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A Regulatory Locus, *pehSR,* Controls Polygalacturonase Production and Other Virulence Functions in *Ralstonia solanacearum*

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We previously identified a locus that regulates production of polygalacturonase (PG), an extracellular plant cell wall–degrading enzyme important in bacterial wilt of plants caused by *Ralstonia (Pseudomonas) solanacearum*. The DNA sequence of this locus, called *pehSR,* was determined and two consecutive open reading frames (ORFs) of 1,905 and 1,680 bp were identified. The amino acid sequences predicted to be encoded by these ORFs are similar to those of regulators of pilin synthesis in *Pseudomonas aeruginosa* and *Myxococcus xanthus* and to a regulator of flagellin synthesis and adhesion in *P. aeruginosa,* as well as to other two-component regulators of the NtrB/C subfamily. *pehSR* mutants produced negligible levels of *endo*-PG activity, while *exo*-PG activity was reduced by 50%. Northern (RNA) blot analysis showed that PehSR regulates *endo*-PG expression at the transcriptional level. *pehSR* mutants grew normally in culture and in planta but were dramatically reduced in virulence; this loss of virulence was substantially greater than that observed for *endo*-PG structural gene mutants, suggesting that *pehSR* regulates additional factors important in virulence. Although *pehSR* mutants were essentially nonmotile, like the wild-type strain, multiple copies of *pehSR* conferred motility on the bacterium. Reporter gene studies indicated that *pehSR* expression increased when bacteria grew in plant tissue, and that the *pehSR* locus was itself negatively regulated by the global virulence gene regulator PhcA.

Ralstonia (Pseudomonas) solanacearum is a widespread phytopathogenic bacterium responsible for a lethal wilting disease of over 200 plant species, including such economically important crops as potato, tobacco, pepper, banana, and peanut (Buddenhagan and Kelman 1964; Hayward 1991). *R. solanacearum* enters host plants through the roots, either at wounds or sites of secondary root emergence. Bacteria aggregate on the plant surface at root junctions and then penetrate the cortex, living and multiplying in the interstitial spaces. Eventually the pathogen invades host plant xylem cells and spreads throughout the vascular system, causing severe wilting that ultimately leads to plant death. Xylem tissue from diseased plants is macerated and contains copious amounts of extracellular polysaccharides (EPS); these changes are thought to be largely responsible for bacterial wilt symptoms (Husain and Kelman 1958; Vasse et al. 1995).

The physiological basis of the disease is multifactorial. Biochemical and genetic studies indicate that, in addition to EPS, the secreted plant cell wall–degrading enzymes polygalacturonase (PG) and endoglucanase (Egl) are necessary for full virulence (Allen et al. 1991; Cook and Sequeira 1991; Denny et al. 1990; Roberts et al. 1988). Regulation of these products is complex (Schell 1996) and while none is essential for pathogenicity, each contributes measurably to the expression of wild-type virulence. Extracellular proteins of unknown function, as well as plant hormones, may also play a role in disease but have been less intensively studied (Bonn et al. 1975; Denny et al. 1996; Phelps and Sequeira 1968).

K60, a race 1, biovar 1 strain of *R. solanacearum,* produces three PGs: an *endo*-PG (PehA) and two *exo*-PGs (PehB and C). The equivalents of PehA and PehB have also been found in another race 1 strain, AW1, and named PglA and PglB, respectively. The *endo*-PG structural genes have been cloned and mutated (Allen et al. 1991; Huang and Schell 1990). PehA and PglA mutants were substantially reduced in virulence when stem inoculated into eggplants and tomatoes, respectively; this loss of virulence was even more pronounced when the *pehA* mutant was root inoculated. In stem and root inoculation experiments *exo*-PG PehB mutants were significantly less virulent than wild type, but not as strongly attenuated as the PehA mutant (Huang and Allen, *in press*).

Total PG activity is low when *R. solanacearum* is grown in rich medium, but increases fourfold when bacteria grow in minimal medium and 100-fold when bacteria grow in tobacco leaves, suggesting that PG production is repressed by a component of the rich medium and also induced by a factor present in the plant (Allen et al. 1991). The precise role of PGs in virulence is not clear, but it is plausible that these enzymes play a part in several stages of the infection process, including invasion, spread, and nutrition of the bacterium.

Production of EPS, Egl, and the PGs is regulated in re-

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Nucleotide and/or amino acid sequence data are to be found at GenBank as accession no. AF001171.

sponse to bacterial population density via a quorum-sensing mechanism. This regulation is mediated by an endogenous elicitor, 3-hydroxypalmitic acid methylester (3-OH-PAME), that accumulates intercellularly and reaches induction levels at a cell density of about 10^7 CFU/ml in culture (Clough et al. 1997a). 3-OH-PAME regulates virulence genes via PhcA, a LysR-type global regulator that directly or indirectly controls many virulence genes, including a number of other regulators. PhcA positively controls EPS and Egl expression, but negatively regulates PG production and motility (Brumbley et al. 1993; Huang et al. 1995; Schell et al. 1994). In contrast to the wild-type strains, which are only transiently motile at low population density, *phcA* mutants retain motility at high population densities (Clough et al. 1997b). Not surprisingly, *phcA* mutants are avirulent.

While screening for transposon insertion mutations in PG genes, we isolated a group of mutants that had a 50% reduction in *exo*-PG activity and produced only trace *endo*-PG activity. We named the locus interrupted by these insertions *pehR,* suspecting it to have a role in the regulation of PGs (Allen et al. 1991). We report here further characterization of *pehR* with transposon mutagenesis, DNA sequence analysis, and in planta virulence assays. We found that this locus encodes an apparent two-component regulator of the NtrB/C family; henceforth we will refer to it as *pehSR*. *pehSR* mutants are reduced in virulence on eggplant to a greater extent than either *pehA* or *pehB* mutants or a *pehA/B* double mutant, although their ability to grow in planta is not compromised. We present evidence that *pehSR* positively regulates *pehA* and *pehB* expression, although differently, and that *pehSR* positively regulates motility. We also have evidence that *pehSR* is itself negatively regulated by *phcA*.

RESULTS

Twenty-one chromosomal *pehSR*::Tn*3-gus* reporter gene mutants were obtained.

Out of 206 transposon insertions in the *pehSR* locus, 21 were chosen for further study (Fig. 1). These insertion constructs were recombined into the wild-type chromosome to create *pehSR*::Tn*3-gus* chromosomal mutants; gene replacement was confirmed by Southern blot (data not shown). Interestingly, no transposon insertions were found in the orientation opposite to that of *pehSR* transcription. We can offer no explanation for this observation.

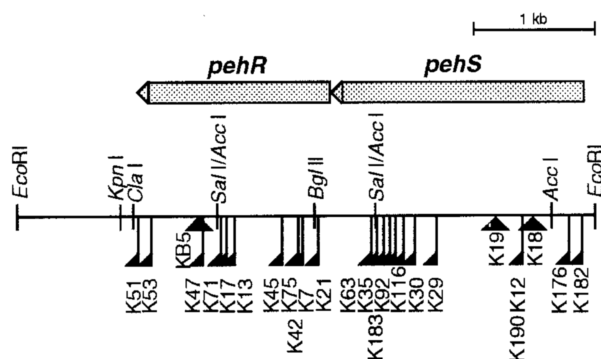

Fig. 1. Physical map of the *pehSR* locus showing Tn*5* (triangles) and Tn*3::gus* (flags) insertions that were recombined into the chromosome to generate mutant strains. Names of the corresponding mutant strains are given beneath each insertion site.

pehSR mutants grow normally in culture and in planta.

The growth curves of *pehSR* mutants in CPG, defined medium, and tobacco leaves were not significantly different from those of wild-type strain K60 (data not shown). On CPG plates these mutants had a normal mucoid colony morphology and were indistinguishable from wild type.

Virulence of *pehSR* mutants is severely attenuated on eggplant.

Eggplants inoculated with *pehSR* mutant strain K71 exhibited later disease onset and reduced wilt symptom severity, compared with plants inoculated with either wild-type strain K60 or the *pehA endo*-PG mutant, K60-06 (Fig. 2). Some plants with limited wilting outgrew the disease and some never developed symptoms. However, all plants inoculated with the wild-type strain rapidly wilted and died. Similar results were obtained with other *pehSR* mutant strains (data not shown). Differences among the three strains were analyzed by analysis of variance at days 7 and 12 after inoculation. The virulence levels were significantly different at $P = 0.001$.

PehSR regulates *endo*- and *exo*-PGs differently.

Concentrated culture supernatants from wild-type strain K60 reduced the viscosity of a polygalacturonate substrate solution (a measure of *endo*-PG activity) by 50% after 50 min whereas a comparable supernatant from a *pehSR* mutant reduced viscosity by 2.5 to 5% in the same time period (Table 1). These *endo*-PG activity levels were in the range of those observed for the *pehA endo*-PG structural gene mutant K60-06 (2%), implying that *pehA* was expressed at negligible levels in a *pehSR* mutant background. In contrast, *exo*-PG activity in *pehSR* mutants was 50% of that observed in wild-type strain K60. Data obtained from isoelectric focusing gels and from a reporter gene construct in the *pehB exo*-PG structural gene

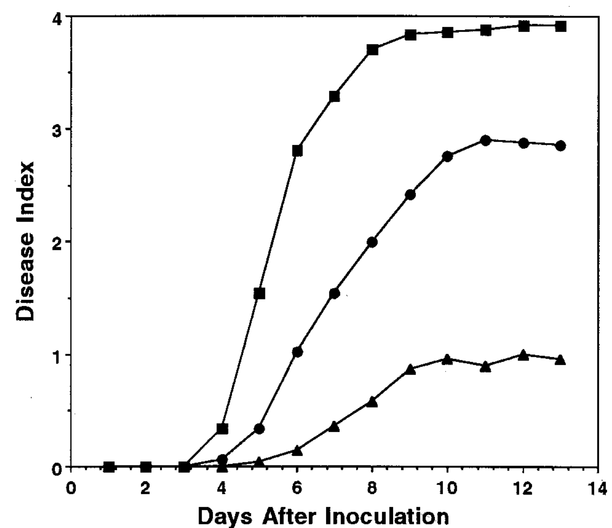

Fig. 2. Disease progress curves of 21-day-old eggplants in soil inoculated with 5×10^5 CFU of various bacterial strains per g of soil. Inoculant strains were K60 (wild type; squares), K60-06 (PehA$^-$; circles), and K71 (PehR$^-$; triangles). Plants were rated daily on a 0 to 4 disease index scale; points shown are the means of 48 plants in three replicates.

indicate that this loss of activity was associated with a 50% reduction in both PehB and PehC activity (data not shown), suggesting that expression of the two *exo*-PG structural genes was similar in a *pehSR* mutant background.

PehSR regulates *pehA* at the transcriptional level.

PehA is an abundantly expressed protein in strain K60 and Northern (RNA) blots reveal a single intense band when probed with a *pehA* probe. However, no *pehA* transcript was detected in Northern blots of *pehSR* mutant K71 (Fig. 3). This result is consistent with the absence of *endo*-PG activity in *pehSR* mutants and suggests that the *pehSR* locus is necessary for transcription of the *pehA endo*-PG structural gene.

Multiple copies of *pehSR* confer motility.

When the nonmotile, wild-type strain K60 was transformed with pKH19, a low-copy-number plasmid carrying *pehSR,* it became motile, as observed qualitatively in a stab assay. The motility conferred by multiple copies of *pehSR* was similar to that observed in K60-*phcA* or in KS5, a spontaneous, nonmucoid, avirulent mutant of K60. Neither K60 nor *pehR* mutants K7, K35, and K71 were motile under these conditions. Interestingly, the *phcA*//*pehR* double mutant strain P71 was not motile, either (Table 1). Thus, a mutation in *pehR* eliminates the *phcA* motile phenotype, suggesting that *phcA* affects motility via *pehSR*.

***pehSR* is negatively regulated by PhcA.**

The *pehSR*::Tn*3-gus* insertion constructs used to make strains K71, K7, and K35 (Fig. 1) were individually exchanged into the K60-*phcA* chromosome to create three separate *pehSR*//*phcA* double mutant strains, P71, P7, and P35. *pehSR* expression was measured as β-glucuronidase activity, and did not vary with the insertion mutant used. β-glucuronidase activity in the *pehSR* strains was on average 2.2 nmol methyl umbelliferone per min per CFU, but this rose to 27 nmol/min, a 12-fold increase, in the *pehSR*/*phcA* double mutant strains. Since the absence of PhcA resulted in a significant increase in expression from the *pehSR* promoter, we concluded that PhcA directly or indirectly represses expression of *pehSR* during growth in defined medium.

PehSR expression is not auto regulated.

Several regulatory loci in plant-associated bacteria can directly or indirectly regulate their own transcription (Schlaman et al. 1992; Winans 1992). To determine if *pehSR* is auto regulated, three different *pehSR*::Tn*3-gus* chromosomal mutants (K7, K35, and K71) were transformed with either plasmid pKH19, which contains an intact *pehSR* locus, or the vector pLAFR3 alone. If *pehSR* were auto regulated, the presence of 3 to 5 plasmid-borne copies of *pehSR* would be expected to alter expression of the *pehSR*::Tn*3-gus* fusion. However, β-glucuronidase activity was the same whether strains were transformed with a wild-type *pehSR* locus or with the vector alone, so we concluded that this locus does not regulate its own expression, at least in defined medium.

Expression of PehSR increased when bacteria grew in minimal medium or plant tissues.

pehSR::Tn*3-gus* strain K71 growing in rich culture medium produced low levels of β-glucuronidase. However, *pehSR* expression increased fivefold when K71 grew in defined medium, and 10-fold when K71 was infiltrated into tobacco leaves (Fig. 4). This is the same trend previously observed for PG activity, which increased 10-fold when bacteria grew in

Table 1. Motility and *endo*-polygalacturonase (PG) activity of various strains

Strain	Genotype	Motility[a]	*endo*-PG[b]
K60	Wild-type race 1, biovar 1	–	100%
KS5	Spontaneously avirulent mutant	+	165%
K71	*pehR*::Tn*3gus*	–	5%
K60/pKH19	Two to five copies *pehSR* in *trans*	+	97%
K60-*phcA*	*phcA*::Ω	+	180%
K71-*phcA*	*pehR*::Tn*3gus, phcA*::Ω	–	5%

[a] Determined by motility tube stab assays. Cultures were stabbed into CPG medium (Hendrick and Sequeira 1984) containing 0.3% agar and rated after 24 h at 28°C.

[b] Measured as loss of viscosity of a 1% sodium polypectate solution at 28°C and given as percent wild-type activity.

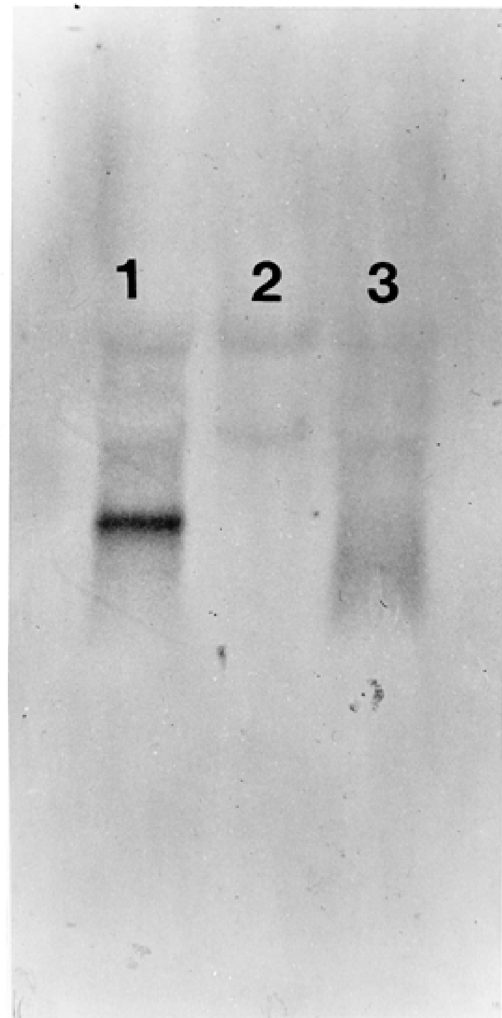

Fig. 3. Northern (RNA) blot showing total bacterial RNA from strain K60 (wild type; lane 1), K71 ($PehR^-$; lane 2), and K60-06 ($PehA^-$; lane 3) probed with a ^{32}P-labeled, 2.4-kb *Cla*I-*Eco*RI DNA fragment containing *pehA* (Allen et al. 1991).

defined medium instead of rich medium, rising to 100-fold greater in bacteria growing in tobacco (Allen et al. 1991).

Sequence analysis suggests PehSR is a two-component regulator.

The nucleotide sequence of more than 4 kb of the *pehSR* locus was determined and has been deposited in GenBank, accession number AF001171 (Fig. 5). This included almost 500 bp 5′ of the *Eco*RI site of pKH19, since it was anticipated that this region contained control elements. The sequence contained two consecutive open reading frames (ORFs) with a high GC codon bias typical of *R. solanacearum* (C. Allen, *unpublished data*). These ORFs were separated from each other by 26 bp, and therefore were in different reading frames. The two putative genes were predicted to encode proteins of 635 and 560 amino acid residues with molecular masses of about 70 and 60 kDa, respectively. The GCG package FastA program revealed that the *pehSR* locus has similarity to many genes of two-component regulators belonging to the NtrB/C family (reviewed in Stock et al. 1995 and Stock et al. 1989). This family includes genes involved in diverse metabolic pathways, including nitrogen assimilation, catabolism, and production of pilin and flagellin. These regulators all require the alternative sigma factor RpoN to activate transcription. RpoN itself cannot initiate transcription without the binding of an additional transcription factor (Kustu et al. 1989). It is not clear why regulators of such diverse function are so similar at the nucleotide level, but it may simply indicate a common evolutionary origin.

PehSR most closely resembled two versions of PilSR, which regulates pilin production in the opportunistic human pathogen *Pseudomonas aeruginosa* and in *Myxococcus xanthus* (Boyd and Lory 1996; Hobbs et al. 1993; Wu and Kaiser 1995) and a regulator of flagellin synthesis, *fleSR,* from *P. aeruginosa* (Ritchings et al. 1995) (Table 2). On the strength of these relationships these ORFs were presumed to encode a two-component regulator and were named *pehS* and *pehR,* respectively. The predicted PehSR amino acid sequences contain all the conserved regions common to two-component regulators (Figs. 6 and 7). These include conserved C-terminal regions in the PehS sensor, including the presumably phosphorylated histidine residue followed about 100 amino acids downstream (128 in PehS) by an asparagine residue. These two invariant residues reside in regions designated I and II, respectively (Fig. 6; Parkinson and Kofoid 1992). Region III consists of two groups of conserved residues, the first of which is not well conserved in PehS. Some sensors have a hydrophobic N-terminal region that probably allows them to be anchored in the cytoplasmic membrane; this region is present in PehS (Fig. 6). DNA similarity between *pehS* and *pilS* extends back to the proposed translation start site of *pehS,* an ATG at position 496. Although the potential ORF for *pehS* extends some distance upstream of this, the position 496 start site seemed most probable because it was preceded by a run of codons rarely used by *R. solanacearum.* In addition there is also a possible Shine-Dalgarno ribosome-binding sequence 6 bp upstream (Fig. 5). Furthermore, complementation studies indicated that the 4.5-kb *Eco*RI fragment that begins just upstream of this proposed start was sufficient to restore normal *endo*-PG expression to a *pehS* mutant. There are several possible σ54 binding sites upstream from the ATG, though without knowing the transcriptional start site we cannot suggest which one, if any, is functional.

Response regulators typically contain three highly conserved regions, which probably have important functions. An N-terminal region of about 100 residues contains two conserved aspartate residues (located at positions 15 and 59 in PehR) and a conserved lysine (position 139). A central domain that interacts with σ54 RNA polymerase in NtrC homologs is also well conserved in PehR. Two possible ATP-binding sites are indicated in Figure 7; these occur within the domain that is thought to interact with σ54 when hydrolysis of ATP is required to produce an open complex. The third region is a possible DNA-binding domain in the form of a helix-turn-helix motif in the C-terminal region (Fig. 7). Although reporter gene insertions in *pehR* such as K71 are well expressed, there is no obvious ribosome-binding site upstream of the proposed *pehR* start site.

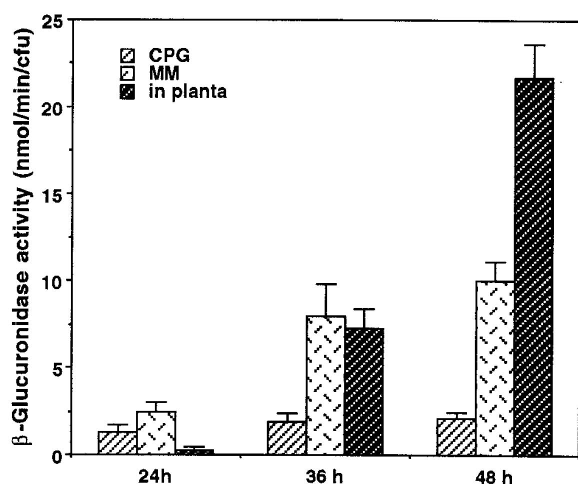

Fig. 4. β-glucuronidase activity per cell produced by *pehR*::Tn*3-gus* fusion strain K71 at different times after inoculation of bacteria into various media. CPG = rich medium; MM = Boucher's minimal medium plus 0.2% citrate; in planta = bacteria infiltrated into tobacco leaves. Values are means of three experiments; standard error is represented by the error bars.

DISCUSSION

The previously described *pehR* locus was found to encode an apparent two-component regulator, named PehSR, that controlled expression of *endo*-PG in *R. solanacearum* at the transcriptional level and also affected expression of *exo*-PG and motility in an undetermined manner. In addition, *pehSR* mutants were dramatically less virulent than the wild-type strain. Control of *pehSR* expression is multifaceted; we found that it was down-regulated by global regulator PhcA and factor(s) present in rich medium, but induced when the bacterium was associated with plant tissue.

The *pehSR* locus was shown to positively regulate both *endo-* and *exo*-PG production in *R. solanacearum,* though not to the same extent. In the absence of *pehSR, endo*-PG production was negligible (around 5%), whereas *exo*-PG activity was reduced to 50% of wild type. This result suggests that either *exo*-PG is expressed at a high constitutive level or that other regulatory elements also affect *exo*-PG gene expression.

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1     CTATGTCGCAGCGTTCGTGCTGGAAGTCATTTGCATCGCGTATAGCGCGCCCGCCCGCGGTTTGTCCTTCGCTTCTCCAC
81    CATGGCCCGTCCCGTCCTGCTGATCCTGCTGCTGCTTGCCGGCCTGTGGTGGCTCAGCCGCCAAGGCCTGCGCAATGCGC
161   GGCCGACGCCGTCGGCACCCGCCGCCCGCCAGCGCGATCAGGAGGCACCTGGCACGGCGCAGCCGATCGAGCAATGCGCC
241   GTCTGCGGCGTGCATGCGCCGCGCACCGGCTTAGTCGCCCTGTCTGGCGGGCGCTATTGCTGCGCCGAACACGCCGACCA
321   GGCCGGGCGGGGGCGGCGCATGATGCGCGACGCATCCGGCTCGGCACAGTCCAAGGCCGGCGGTAGCGACGCTGCCGGCG
401   CCCGGTCGACCGGCATGCGCTTGTGGTCGCGCCTGAATGAATGCCTGGCGCCCGGTGCGCTCGGTGTGGCTGGAGCCGGA
            EcoRI
481   TCCGCCGGAATTCCAATGCGCCTGCTGCGCTACTTCGCCTTCAGCCGGGCGGCGGTGGCGCTGGTGCTGCTGCTGTTCATC
                      M  R  L  L  R  Y  F  A  F  S  R  A  A  V  A  L  V  L  L  L  F  I
562   TCGATCCCGCGCGAGCATGCGACCGACTTGCCGGCGTCCGTCGGCGACGCCATGCTCAGCCTGACGCTGCCGTACTTGGCG
      S  I  P  R  E  H  A  T  D  L  P  A  S  V  G  D  A  M  L  S  L  T  L  P  Y  L  A
643   CTCGCGCTGCTGATCCTGGCGGCGGCAGGCTGGTGGCGCGCGCGTTTCCAGTTCCGGGTGCGGCTGGACGTGCTGCTGGAC
      L  A  L  L  I  L  A  A  A  G  W  W  R  A  R  F  Q  F  R  V  R  L  D  V  L  L  D
724   CTGCTCTTCCTGGGGCTGGCCTATACGACGCTGTCGCGCCTGTCGGCCAGCGTGGCGATGGTGTTCCTGATGCCGGTGCTG
      L  L  F  L  G  L  A  Y  T  T  L  S  R  L  S  A  S  V  A  M  V  F  L  M  P  V  L
805   GCGGCCGGTGCGCTGACCAGCCTGCTGTTCGCGCTGTTCACGGCGGCGGTGGCGTCGATGGTGGTGCTGGCCGAGCCCTTC
      A  A  G  A  L  T  S  L  L  F  A  L  F  T  A  A  V  A  S  M  V  V  L  A  E  P  F
886   CTGCGCATGCTGGGCGACGGCACGATCGACTCGGGGCTGGCCTCCGCCGGGCTGTATGGCTTGGTCTACATGATGGCCGCG
      L  R  M  L  G  D  G  T  I  D  S  G  L  A  S  A  G  L  Y  G  L  V  Y  M  M  A  A
967   CTGATGATGTACGGCCTGTCGCACCGGCAGGTGGCGCAGGAGCGCCTGACGCTGGCCCGCGAGCGCGAACTGCGCCTGCAG
      L  M  M  Y  G  L  S  H  R  Q  V  A  Q  E  R  L  T  L  A  R  E  R  E  L  R  L  Q
1048  CAGCTGGTGAACCGGCTGATGGTCTACGACATGCAGGACGGCGTGATGCTGGTGCGCGCCGACGGCCGCGTGGTGGCCGCC
      Q  L  V  N  R  L  M  V  Y  D  M  Q  D  G  V  M  L  V  R  A  D  G  R  V  V  A  A
1129  AATCCCGCCGCGGCGATGCTGCTGGGCGTGCCGCAGAATGCATTCGTCGGCAGCGGCTCCGTGCTGTTCGACCTGAAGGGG
      N  P  A  A  A  M  L  L  G  V  P  Q  N  A  F  V  G  S  G  S  V  L  F  D  L  K  G
1210  ATTCCTCACCTGCATCCGCTGCTGGAAACGTTGCGCCAATGGCTGCGCCGCCAGAGCCGGCATCCGGGCAGCGGCACCGAC
      I  P  H  L  H  P  L  L  E  T  L  R  Q  W  L  R  R  Q  S  R  H  P  G  S  G  T  D
1291  GCGGGCGATGACGATGCCACGCGCATCCTCGACCTGCAGCCGATCGCCCCGGGCGGTCGTGCCGCGCTGCGTGCCCGCCTG
      A  G  D  D  D  A  T  R  I  L  D  L  Q  P  I  A  P  G  G  R  A  A  L  R  A  R  L
1372  CGCTTGCGCTTCATCCTGCCGAGCCTGGCAAACCTGCGCACGGTCTATCTGGACAGCCTGGTCGGCGCGATCGGCCTGGGC
      R  L  R  F  I  L  P  S  L  A  N  L  R  T  V  Y  L  D  S  L  V  G  A  I  G  L  G
1453  CTGCCGGGCGAGGCCGTGGGCGAGCCGCCGCGTCCGCGCGGCCCCGCGACCGAGACCGTCGCGCAAGGCTGGTCCGCCGAT
      L  P  G  E  A  V  G  E  P  P  R  P  R  G  P  A  T  E  T  V  A  Q  G  W  S  A  D
1534  GACGAGGTCTTTCTGCGCCACGAGCTGCACGATACCGTGCTGGTGCACGTGGAGAGCTGGGAGCGCGTGACCGAGCAGGCT
      D  E  V  F  L  R  H  E  L  H  D  T  V  L  V  H  V  E  S  W  E  R  V  T  E  Q  A
1615  CAGCAGGAAAAGCTGGCCTCGATGGGGCGGCTGGTGGCGAGCGTGGCGCACCAGATCCGCAATCCGCTCGCGGCCATCAGC
      Q  Q  E  K  L  A  S  M  G  R  L  V  A  S  V  A  H  Q  I  R  N  P  L  A  A  I  S
1696  CAGGCGGCCGAACTGCTGGACGACCCGGGCGAGGGCGGCGAGCCACTGCGCCCCGAGGGCCGCGGCGTGGAGACGCGCCTG
      Q  A  A  E  L  L  D  D  P  G  E  G  G  E  P  L  R  P  E  G  R  G  V  E  T  R  L
1777  CTGCGCATCATCCGCGACAACGTGCGCCGCCTCGACCAGGTGGTCGCCGACGTGCTGATGCTGTCGCGCCGGCCGCGCGGC
      L  R  I  I  R  D  N  V  R  R  L  D  Q  V  V  A  D  V  L  M  L  S  R  R  P  R  G
1858  GAGCGCGTGCGGGTGCAGCTCGCGCAGGTGCTGCCCGAGGTGGTGGAGCGCTGGCGTGCCGAGGCGCTGCGCCGCGCGGGC
      E  R  V  R  V  Q  L  A  Q  V  L  P  E  V  V  E  R  W  R  A  E  A  L  R  R  A  G
1939  GAGGCGACCGAGATCCACGCCGATCTGGTGCGCGTGGCCGTCGACCTGCCGGGGCCGGTGCTGTTCGATCCGGCCCATCTG
      E  A  T  E  I  H  A  D  L  V  R  V  A  V  D  L  P  G  P  V  L  F  D  P  A  H  L
2020  CAGCAGGTGGCCGGTAACCTGCTCGACAACGCGCTGCGCTATTGCCGCCGTGTGCCGGGCTCGATCCTGCTGGCAGCCTAT
      Q  Q  V  A  G  N  L  L  D  N  A  L  R  Y  C  R  R  V  P  G  S  I  L  L  A  A  Y
2101  CCGCTGGATGATACCCATGCCGAACTGGTGATCTGGAACGACGGCCCCGAGGTTTCCGCCGAGCAGCAGCGCAGCCTGTTC
      P  L  D  D  T  H  A  E  L  V  I  W  N  D  G  P  E  V  S  A  E  Q  Q  R  S  L  F
2182  GAGCCGTTCTTCACCAACGACGCGCAGGGCACGGGGCTCGGCCTCTACATGGCGCGCGAGTTGTGCGCTGCCAACGACGCG
      E  P  F  F  T  N  D  A  Q  G  T  G  L  G  L  Y  M  A  R  E  L  C  A  A  N  D  A
2263  CAGATCCGCTACGGCGACATCGCGCTCGAATCCTTGCTCGATCGCACCGGAGCGTTGACCATGGTGGCGCGCGAGGCCCTG
      Q  I  R  Y  G  D  I  A  L  E  S  L  L  D  R  T  G  A  L  T  M  V  A  R  E  A  L
2344  CCGCGCCGCGCCTTCGTCATCACCCTGATGTTCGACCAACCCGCTGTGCCGGCGGAATGATCCGCCAGCCGTTTTTCCAGT
      P  R  R  A  F  V  I  T  L  M  F  D  Q  P  A  V  P  A  E  *
2425  CGCCCATGTCCAAAGCCGCCATCGTTCGCGAACCCATTCTCGTCGTCGATGACGAGGCCGACCTGCGCGAGCTGCTGGAGA
         M  S  K  A  A  I  V  R  E  P  I  L  V  V  D  D  E  A  D  L  R  E  L  L  E  I
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Fig. 5. Nucleotide sequence of the *pehSR* locus with translation of putative open reading frames. Possible ribosome binding site is underlined.

Figure 5 continued on following page.

Figure 5 continued from previous page.

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2506 TCTCCCTGCGGCGCATGGGGCACGACGTGGTACTGGCCGCTGGCCTGGGCGAGGCGCGCGAGGCGCTGGCGCGGCAACGCT
       S  L  R  R  M  G  H  D  V  V  L  A  A  G  L  G  E  A  R  E  A  L  A  R  Q  R  F
2587 TTGCGCTGGTGCTGACCGACATGCGCTTGGGCGATGGCCTGGGCATCGATCTGGTGCGCCAGCTTTCGGCCACCGCCGACC
       A  L  V  L  T  D  M  R  L  G  D  G  L  G  I  D  L  V  R  Q  L  S  A  T  A  D  R
2668 GCACGCCGGTGGCCGTCATCACCGCCTACGGCAGCGCCGAGAACGCGGTGGAGGCGCTCAAGGCGGGGGCGTTCGACTATA
       T  P  V  A  V  I  T  A  Y  G  S  A  E  N  A  V  E  A  L  K  A  G  A  F  D  Y  I
2749 TTGCCAAGCCGCTGTCGCTCGATCAGTTGCGCAGCCTGGTGCTCAACGCGCTGGGTCGCCAGCAGCGCGATCCCGATCCCG
       A  K  P  L  S  L  D  Q  L  R  S  L  V  L  N  A  L  G  R  Q  Q  R  D  P  D  P  G
2830 GCAGCGCTGATCTCGCCGAGCGCACCAATGCGCTGTTGCCCGGCCATTCCGCCGCCATGCAGGAGGTGCGCCGCTCGCTGC
       S  A  D  L  A  E  R  T  N  A  L  L  P  G  H  S  A  A  M  Q  E  V  R  R  S  L  L
2911 TGCGGCTGGCGCGCAGCATGGCCCCGGTGGTGATCAGCGGCGAGTCGGGCAGCGGCAAGGAGCGTGCGGCGCGCGCCATCC
       R  L  A  R  S  M  A  P  V  V  I  S  G  E  S  G  S  G  K  E  R  A  A  R  A  I  H
2992 ATGCGCTGTCGGCACGCTCGCCGCGGCCGTTCGTGGCGGTCAACTGCGGCGCGATTCCCGAGAACCTGATGGAAGCCGAGT
       A  L  S  A  R  S  P  R  P  F  V  A  V  N  C  G  A  I  P  E  N  L  M  E  A  E  F
3073 TCTTCGGCTATGTGAAAGGCGCCTTCACCGGCGCCGACAGCGACCGCCAGGGTTTCTTCCAGGCAGCCCATGGCGGCACGC
       F  G  Y  V  K  G  A  F  T  G  A  D  S  D  R  Q  G  F  F  Q  A  A  H  G  G  T  L
3154 TCATGCTCGACGAGGTGGCCGACCTGCCGCTGACCATGCAGGTCAAGCTGCTGCGCCGGCTGCAGGACGGCCGCGTGCGCA
       M  L  D  E  V  A  D  L  P  L  T  M  Q  V  K  L  L  R  R  L  Q  D  G  R  V  R  K
3235 AGATCGGCGAGAGCCGCGAAGACCCGGTCGACGTGCGCGTGGTGTGCGCGACGCACCAGAATCTTGCGCGCCTGGTTGCCG
       I  G  E  S  R  E  D  P  V  D  V  R  V  V  C  A  T  H  Q  N  L  A  R  L  V  A  A
3316 CCGGACGTTTCCGCGAAGACCTGTTTTACCGGCTGAACGTGCTGGAGCTGCGCATGCCGACGCTGCGCGAGCGTGCCGAGG
       G  R  F  R  E  D  L  F  Y  R  L  N  V  L  E  L  R  M  P  T  L  R  E  R  A  E  D
3397 ATGTGCCGGTCCTGGCGGGCGTGCTGCTGGAGCAACTGGCCACGCGTTACGGCGATCCGCGGCCGAAGCGGCTCACGCGCC
       V  P  V  L  A  G  V  L  L  E  Q  L  A  T  R  Y  G  D  P  R  P  K  R  L  T  R  Q
3478 AGGCGCTGCAGCAGTTGTGCGCCTATCCGTTCCCCGGCAACGTGCGCGACGTGGACAACCTGCTGGAGCGTGCCTACGCCT
       A  L  Q  Q  L  C  A  Y  P  F  P  G  N  V  R  D  V  D  N  L  L  E  R  A  Y  A  F
3559 TCGCCGAGGGCGAGTCGATCGACGTGGACCACCTCGGCGCGCTGGGTTCAGACATCGAGCGCTCGCCGCTGTTCCACCGAG
       A  E  G  E  S  I  D  V  D  H  L  G  A  L  G  S  D  I  E  R  S  P  L  F  H  R  A
3640 CGCGCGAGGCGCACGCCGGCCATCCGGTACATCCGGCGCACCTCCCGCCGGTGCCGGCGCCGGGCCATCCGGCCCATCCGG
       R  E  A  H  A  G  H  P  V  H  P  A  H  L  P  P  V  P  A  P  G  H  P  A  H  P  G
3721 GGCATTCCGGCCATGTCGCGCATCCGCTCGGCGTGCCGCAGCCGGTGGGCGGGTGGCCCGATGCCGCCGCCTACATTCCCG
       H  S  G  H  V  A  H  P  L  G  V  P  Q  P  V  G  G  W  P  D  A  A  A  Y  I  P  V
3802 TGCCCGGCCCGATGGGCCTGGTGCCGCATCCCGCCGCCGTGCCGTTCGAGCCCGCGCCGGCCGCCGAACCGGCCGCGTCGC
       P  G  P  M  G  L  V  P  H  P  A  A  V  P  F  E  P  A  P  A  A  E  P  A  A  S  P
3883 CCATGCCCGCCGTGTCCTTGCCGGTGGACCTGCCGGCCTACCTCGAATCGGTGGAGCGCAGCGTGATCCTGGCCGCACTGG
       M  P  A  V  S  L  P  V  D  L  P  A  Y  L  E  S  V  E  R  S  V  I  L  A  A  L  A
3964 CGCAGACCGGCTTCAATCGGACGGCGGCCGCCAAGCTGCTCGGCCTGAGCTTCCGGCAGTTGCGTTACCGGATGCAGCAGC
       Q  T  G  F  N  R  T  A  A  A  K  L  L  G  L  S  F  R  Q  L  R  Y  R  M  Q  Q  L
4045 TGGACATCCGCGATCCGCGCGATATTGACGCCGCGGCGGGCAACGGCGGGGTGGGCGATGCCTGAGGCGGCCGGGCCGGTG
       D  I  R  D  P  R  D  I  D  A  A  A  G  N  G  G  V  G  D  A  *
                                                                 ClaI
4126 CGCGTTGGCGCGGACGGCTGGGTGGAGGGGGTGCACCTCCATCCTTCGCCCAATATCGAT
```

The role of motility in bacterial wilt virulence is not known. Most cells of *R. solanacearum* are not motile at cell densities up to 10^6 CFU/ml, and 30 to 50% of cells are motile at 10^7 to 10^8 CFU/ml. As the population grows past this point, motility levels ordinarily decrease; fewer than 5% of wild-type cells are motile at 10^9 CFU/ml. In contrast, about 60% of *phcA* global regulatory mutant cells are still motile at 10^9 CFU/ml (Clough et al. 1997b). The stab motility assays used in this work reveal motility at high population densities, when the PhcA-mediated, quorum-sensing system has normally reduced frequency of motility. We found that although *pehSR* mutants are qualitatively nonmotile, like the wild-type strain, multiple copies of the *pehSR* locus conferred motility. Thus, multiple copies of *pehSR* mimicked the effect of a *phcA* mutation on motility. Furthermore, a *pehSR*/*phcA* double mutant strain was not motile. This suggested that *pehSR* is downstream from *phcA* in the regulatory cascade but upstream from motility and PG production.

In the absence of global virulence gene regulator PhcA, *pehSR* expression increases 12-fold, suggesting that *pehSR* itself is normally negatively controlled by PhcA. Release of this repression probably explains the over-expression of *endo*-PG and the acquisition of motility observed in *phcA* mutants, since the latter (but no other effects of a *phcA* mutation) can be duplicated with multiple copies of the *pehSR* locus. These results support a model in which PehSR positively regulates motility, production of PehA, and to some degree PehB and C at low population densities. As bacterial populations increase to 10^7 CFU/ml, PhcA reduces *pehSR* expression, which in turn results in reduced PG expression and motility.

Plant virulence assays demonstrated that *pehSR* is required for wild-type bacterial wilt virulence. However, since this locus is negatively regulated by PhcA at high bacterial population density, the role of *pehSR* and the genes it positively regulates may be limited to the early stages of infection when bacterial population densities are low. Strange as it may seem for the bacterium to restrict maximal expression of PGs to the early stages of infection, it is not difficult to imagine a role for

them there, perhaps in the production of elicitor fragments from plant cell walls, or facilitating the initial entry of bacteria into plant tissue. Production of *endo-* and *exo-*PG is required for wild-type virulence, suggesting that these *pehSR*-regulated genes play an important role at some point in disease development. The bacterium produced 50% of wild-type *exo*-PG activity in the absence of *pehSR,* which may be sufficient to release sugars for nutritive or signaling purposes. Further, we cannot ignore the possibility that the *exo*-PGs are also multiply regulated and may be induced or repressed independently of PehSR during disease. Obvious roles for these degradative enzymes in virulence would be in penetration, cell wall maceration, and release of sugar monomers as an energy source to the rapidly multiplying bacteria.

It therefore seems counter-intuitive for the bacterium to down-regulate these genes just as disease is getting underway. However, this anomaly may reflect the complexity with which pathogenicity factors are regulated in *R. solanacearum*. PehA expression is now known to be affected by cell density (via PhcA), PehSR, an unidentified plant signal(s), and at least one other regulatory pathway (VsrB/C) (Huang et al. 1993; Schell 1996). Further, regulatory studies to date have been conducted in culture and the in planta picture may well be much more complex. We do not know how *pehSR* is regulated over the course of infection. The in planta expression study reported here found high levels of *pehSR* expression at 48 h postinoculation but this was not representative of natural infection as we artificially infused areas of tobacco leaf with concentrated bacteria and then assayed for reporter gene expression over time. We therefore cannot draw strict parallels between gene expression in bacteria under these conditions and during normal invasion and colonization.

Two ORFs with typical *R. solanacearum* codon usage bias were identified and named *pehS* and *pehR* on the basis of their

Table 2. Proteins resembling PehS and PehR from *Ralstonia solanacearum*

Protein	(source)	Function	Similarity (%)	Identity (%)
PehS	PilS *(Pseudomonas aeruginosa)*	Regulates pilin synthesis	56	32
	PilS *(Myxococcus xanthus)*	Regulates pilin synthesis	48	28
	FleS *(P. aeruginosa)*	Regulates flagellin, adhesion	53	31
PehR	PilR *(P. aeruginosa)*	Regulates pilin synthesis	73	55
	PilR *(M. xanthus)*	Regulates pilin synthesis	63	43
	FleR *(P. aeruginosa)*	Regulates flagellin, adhesion	59	40

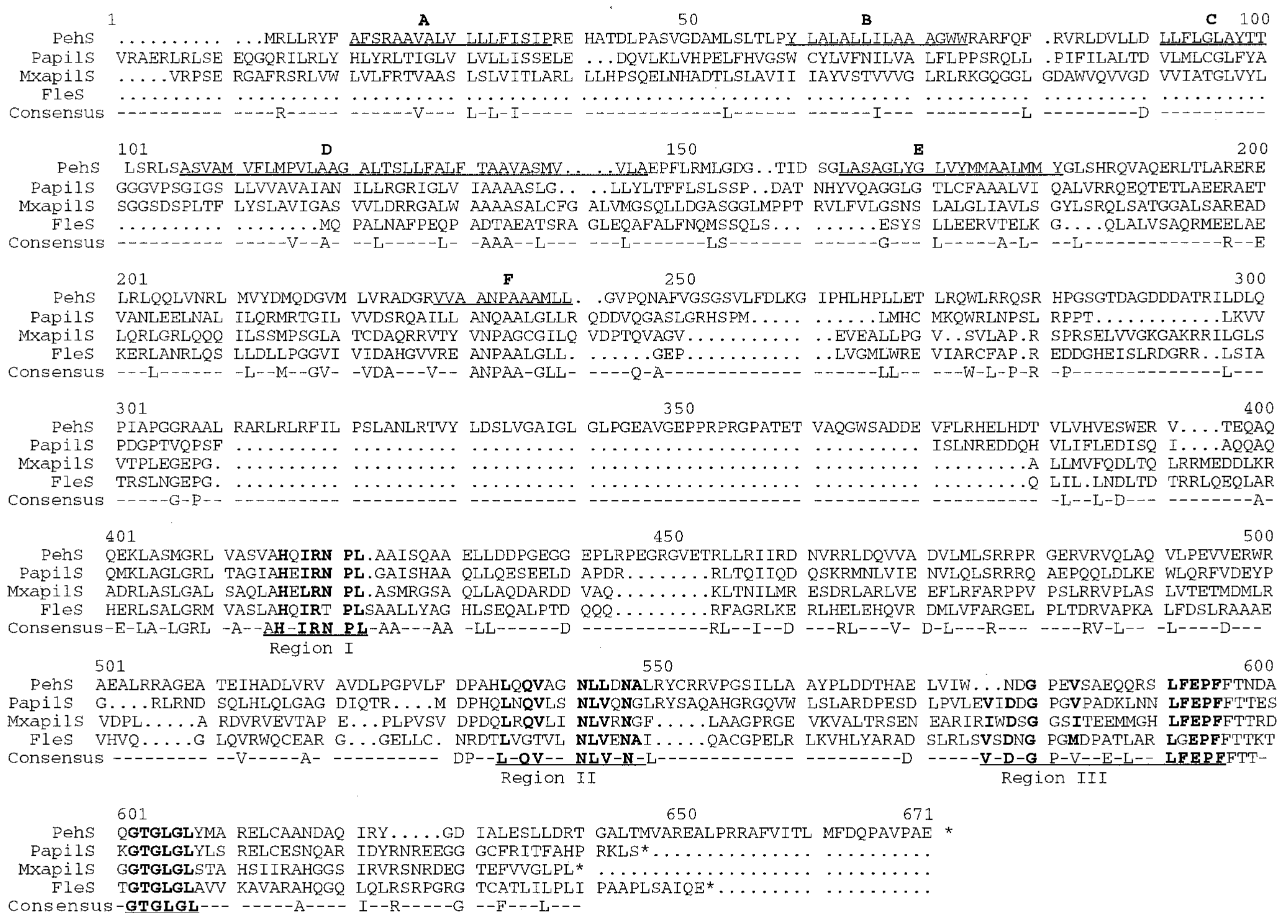

Fig. 6. Pileup of amino acid sequences showing strong similarity to *Ralstonia solanacearum* PehS. Potential membrane-spanning domains in PehS are underlined and marked with letters A to F. Highly conserved amino acids are in bold. PapilS, *Pseudomonas aeruginosa* PilS; MxapilS, *Myxococcus xanthus* PilS; FleS, *P. aeruginosa* FleS.

similarity to bacterial two-component regulators of the NtrB/C family, which consists of a histidine protein kinase sensor and a response regulator. The close proximity of these ORFs (26 bp) and the absence of any obvious promoter elements upstream of the second one suggests that the two genes are cotranscribed. However, this inference is tenuous at present.

The C terminus of PehS closely resembles histidine kinases of the NtrB family, but there is similarity along its entire coding sequence to PilS pilin regulators from *P. aeruginosa* and *M. xanthus,* and also to FleS, a regulator of flagellin synthesis and adhesion in *P. aeruginosa* (Boyd and Lory 1996; Hobbs et al. 1993; Ritchings et al. 1995; Wu and Kaiser 1995) (Fig. 6). Similarly, the N terminus of PehR resembles those of the entire NtrC family of response regulators, but there is significant similarity and identity to the two PilRs and FleR along the full sequence length (Fig. 7). This relationship does not necessarily indicate that *PehSR* is also a regulator of pilin production or flagellin synthesis or adhesion, although the involvement of PehSR in motility and its possible role in the early stages of plant colonization is suggestive. Nevertheless, it is entirely consistent with the idea that PehSR also regulates other virulence factors(s) besides the PGs.

Six regions in the N terminus of PehS were identified as possible membrane-spanning segments on the basis of charge, polarity, and hydrophobicity of the amino acid residues. These segments correspond closely to the sequence positions of the six putative membrane-spanning segments of the two PilS proteins. We suspect that PehS is toxic to *Escherichia coli* cells when present on a high-copy-number plasmid since throughout the cloning of *pehSR* we have never retrieved clones in which *pehSR* was under the control of a strong plasmid promoter. Although the *P. aeruginosa* PilS was successfully overexpressed in *E. coli,* it was as a truncated version of the protein lacking the hydrophobic, putative membrane-spanning segment. This was apparently because *E. coli* did not recognize the TTG start site (Boyd and Lory 1996). The putative PehS start site is an ATG, which presumably poses no problem to *E. coli* RNA polymerase. Therefore, we speculate that the intact PehS protein may integrate into the *E. coli* cytoplasmic membrane and disrupt normal cell signaling or other essential functions.

Pili have been shown to play an important role in the initial stages of infection in *P. aeruginosa* by mediating adhesion to human epithelial cells (Farinha et al. 1994; Irvin et al. 1989). Evidence is also accumulating that the pili of other Type IV fimbriae producers specifically recognize and bind the cells of their respective hosts (Rudel et al. 1995). Fimbriae of *R. solanacearum* bind plant cell wall fragments as well as the fimbriae of fellow bacteria, leading to agglutination (Stemmer and Sequeira 1987). Interestingly, flagella of *P. aeruginosa* are also associated with adhesion, but to host mucins, not cells. This mucin adhesion is thought to be instrumental in host colonization but it is not a property of the flagellum itself. Mutations in the flagellar structural gene that result in the absence of flagella do not affect adhesion. However, a mutation in *fleSR* affects both adhesion and motility (Ritchings et al. 1995). If *pehSR* should be involved in the positive regulation of pilin and/or flagellin, it may be significant that its repression coincides with the induction of EPS, which is thought to prevent bacterial cell agglutination and plant cell wall attach-

Fig. 7. Pileup of amino acid sequences showing strong similarity to *Ralstonia solanacearum* PehR. Highly conserved amino acids are in bold. PapilR, *Pseudomonas aeruginosa* PilR; MxapilR, *Myxococcus xanthus* PilR; FleR, *P. aeruginosa* FleR.

ment, in which case EPS may have a role in the mobilization of bacteria throughout the plant.

Type IV pili of *P. aeruginosa* give the bacterium a twitching motility that may be important in pathogenesis and allow the bacterium to move across surfaces; similarly, pili in *M. xanthus* are associated with social gliding motility. We are not aware that *R. solanacearum* has pilus-mediated motility, but many bacteria, including *P. aeruginosa* and *M. xanthus,* sport more than one type of motility, facilitated by different sets of genes and operating via different structures. Ongoing studies of type IV pilus production and regulation have unearthed a large number of genes essential for normal pilus production in *P. aeruginosa* (Alm et al. 1996a, and references therein). Some of these have significant structural and biochemical homology to genes involved in the chemotactic behavior of enterics (Darzins 1994). Some pilin genes are also related to genes of the general protein secretion pathway (Alm et al. 1996b). It is not inconceivable that a similar array of genes also exists in *R. solanacearum* and contributes to virulence. We need to address the question of whether *pehSR* regulates pilin production in *R. solanacearum* and, if so, the possibility that it may have control over a complex set of behaviors that could include a positive chemotactic response of *R. solanacearum* to plant root exudates, subsequent epithelial cell binding, and bacterial aggregation at root junctions.

Equally important is the study of what role, if any, *pehSR* plays in flagella-mediated motility. Studies of motility in *R. solanacearum* involving flagella found that motility varies with cell density (Clough et al. 1997b; Kelman and Hruschka 1973), and that flagella of nonmotile and motile bacteria had different conformations (Kelman and Hruschka 1973). Pili were also noted on both nonmotile and motile cells, and did not appear to be visibly different (Stemmer and Sequeira 1987). The motility loss observed in *pehSR* mutants was most probably associated with flagella. Interestingly, flagella are also important in the early stages of colonization by *P. aeruginosa* (Drake and Montie 1988) but later expression interferes with disease development. There is insufficient evidence at present to speculate about the role of *pehSR* in pilin or flagellin production. However, it is tempting to note the parallel between the active repression of *pehSR* during full disease and inhibition of flagella in *P. aeruginosa* pathogenesis. In *Salmonella typhimurium,* genes important for one stage of pathogenicity have been shown to be antagonistic to virulence at other stages, and are therefore repressed at those times (Miller and Mekalanos 1990). Placing *pehSR* under the control of a constitutive promoter would reveal any significant effect its continuous expression may have on virulence, and if virulence should be attenuated in a *pehSR*^{C} strain, then constitutive expression of the individual genes that *pehSR* controls may reveal which product, if any, interferes with disease.

The study of genes involved in the initial stages of pathogenicity can be invaluable in the development of effective preventative control measures for any pathogenic organism. However, it is of particular significance in *R. solanacearum,* because recent widespread outbreaks of brown rot in potatoes have been attributed to latent infection of contaminated seed. Study of *pehSR* and the genes it regulates may shed some light on the factors that determine successful colonization of a plant by *R. solanacearum* and perhaps open up new avenues for disease control.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Strains and plasmids together with relevant characteristics are listed in Table 3.

Culture media and growth conditions.

R. solanacearum strains were cultured at 28°C in CPG broth or on CPG plates containing 0.05% 2,3,5-triphenyltetrazolium chloride (Hendrick and Sequeira 1984). Boucher's minimal medium (BMM) supplemented with 0.1% citric acid and 0.1% galacturonic acid was used as a defined medium (Boucher et al. 1985). Cultures were grown in BMM for virulence assay inoculation, RNA purification for Northern blot analysis, and reporter gene expression studies, except those conducted in planta. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (LB) (Ausubel et al. 1987). The following antibiotics were used as required: ampicillin, 50 µg/ml; chloramphenicol, 25 µg/ml; kanamycin, 25 µg/ml; nalidixic acid, 75 µg/ml; streptomycin, 50 µg/ml; and tetracycline, 25 µg/ml.

Chemicals.

Growth media components were purchased from Difco Laboratories (Detroit, MI). Citrus pectin was from Fluka Laboratories (Biochemika, Switzerland). Restriction and modification enzymes were from Promega (Madison, WI). T1 RNAse was from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Table 3. Bacterial strains (*Ralstonia solanacearum)* and plasmids used in this study

Bacterial strains or plasmids	Relevant characteristics[a]	Reference
Strains		
K60	Wild-type race 1, biovar 1, isolated from tomato	Kelman 1954
K60-06	*pehA*::Ω Sm^r	Allen et al. 1991
K12, K29, K30, K35, K63, K92, K116, K176, K182, K183	*pehS*::Tn*3-gus* Km^r	This study; see Figure 2 for insertion locations
K7, K13, K17, K21, K42, K45, K47, K51, K53, K71, K75, K81	*pehR*::Tn*3-gus* Km^r	This study; see Figure 2 for insertion locations
K60-*phcA*	*phcA*::Ω Sm^r	This study
P7	*phcA*::Ω, *pehR*::Tn*3-gus* 7 Km^r Sm^r	This study
P35	*phcA*::Ω, *pehS::*Tn*3-gus* 35 Km^r Sm^r	This study
P71	*phcA*::Ω, *pehR*::Tn*3-gus* 71 Km^r Sm^r	This study
KS5	Spontaneously avirulent mutant	This study
Plasmids		
pLAFR3	Inc P RK2-derived cosmid vector; Tc^r	Staskawicz et al. 1986
pKH19	4.5-kb *Eco*RI fragment containing *pehSR* in pLAFR3; Tc^r	Allen et al. 1991

[a] Nal, nalidixic acid; Ap, ampicillin; Tc, tetracycline; Km, kanamycin; Sm, streptomycin.

Random primer labeling kits were purchased from Bethesda Research Laboratories (Gaithersburg, MD) and used according to the supplier's directions. Electrophoresis chemicals and nylon transfer membranes were from Bio-Rad Laboratories (Richmond, CA). Radio nucleotides were from DuPont-NEN (Boston), and custom-made primers were from the University of Wisconsin Biotechnology Center. Sequenase DNA sequencing kits were from USB (Cleveland, OH). Other chemicals were from Sigma Chemical (St. Louis, MO).

General procedures for DNA manipulation.

Plasmid DNA isolation, agarose gel electrophoresis, transformation of *E. coli* strains, and Southern and Northern blot hybridization were performed as previously described (Ausubel et al. 1987). Chromosomal DNA was isolated from *R. solanacearum* as previously described (Cook et al. 1989). *R. solanacearum* strains were transformed by electroporation as previously described (Allen et al. 1991). Transposon mutagenesis of the 4.5-kb *Eco*RI fragment harboring the *pehSR* locus was performed with the Tn*3-gus* transposon (Bonas et al. 1989) according to the procedure of Stachel (Stachel et al. 1985). This construct contains a promoterless β-glucuronidase gene and encodes kanamycin resistance. Northern blots were probed with a ^{32}P-labeled, 2.4-kb *Eco*RI-*Cla*I fragment of *pehA* (Allen et al. 1991).

Complementation analysis.

Mutant strains were transformed with cosmid pKH19, carrying the wild-type *pehSR* locus, or with selected Tn*3-gus* derivatives. Complementation was defined as the restoration of the wild-type level of *endo-* and *exo*-PG activity.

Enzyme assays.

β-glucuronidase activity was measured fluorimetrically by a modification of Jefferson's procedure (Cook and Sequeira 1991; Jefferson 1987). For in vitro expression studies, Tn*3-gus* mutant strains were grown in BMM for 3 days. In planta β-glucuronidase activity assays were conducted with filtered homogenates of tobacco leaves (cv. Bottom Special) that had been syringe-infiltrated 18 h previously with a 5×10^8 CFU/ml suspension of washed bacteria grown in BMM. Samples were sonicated and assayed for β-glucuronidase activity. Culture and homogenized leaf samples were also dilution plated to determine β-glucuronidase activity per CFU, expressed as nmoles of 4-methyl umbelliferone produced per minute per CFU. Polygalacturonase activity was measured by concentrating cleared supernatants from cultures grown 4 days in defined medium 50-fold in Centricon-30 microconcentrators (Amicon, LOCATION). *endo*-PG activity was measured as loss of viscosity of a polygalacturonate substrate solution (Keen et al. 1984). *exo*-PG activity was measured as reducing sugar ends generated (Nelson 1944).

Virulence assays.

These were performed by root inoculating 3-week-old eggplants (cv. Black Beauty) grown in 80 g dry weight of Jiffy Mix. Roots were severed by cutting vertically down across the pot, 1 cm away from the stem. Then, 50 ml of a water suspension of washed bacteria was poured over the soil to give a final bacterial concentration of 5×10^5 CFU per g of soil. Pot labels were coded so that the person scoring was unaware of the treatment identity. Plants were inspected daily for signs of wilting and rated on a 0 to 4 disease index scale in which 0 = no wilting, 1 = 1 to 25%, 2 = 26 to 50%, and 3 = 51 to 75% of leaves wilted, and 4 = 76 to 100% wilted or dead. Each treatment contained 16 plants and each assay was performed in triplicate. Results were analyzed with analysis of variance and Tukey's honestly significant differences test.

In planta growth.

In planta growth of *R. solanacearum* mutants was measured by infiltrating tobacco leaves with bacterial suspensions as previously described (Sequeira and Hill 1974).

DNA sequencing.

Sequencing of both strands was performed by the dideoxy-chain termination method. Deaza-GTP was used to resolve compressions. Computer analyses were carried out with the Genetics Computer Group (GCG) software package (Devereux et al. 1984).

Strain motility.

Motility was measured qualitatively by a stab assay; cultures were inoculated by stabbing a needle 4 cm into a tube containing 10 ml of CPG medium plus 0.3% wt/vol agar. Presence or absence of motility was rated at 48 h. Strain names were encoded so that the person rating motility was unaware of the treatment identity.

Construction of a K60 *phcA* mutant.

A cloned *phcA* structural gene interrupted by an Ω fragment containing a streptomycin resistance gene (a gift from Mark Schell, University of Georgia) was electroporated into wild-type strain K60 and *pehR*::Tn*3-gus* mutant strain K71. Genomic DNA from strep-resistant transformants with non-mucoid colony morphology was analyzed by Southern blot to confirm allelic replacement of the wild-type *phcA* locus.

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