Hess et al. 1988) and nitrogen regulator I (NR_I) autophosphorylation (Weiss and Magasanik 1988), respectively. Together, these comparisons demonstrate the close structural similarity of the PehR and PehS polypeptides to the two-component regulator PhoP-PhoQ of *E. coli* and *S. typhimurium* and indicate that *pehR-pehS* encodes a two-component regulator system.

In addition to the homology of the *pehR-pehS* and *phoP-phoQ* genes, the flanking regions also appeared similar. The *pehR* gene is preceded by a short ORF encoding the C terminus of adenylosuccinate lyase; the ORF distal to *pehS* encodes the YcfD hypothetical protein. This organization is similar to that of the *phoP-phoQ* flanking regions from *E. coli* K12 as characterized by Kasahara and co-workers (1992).

Table 1. Virulence of *pehR* and *pehS* mutants on axenic tobacco (*Nicotiana tabacum* cv. Samsun) seedlings and complementation of mutants

Strain	Genotype	Virulence	
		Plants infected ^a	%
SCC3193	Wild type	71/72	98
SCC1935	<i>pehR</i> mutant	16/72	22
SCC4194	<i>pehS</i> mutant	5/72	7
SCC4080	<i>pehA</i> mutant	17/72	23
SCC3193 pMW118	Wild type	37/48	77
SCC1935 pMW118	<i>pehR</i> mutant	0/24	0
SCC1935 pREM200	<i>pehR</i> mutant (<i>pehR</i> ⁺ - <i>pehS</i> ⁺)	18/24	75
SCC4194 pMW118	<i>pehS</i> mutant	0/24	0
SCC4194 pREM200	<i>pehS</i> mutant (<i>pehR</i> ⁺ - <i>pehS</i> ⁺)	9/24	37

^a Results show ratio of plants infected to those inoculated.

PehR-PehS is functionally related to PhoP-PhoQ of *E. coli* and *S. typhimurium*.

The structural similarity of PehR-PehS of *E. carotovora* subsp. *carotovora* and PhoP-PhoQ of *E. coli* and *S. typhimurium* prompted us to test whether they were also functionally related. To this end, we transferred a plasmid (pDFL2) harboring a *pehA-lacZ* transcriptional fusion to the *E. coli* wild-type (with respect to PhoP-PhoQ) strain MC4100 and to its *phoP* mutant FS1002 and measured β -galactosidase activity. As a control, the β -galactosidase activity from pDFL2 was measured in the *E. carotovora* subsp. *carotovora* wild-type strain SCC3193, the *pehR* mutant SCC1935, and the *pehS* mutant SCC4194. As expected, β -galactosidase activity was almost abolished in the *pehR* and *pehS* mutants, compared with the activity found in the *E. carotovora* subsp. *carotovora* wild-type strain SCC3193 (Fig. 4). Interestingly, the β -galactosidase activity in the *E. coli* wild-type strain MC4100 was relatively high, about 42% of the activity in *E. carotovora* subsp. *carotovora* wild type, showing that *E. coli* can support the expression of the *pehA* gene. This activity was abolished in the *phoP* mutant FS1002, indicating that intact *phoP* is required for *pehA* expression in *E. coli*. Our previous work (Flego et al. 1997) has indicated that calcium regulation of *pehA* is mediated by *pehR*. Similarly, the PhoP-PhoQ system is involved in

Fig. 4. PhoP-dependent expression of *pehA* in *Escherichia coli*. **A**, β -Galactosidase activity of pDFL2 carrying transcriptional *pehA-lacZ* fusion was determined in *E. coli* strains MC4100 (wild type) and FS1002 (*phoP* mutant), *Erwinia carotovora* subsp. *carotovora* strains SCC3193 (wild type), SCC1935 (*pehR* mutant), and SCC4194 (*pehS* mutant). The strains are indicated as follows: SCC3193(pDFL2) (■), SCC1935(pDFL2) (▲), SCC4194(pDFL2) (●), MC4100(pDFL2) (◆), FS1002(pDFL2) (★). **B**, Corresponding growth curves; strains were grown in L medium supplemented with ampicillin, *E. coli* strains were grown at 37°C and *E. carotovora* subsp. *carotovora* strains at 28°C.

A

PehR <i>Ecc</i>	M-RILVVEVNVLLHHLTVQMNMGHQVDAASDAKEADYFLHNMPIAI	49
PhoP <i>Ec</i>	M-RVLVVEVNVLLHHLTVQDAGHQVDAEDAEADYFLNEHIPDAI	49
PhoP <i>St</i>	MMRVLVVEVNVLLHHLTVQDSGHQVDAEDAEADYFLNEHLPDAI	50
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PehR <i>Ecc</i>	V-LGLPDEDEGMSMIRWRANQAKLPILVLTAREGWQTKLPCWK-CDDYV	98
PhoP <i>Ec</i>	V-LGLPDEDEGLSIRWRNSNDVSLPILVLTARESWQDKVEVLSAGADDYV	99
PhoP <i>St</i>	V-LGLPDEDEGLSIRWRNSDVSPLVLTAREGWQDKVEVLSGADDYV	100
	* * * * * * * * * * * * * * *	
PehR <i>Ecc</i>	T-PFHMEEVVARLQALMRNNSGLASQIISLPFVEVDLSR-ELVIHSTPIK	147
PhoP <i>Ec</i>	T-PFHIEEVMARMQALMRNNSGLASQVISLPFQVDLSRRELSINDEVK	149
PhoP <i>St</i>	T-PFHIEEVMARMQALMRNNSGLASQVINIPFQVDLSRRELSVNEEVK	150
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PehR <i>Ecc</i>	LTAFEYTIETLIRNTGKVVSKDSLMLQLYPDAELRESHTIDVLMGRLRK	197
PhoP <i>Ec</i>	LTAFEYTIMETLIRNNGKVVSKDSLMLQLYPDAELRESHTIDVLMGRLRK	199
PhoP <i>St</i>	LTAFEYTIMETLIRNNGKVVSKDSLMLQLYPDAELRESHTIDVLMGRLRK	200
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PehR <i>Ecc</i>	KIQQADAPDVITTVRGQGYRFDIDTPSGSV	227
PhoP <i>Ec</i>	KIQAQYQGVITTVRGQGYLFELR-----	223
PhoP <i>St</i>	KIQAQYPHDVITTVRGQGYLFELR-----	224
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B

PehS <i>Ecc</i>	MSDNDKK-QPFSLRIRFLLATA-IVLALSLAYGTVAIGYSVNEKTSFR	48
PhoQ <i>Ec</i>	MKKLLRLFPPLSLRVRFLATAAVVLVLSLAYGMVALIGYSVSEDKTTFR	50
PhoQ <i>St</i>	MNKFARHFLPLSLRVRFLATAGVVLVLSLAYGIVLVGYSVSEDKTTFR	50
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PehS <i>Ecc</i>	LLRGESNLVYSLAQWRDNQNLNIVTPPDVDINFTPLVLIYDEQGNMLWREK	98
PhoQ <i>Ec</i>	LLRGESNLVYTLAKWENKHLVELPENIDKQSPMTTLIYDENGQLLWAQR	100
PhoQ <i>St</i>	LLRGESNLVYTLAKWENKISVELPENLRMQSPMTTLIYDETGLKLLTQR	100
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PehS <i>Ecc</i>	HVPELEALIKPEWLKNTAYHELDTSSTSAVLTGNLTLLSLRALNGTQ	148
PhoQ <i>Ec</i>	DVPNLMKMIQPDWLKNGFHEIADVNDTLLSLGDSHSIQQLQEVREDD	150
PhoQ <i>St</i>	NIPWLIKSIQPEWLKNTNGFHEIETNVDATSTLLSEDSHAQEKLEVEDD	150
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PehS <i>Ecc</i>	NNA-LTHSIAVN-VYPRTEHLPSITIVVDRIPQELQEQEDVVWVWFSYVF	196
PhoQ <i>Ec</i>	DDAEMTHSVAVN-VYPATSRMPKLTIVVDITPVELKSSYVWVWFSYVYL	199
PhoQ <i>St</i>	DDAEM-HSVAVNNIYPATARMPLQITIVVDITPIELKRSYVWVWFSYVYL	199
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PehS <i>Ecc</i>	IANLLVLPFLWAAWVSLRPIEALAEVRELEHREHRLINPATRELS	246
PhoQ <i>Ec</i>	SANLLVLPFLWAAWVSLRPIEALAEVRELEHREHRLINPATRELS	249
PhoQ <i>St</i>	IANLLVLPFLWAAWVSLRPIEALAEVRELEHREHRLINPATRELS	249
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PehS <i>Ecc</i>	LVKNLNLNLLNNEQRYHKYRTLTDLTHSIKTT-GVLQTTLRALRTGKEI	295
PhoQ <i>Ec</i>	LVRNLNLLKSERERYDKYRTLTDLTHSIKTTPLAVLQSTLRSLRSEK-M	298
PhoQ <i>St</i>	LVRNLNLLKSERERYDKYRTLTDLTHSIKTTPLAVLQSTLRSLRSEK-M	298
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PehS <i>Ecc</i>	TIEQAEPIMLAQISRSISQIGYLLHRASVRAENLLIREVSVPAVLDEL	345
PhoQ <i>Ec</i>	SVSDAEFVMLEQISRSISQIGYLLHRASRG-GTLLSRELHPVAPLLDNL	347
PhoQ <i>St</i>	SVSKAEFVMLEQISRSISQIGYLLHRASRGSGVLLSRELHPVAPLLDNL	348
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PehS <i>Ecc</i>	CSALNKVYQRKGVVLTLDIPPELTFVGEKNDMEVGMGNLDNACKYCLEF	395
PhoQ <i>Ec</i>	TSALNKVYQRKGVNISLDSPEISFVGEQNDFVEVMGNVLDNACKYCLEF	397
PhoQ <i>St</i>	ISALNKVYQRKGVNISLDSPEISFVGEQNDFVEVMGNVLDNACKYCLEF	398
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PehS <i>Ecc</i>	VEISVQYSDHKLHLIIDDDGPGILESKREVIQQRQADRMPPGGIGIA	445
PhoQ <i>Ec</i>	VEISARQTDHLHYVVEDDGGPIPLSKREVIQQRQADTLPFGQGVGLA	447
PhoQ <i>St</i>	VEISARQTDHLHYVVEDDGGPIPLSKREVIQQRQADTLPFGQGVGLA	448
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PehS <i>Ecc</i>	VAVEITEQYQGEIRISDSALGGARVEAIFSRQNLSONEG	484
PhoQ <i>Ec</i>	VAREITEQYQGEIVAGEISLGGARMEVIFGRQHSAPKDE	486
PhoQ <i>St</i>	VAREITEQYQGEIISDLSLLGGARMEVIFGRQHSAPKDE	487
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Fig. 3. Amino acid alignment of PehR-PehS of *Erwinia carotovora* subsp. *carotovora* (*Ecc*) with PhoP-PhoQ of *Escherichia coli* (*Ec*) and *Salmonella typhimurium* (*St*). Alignment of (A) response regulators (PehR and PhoP) and (B) sensor kinases (PehS and PhoQ). Highly conserved amino acid residues in response regulator family are boxed. A potential phosphorylation site is indicated by P; a putative DNA binding domain by overlining. Hydrophobic transmembrane segments in PehS and PhoQ are highlighted by a shaded rectangle; putative autophosphorylation site is boxed. * = identical and “.” = similar amino acids.

divalent cation (including Mg^{2+} and Ca^{2+}) controlled expression of a number of genes in *E. coli* and *S. typhimurium* (García-Véscovi et al. 1996). To test whether the PhoP-PhoQ system was also involved in Ca^{2+} control of *pehA* expression, we characterized the expression of a *pehA-lacZ* fusion in both wild-type (MC4100) and *phoP* (FS1002) strains of *E. coli*. The results of this analysis (Table 2) demonstrate the presence of Ca^{2+} regulation of *pehA* in *E. coli* and show that this regulation is abolished in the *phoP* mutant, suggesting that PhoP is a functional analogue of PehR also in this respect.

PhoP of *S. typhimurium* was originally discovered as a transcriptional regulator of nonspecific acid phosphatase (NSAP). Both *phoP* and *phoQ* mutants lack NSAP activity. To assess whether *pehR-pehS* is able to complement the NSAP-negative phenotype of the *phoP* and *phoQ* mutants we transferred pREM200 (*pehR*⁺-*pehS*⁺), pREM201 (*pehR* mutant-*pehS*⁺) and pREM202 (*pehR*⁺-*pehS* mutant) to the *phoP* mutant strain MS7953s and to the *phoQ* mutant strain MS5996s. The *S. typhimurium* wild-type strain ATCC14028s containing the vector pMW118 was used as a control. Strains were grown on phosphorus free M121 medium plates supplemented with 50 mM KH_2PO_4 and stained for NSAP activity (Fig. 5). The

phoP mutant MS7953s was clearly complemented by plasmids carrying an intact *pehR* gene, pREM200 (*pehR*⁺-*pehS*⁺) and pREM202 (*pehR*⁺-*pehS* mutant). Both plasmids restored the NSAP-positive phenotype to the *phoP* mutant. In contrast, the pREM201 plasmid (*pehR* mutant-*pehS*⁺) carrying a frameshift mutation in the *pehR* gene could not restore NSAP activity to the *phoP* mutant (Figs. 5 and 1). The *phoQ* sensor kinase mutant MS5996s was complemented by the plasmids carrying the *pehS* wild-type allele, pREM200 and pREM201. Surprisingly, NSAP activity was restored even by pREM202, which contains an intact *pehR* gene but a deletion in the *pehS* gene (Fig. 5). This is in contrast to the results in *E. carotovora* subsp. *carotovora*, where the *pehS* sensor mutant SCC4194 was complemented only by plasmids containing the intact *pehS* gene (Fig. 1).

It appears that *E. carotovora* subsp. *carotovora* does not produce NSAP activity. Staining of *E. carotovora* subsp. *carotovora* wild type (SCC3193) and both mutants (SCC1935 and SCC4194) for NSAP activity on induced and non-induced conditions did not give any positive reaction. All three strains have regulated phosphatase activity toward p-nitrophenyl phosphate (data not shown).

In conclusion, these data showing PhoP-dependent expression of an *pehR-pehS* controlled target gene *pehA* in *E. coli* and complementation of *phoP* and *phoQ* mutants by *pehR-pehS* in *S. typhimurium* demonstrate that PehR-PehS is functionally similar to the two-component regulator system PhoP-PhoQ in these bacteria.

Table 2. Effect of PhoP on the calcium modulated expression of *pehA* in *Escherichia coli*

[CaCl ₂]	β-Galactosidase activity ^a	
	MC4100	FS1002 (<i>phoP</i>)
0 mM	4,665	76
10 mM	288	73

^a Both strains harbored a plasmid-borne *pehA-lacZ* transcriptional fusion. The β-galactosidase activity assayed after 8 h of growth is given in Miller units.

DISCUSSION

The *E. carotovora* subsp. *carotovora* wild-type strain SCC3193 produces a number of extracellular plant cell wall-

Fig. 5. Complementation of nonspecific acid phosphatase (NSAP) negative phenotype of *phoP* and *phoQ* mutants of *Salmonella typhimurium* by *pehR-pehS* of *Erwinia carotovora* subsp. *carotovora*. The *S. typhimurium* wild-type strain ATCC14028s carrying the vector plasmid pMW118, and its transposon mutant derivatives MS7953s (a *phoP* mutant) and MS5996s (a *phoQ* mutant) carrying pREM200 (*pehR*⁺-*pehS*⁺), pREM201 (*pehR* mutant-*pehS*⁺), pREM202 (*pehR*⁺-*pehS* mutant), or pMW118 were stained for NSAP activity. Cells producing NSAP are red.

degrading enzymes: an endopolygalacturonase (encoded by *pehA*), at least four Pels (encoded by *pelA* to *pelD*) and two Cels (encoded by *celC*, *celVI*) (Saarilahti et al. 1990; Heikinheimo et al. 1995; Mäe et al. 1995). All of these virulence genes including *pehA* are controlled by at least two global regulatory systems, quorum sensing (Pirhonen et al. 1993) and the two-component regulator ExpA-ExpS (Eriksson et al. 1998). In addition to these global controls, the *pehA* gene seems to be specifically controlled by the *pehR* locus, in contrast to the other major virulence genes.

In this paper, we describe the molecular characterization of the *pehR* locus. By DNA sequence analysis we show that the locus contains two ORFs. The genes designated *pehR* and *pehS* appear to form an operon with *pehR* being the promoter proximal gene. Analysis of the deduced amino acid sequences suggests that PehR and PehS are members of a two-component regulator system, PehS being the sensor kinase and PehR the response regulator. The evidence for the two proteins belonging to the same regulator system comes partly from the structural organization of corresponding genes as an operon, a usual arrangement for cognate partners of two-component systems, and partly from the phenotypic analysis of *pehR* and *pehS* mutants. Our experiments indicated that both *pehR* and *pehS* are required for *pehA* expression and virulence. Mutations in either of the genes resulted in a Peh-negative phenotype and reduced virulence. The mutations could be complemented by introduction of the respective wild-type alleles on low copy plasmids restoring both *pehA* expression and virulence. Surprisingly, even the plasmid pREM202 (*pehR*⁺-*pehS* mutant) restored Peh activity to the *pehR* mutant SCC1935, which has a transposon insertion in the *pehR* gene and should be polar on *pehS*. It is possible that the increased amount of PehR is sufficient to activate transcription of *pehA* in the absence of PehS phosphorylation. Alternatively, the increased PehR concentration could stimulate unspecific phosphorylation. A third possibility is that *pehS*, in addition to being co-transcribed with *pehR*, has its own promoter, leading to low level production of PehS in the *pehR* mutant SCC1935.

The observed effect on virulence of *pehR* or *pehS* mutants could be explained by their effect on *PehA* production. Inactivation of *pehA* alone results in reduced virulence (Saarilahti et al. 1992; Flego et al. 1997). The reduced virulence phenotypes of *pehA*, *pehR*, and *pehS* mutants are similar (Table 1) and the phenotypes of *pehA* and *pehR* mutants could not be distinguished. Interestingly, the *pehS* mutant appeared in repeated tests to be even less virulent. Introduction of the *pehR-pehS* clone restored the Peh activity to both mutants. This activity was apparently sufficient for *pehR* mutant to reach the virulence of wild type, while virulence of *pehS* mutant was only partially restored. Currently, we can only speculate about this difference between the phenotypes of *pehR* and *pehS* mutants. Although in many cases a specific sensor kinase-response regulator pair may function as the primary regulator of a particular response, other kinases and regulators could potentially influence each of the components. Evidence for cross-talk in vivo has consistently been seen in mutants that lack a given kinase. It has been also shown that certain low-molecular-weight phosphorylated compounds such as phosphoramidate, carbamyl phosphate, and acetyl phosphate can transfer

phosphoryl groups to the response regulator in the absence of a kinase protein. Acetyl phosphate is likely to affect the regulation of many targets, such as cyclic AMP (cAMP), and thus might constitute a global signal (McCleary et al. 1993). The required specificity is obtained by control of the phosphorylation of response regulators and, in some cases, by the highly specific control of the dephosphorylation of response regulators by the regulated phosphatase activities. Based on similarity with OmpR-EnvZ and PhoB-PhoR systems, which belong to the same subfamily as PhoP-PhoQ, we can hypothesize that PehS mediates both phosphorylation of PehR and dephosphorylation of P-PehR (Igo et al. 1989; Parkinson and Kofoid 1992; Gunn et al. 1996). Thus, unspecific phosphorylation of PehR, but not dephosphorylation may occur in the *pehS* mutant, causing the overproduction of phosphorylated PehR. The overproduction of the phosphorylated regulator may modulate expression of genes that are directly or indirectly connected with virulence. It is also possible that PehR is not the only response regulator that is phosphorylated by PehS and that the nonphosphorylated response regulator may also have a functional role. We cannot, however, rule out the possibility that some of the observed effects are due to allele copy number. It has been suggested that low concentration of phosphorylated regulator can cause activation of target gene transcription, while high concentration could cause the opposite reaction—repression of these genes (Stock et al. 1989; Parkinson 1993). This could explain why the *pehR-pehS* wild-type allele cloned into a high copy vector pBluescript could not restore the Peh phenotype of *pehR* and *pehS* mutants (data not shown) and the low copy plasmid pREM200 partly restored Peh activity. However, the results could also suggest that Peh activity is not the only cause for reduced virulence in the mutants.

The regulatory operon *pehR-pehS* encodes proteins that are members of a two-component regulatory family. We here demonstrate both structural and functional similarity of PehR-PehS to the products of the *phoP-phoQ* regulatory operon of *S. typhimurium* and *E. coli*. PhoP was clearly required for expression of *pehA* in *E. coli* and it appeared even to be required for Ca²⁺ controlled repression of *pehA*. Such repression of gene expression was described earlier for *pehA* in *E. carotovora* (Flego et al. 1997) and for a number of PhoP-PhoQ controlled genes in *E. coli* and *S. typhimurium* (García-Véscovi et al. 1996).

It is intriguing that in both *E. coli* and *S. typhimurium* *phoP-phoQ* controls production of more than 40 polypeptides (Miller and Mekalanos 1990; Kasahara et al. 1992). In *S. typhimurium* about 25% of these are involved in virulence. A functional *phoP-phoQ* operon is required for expression of an NSAP, survival within macrophages, resistance to antimicrobial peptides, and acid pH, and is also involved in protein export. The *phoP-phoQ* regulator system can be a repressor for some genes and activator for others. The *phoP-phoQ* modulated genes in *S. typhimurium* are induced under very different conditions and respond in different fashions to the same environmental stimuli such as starvation and rich media, acid pH and neutral pH, and anaerobiosis and aerobiosis. Homologues of *phoP-phoQ* have been found in several bacteria species, including non-pathogens, indicating that the *phoP-phoQ* operon is not ex-

clusively involved in virulence and that it probably plays a central role in the physiology of many Gram-negative bacteria (García-Véscovi et al. 1994; Pegues et al. 1995). Thus, the *phoP-phoQ* regulator system appears to be a central global regulator of many processes in bacteria, including virulence. This is clearly contrasted by the role of *pehR-pehS* in *E. carotovora* subsp. *carotovora*, where we so far have only observed a specific reduction of *pehA* expression in the mutants. In addition, we have observed a slight up-regulation of *pelB* expression in the mutant background (not shown). However, the extensive structural and functional similarity between *pehR-pehS* and *phoP-phoQ* suggests that even *pehR-pehS* could control several genes. Thus, it is feasible to assume that additional genes involved directly or indirectly in virulence could be regulated by *pehR-pehS*.

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 its mutant derivatives SCC1935 (*pehR* mutant), SCC4080 (*pehA* mutant) (Saarilahti et al. 1992) have been described. The strain SCC4194 (*pehS* mutant) was isolated in this study (see below). The *E. coli* strain MC4100 (Casadaban 1976) and its *phoP* mutant FS1002 have been described (Groisman et al. 1992). The *S. typhimurium* wild-type strain ATCC 14028s (Fields et al. 1986), its *phoP* mutant MS7953s, and *phoQ* mutant MS5996s have been described (Fields et al. 1989). The pBluescript IISK plasmid was purchased from Stratagene (La Jolla, CA) and the pMW118 from Eurogentec (Seraing, Belgium). The plasmid pHP45 Ω -Cm was described by Fellay et al. (1987). The plasmid pHSK24 has been described by Saarilahti et al. (1990) and pHLU102 by Lång and Palva (1993). The plasmids pREM100, pREM200, pREM201, pREM202, and pDFL2 were constructed in this study (see below). Plasmid preparations were done with Qiagen plasmid kits (Qiagen, Hilden, Germany). Transfer of the plasmids was done by standard transformation technique (*E. coli*) or by electroporation (*E. carotovora* subsp. *carotovora*, *S. typhimurium*) with the Bio-Rad gene pulser (Bio-Rad Laboratories, Richmond, CA).

Media, growth conditions, and chemicals.

E. coli and *S. typhimurium* strains were grown in L medium (Miller 1972) at 37°C and *E. carotovora* subsp. *carotovora* strains at 28°C. When necessary, appropriate antibiotics were added to medium at the following concentrations: chloramphenicol (Cm), kanamycin (Km), and tetracycline (Tc) were added to media at 25 $\mu\text{g ml}^{-1}$ and ampicillin (Ap) at 150 $\mu\text{g ml}^{-1}$. For NSAP assay the *S. typhimurium* strains were grown on phosphorus-free M121 salts medium (Torriani 1966) plates supplemented with KH₂PO₄ (50 mM), thiamin (1 $\mu\text{g ml}^{-1}$) and glucose (0.4%) as a carbon substrate. *E. carotovora* subsp. *carotovora* were grown on M121 salts medium supplemented with KH₂PO₄ (either 50 mM as excess or 0.1 mM as limiting), casamino acids (0.3%) and glycerol (0.8%) as a carbon source. The restriction enzymes, Klenow fragment, T4 ligase, mung bean nuclease, multiprime DNA labeling kit, radiola-

beled dATP, and L-methionine 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). The p-nitrophenyl phosphate (Sigma 104), α -naphthyl phosphate, tetrazotized o-dianisidine, and polygalacturonic acid (PGA) (P-1879) were from Sigma Chemical (St. Louis, MO). 2-Nitrophenyl- β -D-galactopyranoside was from Merck (Darmstadt, Germany).

Cloning techniques and DNA sequencing.

To clone the mutated *pehR* locus, chromosomal DNA of the mini-kan transposon (Way et al. 1984) mutant SCC1935 was cut by restriction enzymes *Pst*I, *Eco*RI-*Xba*I, or *Sal*I and ligated into pBluescript IISK digested with corresponding enzymes, followed by transformation to the *E. coli* strain DH5 α . Mutated allele-containing clones were selected for the kanamycin resistance gene of the transposon. The 3.2-kb *Sal*I-*Eco*RI fragment was further subcloned to smaller fragments suitable for sequencing by the Sanger dideoxy-chain termination method. To sequence both strands, synthetic oligonucleotides designed from the completed sequence were used. For cloning the intact wild-type *pehR-pehS* alleles, the following primers were designed: 5' GGTACCAACCATTTCAGCC TG 3' and 5' CTCGAGATTGAGCTGATAGAC 3'. PCR was performed with the proofreading Vent DNA polymerase (New England Biolabs, Beverly, MA) with wild-type SCC3193 chromosome as a template. The resulting 2.5-kb fragment was ligated to *Eco*RV-digested pBluescript IISK vector to create plasmid pREM100. For generation of *pehS* mutant, *Hind*III-digested and blunted, 3.5-kb, Cm-specific interposon fragment of the plasmid pHP45- Ω was ligated with *Mlu*I-digested and blunted pREM100. Resulting plasmid pDFL6 was electroporated into the *E. carotovora* subsp. *carotovora* wild-type strain SCC3193. For marker exchange, SCC3193/pDFL6 was grown overnight in L medium with Cm selection and left standing at room temperature over two nights. Recombinants were selected on L-Cm plates and true marker exchange mutants were screened for by loss of the plasmid-encoded ampicillin resistance. The *pehS* mutant, named SCC4194, was purified and controlled by Southern analysis. For complementation the 2.5-kb *Bam*HI and *Hind*III fragment carrying *pehR-pehS* in pREM100 was transferred into corresponding sites of the low copy number plasmid pMW118, resulting in pREM200. To generate a mutant of the cloned *pehR*, pREM100 was digested with *Nde*I, the single-strand DNA overhangs removed by mung bean nuclease and the blunt ends ligated. The resulting plasmid pREM101 had lost the *Nde*I cleavage site. Sequencing of both strands showed that two nucleotides T and A (309th and 310th from ATG) were deleted, causing a frameshift in *pehR*. For complementation, the 2.5-kb *Bam*HI-*Hind*III fragment of pREM101, carrying the mutant allele of *pehR* but an intact *pehS* gene, was transferred into pMW118, resulting in pREM201. To generate the *pehR*⁺-*pehS* mutant clone (pREM202), the 859-bp *Mlu*I-*Sph*I fragment was deleted from the *pehS* gene of pREM200. The *pehA-lacZ* transcriptional fusion pDFL2 (Flego et al. 1997) was created by first inserting an 1.6-kb *Hind*III fragment of pHSK24, carrying the *pehA* promoter region, into the *Hind*III site of pBluescript and subsequently moving this promoter as an

EcoRI-KpnI fragment to the corresponding sites upstream of the promoterless *lacZ* gene of pHLU102.

Isolation of chromosomal DNA and total RNA, and Southern and Northern hybridization.

Chromosomal DNA of SCC3193 and SCC4194 was isolated as described by Sambrook et al. (1989). Total RNA was isolated by the RNeasy kit (Qiagen) according to the instructions given by the manufacturer. For Northern analysis, 5 µg of total RNA was denatured in formamide and formaldehyde and separated by formaldehyde gel electrophoresis. The blotting onto nylon membranes (Hybond-N; Amersham International) and Southern and Northern hybridization were as described by Sambrook et al. (1989). The gene-specific probes were the 1.2-kb *EcoRI* fragment of pHSK24 for *pehA* and the 1.3-kb *Clal* fragment of pREM100 for *pehS*. The probe fragments were labeled with ³²P-dATP by random priming. The signals were visualized by autoradiography or by phosphorimager and the data were processed with the Image Quant program (Molecular Dynamics, Sunnyvale, CA).

Assay procedures.

The polygalacturonase (Peh) enzyme activity was measured and units defined as described by Pirhonen et al. (1991). The extracellular enzyme indicator plates were according to Pirhonen et al. (1993). The β-galactosidase activity was determined from samples immediately after collection as described by Miller (1972). The NSAP staining procedure was according to Kier et al. (1979). The *S. typhimurium* strains were grown on non-induced conditions to prevent background caused by low activity of acid hexose phosphatase and cyclic phosphodiesterase toward α-naphthyl phosphate as a substrate (Weppelman et al. 1977). Phosphatase activity toward p-nitrophenyl phosphate was determined as described by Brickman and Beckwith (1975).

Assay of bacterial virulence.

The virulence of SCC3193, SCC1935, SCC4194, and SCC4080 was tested on axenic tobacco seedlings (*Nicotiana tabacum* cv. Samsun) grown on MS medium (Murashige and Skoog 1962) supplemented with 2% sucrose on 24-well tissue culture plates. Bacteria were propagated overnight in L medium at 28°C, diluted in 0.9% NaCl, and used to inoculate 3- to 4-week-old axenic tobacco seedlings. Plasmid carrying strains were diluted in 0.9% NaCl supplemented with ampicillin. The leaves were punctured by a needle and 1 µl of bacterial suspension (10⁴ CFU) was applied to the damaged site on the leaf. One leaf per well was inoculated. After infection, the plants were incubated at 28°C in high humidity at light. The development of disease symptoms (tissue maceration) was followed up to 72 h. Plants that exhibited clear tissue maceration in 72 h were scored as infected.

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