Role of the Nucleoid-Associated Protein H-NS in the Synthesis of Virulence Factors in the Phytopathogenic Bacterium *Erwinia chrysanthemi*

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The ability of the enterobacterium *Erwinia chrysanthemi* to induce pathogenesis in plant tissue is strongly related to the massive production of plant-cell-wall-degrading enzymes (pectinases, cellulases, and proteases). Additional factors, including flagellar proteins and exopolysaccharides (EPS), also are required for the efficient colonization of plants. Production of these virulence factors, particularly pectate lyases, the main virulence determinant, is tightly regulated by environmental conditions. The possible involvement of the protein H-NS in this process was investigated. The *E. chrysanthemi hns* gene was cloned by complementation of an *Escherichia coli hns* mutation. Its nucleotide sequence contains a 405-bp open reading frame that codes for a protein with 85% identity to the *E. coli* H-NS protein. An *E. chrysanthemi hns* mutant was constructed by reverse genetics. This mutant displays a reduced growth rate and motility but an increased EPS synthesis and sensitivity toward high osmolarity. Furthermore, pectate lyase production is dramatically reduced in this mutant. The *hns* mutation acts on at least two conditions affecting pectate lyase synthesis: induction of pectate lyase synthesis at low temperatures (25°C) is no longer observed in the *hns* mutant and induction of pectate lyase production occurs in the late stationary growth phase in the *hns* background, instead of in the late exponential growth phase as it does in the parental strain. Moreover, the *E. chrysanthemi hns* mutant displays reduced virulence on plants. Taken together, these data suggest that H-NS plays a crucial role in the expression of the virulence genes and in the pathogenicity of *E. chrysanthemi*.

Additional keyword: regulation.

H-NS is a small DNA-binding protein that belongs to the class of nucleoid-associated proteins of enterobacteria. This protein is involved in the regulation, primarily at the transcriptional level, of a variety of unrelated genes in *Es-*

cherichia coli and *Salmonella typhimurium* (Atlung and Ingmer 1997; Ussery et al. 1994) and acts as a modulator of virulence-associated gene expression in the human enteropathogenic *Shigella* (Maurelli and Sansonetti 1988), *Salmonella* (Harrison et al. 1994), and enteroinvasive *E. coli* strains (Colonna et al. 1995). Most of the H-NS target genes are regulated by environmental signals such as osmolarity, temperature, growth phase, and pH (Atlung and Ingmer 1997). Several investigations, mostly performed in *E. coli* and *S. typhimurium*, have shown that the *hns* gene is autoregulated (Falconi et al. 1993; Ueguchi et al. 1993) and its nucleoid-associated expression increases several-fold following cold shock (LaTeana et al. 1991).

The gene encoding H-NS or structurally and functionally related proteins has been identified in other gram-negative bacteria such as *Haemophilus influenzae* (Dorman et al. 1999; Fleischmann et al. 1995; Hinton et al. 1992;), *Bordetella pertussis* (Goyard and Bertin 1997), *Vibrio cholerae*, and *Rhodobacter capsulatus* (Bertin et al. 1999). Moreover, a gene with C-terminal homology to H-NS has been found in the plant pathogen *Xanthomonas oryzae* (EMBL X97866) (Dorman et al. 1999). In these bacteria, the H-NS function remains unclear, mostly because of the difficulty of obtaining mutants with an inactivated *hns*-like gene. As a result of indirect studies (e.g., complementation of various *E. coli* H-NS-associated phenotypes and in vitro band-shift assays), it has been suggested that these proteins display transcriptional regulatory functions similar to the *E. coli* H-NS (Bertin et al. 1999). Concerning plant pathogenic bacteria, in the absence of any relevant investigation, the role of H-NS remains unclear, particularly in regard to its implication in the modulation of the expression of virulence genes.

The phytopathogenicity of the soft-rot enterobacteria of the genus *Erwinia* is mainly correlated with their ability to produce and secrete plant cell wall degrading enzymes (pectinases, cellulase, proteases). Among these extracellular enzymes, pectate lyases (Pel) play a predominant role in plant tissue maceration (Collmer and Keen 1986; Hugouvieux-Cotte-Pattat et al. 1996). Nevertheless, plant colonization by pectinolytic *Erwinia* is a multifactorial process, requiring numerous secondary factors such as exopolysaccharides (EPS) (Condemine et al. 1999), lipopolysaccharide (LPS) (Schoonejans et al. 1987), iron assimilation (Enard et al.

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1988), the Hrp system (Bauer et al. 1994), motility (Mulholland et al. 1994), and proteins involved in resistance against plant defense mechanisms (El Hassouni et al. 1999; Lopez-Solanilla et al. 1998).

In *Erwinia* spp., production of pectate lyases, the main virulence determinant, is tightly regulated and responds to various physiological controls, including growth phase-dependent induction, catabolic repression, and variations in environmental conditions such as the presence of pectin and plant extracts, iron starvation, temperature, or high osmolarity (Hugouvieux-Cotte-Pattat et al. 1996). Previous studies allowed the identification of several regulators (KdgR, PecS, PecT, Fur, ExpI-ExpR, Pir, and CRP) that modulate the synthesis of pectate lyases (Franza et al. 1999; Nasser et al. 1998; Nomura et al. 1998; Praillet et al. 1996; Reverchon et al. 1991; Reverchon et al. 1994; Reverchon et al. 1997; Surget et al. 1996). KdgR, PecS, PecT, and Fur are repressors, whereas CRP, Pir, and ExpR mainly act as activators on *pel* expression. Most of these systems are not limited to the control of pectate lyase production but also affect other factors that are, or could be, involved in virulence such as protein secretion, motility, pigment, cellulase and protease production, EPS synthesis, and iron capture. Signals transduced by these regulators are diverse and not always identified. Induction of *pel* gene expression by KDG, an inducer derived from pectin catabolism, is mediated by KdgR (Nasser et al. 1994; Reverchon et al. 1991). Among environmental factors affecting the synthesis of pectate lyase, plant signals other than KDG are important. The chemical nature of the additional inducing factor(s) present in plant extracts, however, is not yet known. Preliminary characterization of one inducing factor from carrot root suggested that it could be an oligosaccharide (Bourson et al. 1993). This plant factor induces the expression of pectinase genes only in synergy with pectic compounds, and the induction by plant extracts is mediated by the Pir activator (Nomura et al. 1998). Induction of pectate lyase production by iron starvation is mediated by Fur, which also controls chrysobactin- and achromobactin-dependent iron transport systems (Franza et al. 1999). Catabolite repression is mediated by the cyclic AMP (cAMP) receptor protein-cAMP complex, which is the main activator of *pel* genes represses EPS production (Condemine et al. 1999; Nasser et al. 1997; Reverchon et al. 1997). ExpR is a quorum-sensing regulator that modulates *pel* gene expression in response to *N*-acyl-homoserine lactones generated by the ExpI synthase (Nasser et al. 1998; Reverchon et al. 1998). Finally, the signals to which the PecS and PecT regulators respond are not yet known. In addition to the *pel* genes, PecS controls cellulase, protease, and indigoidine production (Reverchon et al. 1994), whereas PecT modulates motility and EPS synthesis (Castillo and Reverchon 1997; Condemine et al. 1999). Despite the identification of numerous regulatory proteins, the mechanisms responsible for regulation of *Erwinia* spp. virulence genes by various critical environmental factors such as osmolarity, temperature, or growth phase remain unclear.

To gain further insight into the regulation of virulence gene expression in *E. chrysanthemi*, we looked for the role of the nucleoid-associated protein H-NS in this process. Here we report the cloning of the *E. chrysanthemi hns* gene and the characterization of the corresponding mutant. Interestingly, this mutant displays reduced pectate lyase activity and an attenuated pathogenicity.

RESULTS

Cloning and sequencing of the *E. chrysanthemi hns* homolog.

In *E. coli*, mutations in the *hns* gene abolish motility but confer the ability to catabolize salicin (Bertin et al. 1999; Defez and De Felice 1981). The *E. chrysanthemi hns* gene was cloned by analysis of salicin catabolism in an *E. coli hns* mutant (TP504Δ*hns*) transformed with an *E. chrysanthemi* gene library constructed in pBR322. Out of 5,000 transformants, three plasmids were isolated because they deprived the corresponding clones of the ability to catabolize salicin. They also were able to restore swarming of the *hns* mutant. We first focused on one of these plasmids, pWN3094, which contained a 4.2-kb insert (Fig. 1). Subcloning revealed that the smallest subclone conferring complementation of the *hns* defects contained a 1.1-kb *ClaI*–*HindIII* fragment (Fig. 1). This fragment, used as a probe in hybridization experiments, showed that the two other selected plasmids contain the same DNA fragment. Identification of a single 6.6-kb *SalI* hybridizing fragment in the chromosomal DNA of *E. chrysanthemi* indicated that a single copy of the corresponding gene is present in the 3937 genome (data not shown).

The nucleotide sequence of the 1.1-kb *ClaI*–*HindIII* fragment containing the *E. chrysanthemi hns* gene was determined (GenBank accession no. X89444). An open reading frame (ORF) of 405 bp, extending from position 217 to 621 and showing similarity to the *E. coli hns* gene, was identified. The ATG codon is preceded by a putative Shine-Dalgarno sequence, GAGA. Computer analysis indicated that the presence of a putative σ^{70} -type promoter –35 (TTGCAG) and –10 (TATTAT) were located 42- and 59-bp upstream from the translation start codon, respectively. At the 3' end of the *hns* gene, an inverted repeat may form a 18-bp hairpin that could act as a rho-independent transcription terminator. Moreover, downstream of the *hns* gene and convergently transcribed, the 3' end of a partial ORF corresponding to the *galU* gene was detected. By contrast, this complex gene is transcribed in the opposite direction of *hns*, in accordance with the organization in *E. coli* and *S. typhimurium*.

The H-NS ORF encodes a 135 amino acid (aa) protein exhibiting high relatedness with H-NS proteins from *Serratia marcescens* (91% aa identity), *E. coli* and *Salmonella typhimurium* (85%), *Shigella flexneri* (83%), *Proteus vulgaris* (80%), and *Haemophilus influenzae* (48%) (Atlung and Ingmer 1997), with StpA proteins from *E. coli* (58%) and *S. typhimurium* (54%) (Zhang and Belfort 1992) and the Vich protein from *Vibrio cholerae* (55%). A multiple sequence alignment of H-NS bacterial homologs, including the *E. chrysanthemi* protein, performed by Dorman et al. (1999) revealed a high conservation within the three functional domains (oligomerization, linker, and nucleic acid binding domains).

Construction and phenotypes of an *E. chrysanthemi hns* mutant.

To study the function of H-NS in *E. chrysanthemi*, the *hns* gene was inactivated by insertion of a kanamycin resistance (Kan^r) cartridge into the unique *Afl*III site located at the beginning of the coding region. Attempts to recombine this *hns::Kan* insertion into the *E. chrysanthemi* chromosome were

unsuccessful. This difficulty was overcome by performing the homologous recombination in a merodiploid strain harboring the native *hns* gene on a low-copy number plasmid pRK767. After successful integration of the *hns*::Kan construct into the chromosome, the pRK-*hns* plasmid was eliminated by successive cultures without antibiotic, and mutants were analyzed by Southern blotting with the 1.1-kb *Hind*III-*Cla*I fragment of plasmid pWN3096 as the probe. We retained mutants in which the *Sal*I hybridizing fragment was shifted from 6.6 to 8 kb as expected (data not shown).

The *E. chrysanthemi* *hns* mutant displays a mucoid phenotype on agar plates (suggesting an overproduction of EPS in this mutant), is nonmotile on semisolid medium (Fig. 2A), has a reduced growth rate (Fig. 3B), and is sensitive toward high osmolarity (0.3 M NaCl) (data not shown) compared with the wild-type strain. Such phenotypes are similar to those reported for *E. coli* and *S. typhimurium* *hns* mutants (Atlung and Ingmer 1997; Barth et al. 1995; Bertin et al. 1999; Hinton et al. 1992; Sledjeski and Gottesman 1995). In contrast with *E. coli*, wild-type *E. chrysanthemi* can already catabolize β -glucosides

(Barras et al. 1989). With regard to extracellular enzyme production, plate assays revealed that pectate lyase activity was reduced, whereas cellulase activity was increased in the *hns* mutant compared with the parental strain. All the phenotypes associated with the *hns* mutation were successfully complemented by the presence of pRK767-*hns* plasmid (data not shown), demonstrating that these phenotypes are specifically linked to H-NS.

The *hns* locus was localized on the *E. chrysanthemi* genetic map by chromosomal mobilization mediated by the plasmid pULB110. The *hns*::Kan^r mutation appeared to be located between the markers *trp-1* and *his-1* with cotransfer frequencies of 62 and 8%, respectively. This localization is similar to the *hns* position in the *E. coli* map. The *hns* mutation could be transduced with phage phiEC2 into various genetic backgrounds of *E. chrysanthemi*, although the transduction efficiency was very low in comparison with other markers (data not shown). As for the *crp* mutant, such reduced phiEC2 sensitivity is probably related to the overproduction of EPS that masks the LPS-receptor access to phiEC2 phages.

Fig. 1. Physical map of the 4.2-kb *Sau*3A DNA fragment containing the *Erwinia chrysanthemi* *hns* gene. Localization of the *hns* gene on plasmid pWN3094 was determined by complementation of the *Escherichia coli* *hns* mutant TP504 with the use of deletion derivatives such as pWN3095 and pWN3096. The *Afl*III insertion site of the Kan^r cartridge is shown by an arrowhead.

Transcription of various genes in the *E. chrysanthemi* *hns* mutant.

In *E. coli*, one of the best studied systems whose transcription is strongly affected by H-NS is the osmotically controlled *proU* operon (*proVWX*) that encodes a transport system for the osmoprotectant glycine betaine (Dattananda and Gowrishankar 1989). To determine whether *proU* also is an H-NS target in *E. chrysanthemi*, we screened a genomic library by hybridization with the *E. coli* *proX* gene as probe. One selected plasmid, pSR2489, contains an 8.8-kb insert, including the *proV-proW-proX* genes, as established by partial sequencing of this insert. To monitor *proU* expression, we constructed a genetic fusion by introducing a *uidA*-Cm^r cassette into the unique *MamI* site localized inside *proV*. This *proV::uidA* fusion was then introduced by recombination into the *E. chrysanthemi* genome. Expression of the *proV::uidA* fusion was

analyzed in wild-type and *hns* backgrounds (Fig. 2B). As observed in *E. coli* and *S. typhimurium* (Higgins et al. 1988), inactivation of the *E. chrysanthemi* *hns* gene results in a strong increase in *proV* expression demonstrating that *proU* also is an H-NS target in *E. chrysanthemi*. The extent of induction of *proU* activity by osmotic pressure was studied by measuring β -glucuronidase activity from *proV::uidA* operon fusion in the presence of 0.3 M NaCl. In strain 3779, expression of the *proV::uidA* fusion increased approximately 50-fold in the high-osmolarity medium (Fig. 2B). Supplementation of the high-osmolarity medium with 1 mM glycine betaine repressed *proV::uidA* expression to 25% of the salt-induced activity (Fig. 2B). Although strain A3779 lacks the ProU glycine betaine transport system, it can still utilize glycine betaine as an osmoprotectant, presumably through the low-affinity transport system OusA, which is homologous to the *E. coli* ProP system

Fig. 2. Behavior of the *Erwinia chrysanthemi* *hns* mutant. **A**, Motility assay on semisolid medium. **B**, Expression of the chromosomal *proV::uidA*, *pelE::lacZ*, and *eps::lacZ* fusions in H-NS⁺ and H-NS⁻ strains. Open, gray and black bars = for *proV::uidA*, data obtained in minimal M63 medium two-fold diluted with glycerol as carbon source in the absence and presence of NaCl 0.3M and in the presence of NaCl 0.3 M and glycine betaine 1 mM, respectively; for *pelE::lacZ*, data obtained in minimal M63 medium with glycerol as carbon source in the absence and presence of polygalacturonate, respectively. Bacteria were grown at 30°C in minimal M63-glycerol medium, except for *proV::uidA* fusion strains, which were grown in twofold diluted M63-glycerol medium. Results are representative of three independent experiments. Standard deviations were less than 15%. **C**, Representative examples of Northern blot analysis of *celZ* mRNA in H-NS⁺ and H-NS⁻ strains. *kdgR* RNA is shown as a control. **D**, Electrofocusing of pectate lyases produced by the *E. chrysanthemi* A350 (parental strain) and A3379 (*hns*::Kan). Strains were grown at 30°C in M63 glycerol minimal medium. Pectate lyase activity was revealed by the sandwich technique, as described (this paper).

(Gouesbet et al. 1996). In the *hns* background, the basal transcription is strongly increased, but it is still inducible by high osmolarity. This pattern of *proV* expression in *E. chrysanthemi* is similar to that observed in *E. coli* and *S. typhimurium* (Barron et al. 1986; Lucht et al. 1994).

To determine whether the reduced pectate lyase production and the increased cellulase and EPS production observed in

the *hns* background are caused by transcriptional control of H-NS on these virulence factors, we analyzed expression of the *eps* operon and *pelE* and *celZ* genes with chromosomal gene fusions for *eps* and *pelE* genes and RNA analysis for the *celZ* gene. The *celZ* mRNA increased at least fivefold in the H-NS⁻ strain, whereas the *kdgR* transcript was not noticeably affected by the *hns* inactivation (Fig. 2C). Furthermore, the *eps* expres-

Fig. 3. Effect of *hns* mutation on the modulation of pectate lyase synthesis by temperature and growth phase. **A**, Bacteria were grown in minimal M63-glycerol-polygalacturonate medium at various temperatures. Pectate lyase activities were assayed at the end of the exponential growth phase. **B**, Bacteria were grown in minimal M63-glycerol-polygalacturonate medium at 30°C. Pectate lyase activity was monitored during 24 h of growth. White squares = values of OD_{600nm}; black diamonds = pectate lyase-specific activities. Results are representative of three independent experiments; standard deviations were less than 15%.

sion increased threefold in the *hns* mutant (Fig. 2B). In contrast, *pelE* expression decreased three- and sixfold in noninduced and induced conditions, respectively (Fig. 2B). These results confirm that H-NS acts as a modulator of virulence-associated gene expression in *E. chrysanthemi*.

Pectate lyase production in the *hns* mutant.

The *E. chrysanthemi hns* mutant was tested for pectate lyase production after growth under induced and noninduced conditions. In this genetic background, pectate lyase activity was reduced by 9- and 22-fold in the absence and presence of polygalacturonate, respectively (data not shown). Zymogram analysis after isoelectrofocusing revealed that the synthesis of the five major pectate lyases (PelA, B, C, D, and E) is equally affected (Fig. 3D). Pectate lyase synthesis is regulated by many environmental conditions. We compared pectate lyase production in the wild-type strain and the *hns* mutant under conditions in which H-NS may play a role in *E. coli*, *Salmonella*, and *Shigella* (osmolarity, temperature, and growth phase) (Atlung and Ingmer 1997). Pectate lyase regulation by osmolarity was not affected in the *hns* mutant, indicating that H-NS is not involved in this form of control (data not shown). In contrast, induction of pectate lyase synthesis at a low temperature (25°C) was not observed in the *hns* mutant (Fig. 3A). Furthermore, in the *hns* background, induction of pectate lyase production occurs in the early stationary phase instead of in the late exponential growth phase as it did for the parental strain (Fig. 3B). Thus, H-NS seems to be involved in the modulation of the pectate lyase synthesis by temperature and growth phase.

Plant tissue maceration and pathogenicity on *Saintpaulia ionantha* plants.

The tissue-macerating ability of the *hns* mutant was compared with that of the wild-type strain 3937 by inoculation of chicory leaves. The macerating capacity of the *hns* mutant was strongly reduced in this plant organ (Fig. 4). Bacterial multiplication was monitored by estimation of the bacterial concentration in the rotted tissue 2 days after inoculation. The *hns* mutant displays at least a twofold reduced growth compared with the parental strain (1.3 versus 2.1×10^{10} CFU/g of rotted tissue, as calculated from values obtained in five independent experiments).

The pathogenic behavior of the *hns* mutant was compared with that of the parental strain 3937 after inoculation of potted *S. ionantha* plants. In all plants, inoculation of strain 3937 resulted in maceration of the whole leaf after 3 days. The rotting symptoms continued to spread throughout the petiole and, after 6 days, maceration expanding to a second petiole was detected in 25% of the infected plants. In contrast, the *hns* mutant failed to induce any symptoms in 17% of the plants, whereas in other plants it gave rise to a slow maceration that remained confined to the inoculated leaf (Fig. 4). Thus, the *hns* mutant was clearly less efficient than the wild-type strain in initiating maceration and inducing systemic soft-rot symptoms.

DISCUSSION

Virulence factor synthesis in *E. chrysanthemi* is regulated by various environmental conditions. Some of these signals (e.g., temperature, osmolarity, and growth phase) are mediated

in *E. coli* and in related bacteria, *Salmonella* and *Shigella*, by the nucleoid-associated protein H-NS (Atlung and Ingmer 1997). Because in vivo random mutagenesis strategies in *E. chrysanthemi* were not effective in identifying the regulators responding to these environmental signals, we have initiated the cloning of the *E. chrysanthemi hns* gene to determine whether H-NS is involved in such control. The *E. chrysanthemi* H-NS protein shows a high identity, ranging from 85 to 91%, with its *E. coli*, *Salmonella*, *Shigella*, and *S. marcescens* counterparts. The failure to clone this gene on a multicopy plasmid (Hulton et al. 1990), together with the difficulty in constructing an *hns* mutant and transducing the corresponding mutation, suggest that H-NS has an important role in the cell physiology of *E. chrysanthemi*, as established for its *E. coli* homologue (Atlung and Ingmer 1997). The *E. chrysanthemi hns* mutant displays most of the phenotypes associated with the *E. coli hns* mutation, e.g., loss of motility, reduction in growth rate, mucoidy, sensitivity toward high osmolarity, and overproduction of the osmoprotectant glycine betaine transport system. Synthesis of the *E. chrysanthemi* H-NS protein in *E. coli* leads to the complementation of all these phenotypes and vice versa. These results suggest that H-NS molecular regulatory mechanisms governing these pathways are globally conserved between these two enterobacteria.

Regulation of the *E. chrysanthemi* genes involved in pathogenesis was analyzed more carefully. Inactivation of the *hns* gene results in a strong increase in cellulase and EPS production. With RNA analysis and genetic fusion, we clearly established that the H-NS protein exerts its control at the transcriptional level, demonstrating that H-NS acts as a repressor of cellulase and EPS synthesis. In *E. coli*, H-NS negatively controls the EPS production in a cascade manner via repression of the specific RcsA activator synthesis (Sledjeski and Gottesman 1995). The mechanism in *E. chrysanthemi* could be similar, in view of the parallel control exerted by the environmental factors on the EPS synthesis in these two bacteria (Condemine et al. 1999; Gottesman 1995). H-NS is the third repressor of the EPS synthesis identified in *E. chrysanthemi* in addition to PecT and CRP. Preliminary investigations suggest that the action of H-NS and PecT is cumulative (W. Nasser, unpublished data). These data reveal the complexity of EPS synthesis in *E. chrysanthemi*. The multiplicity of these negative regulations might help bacteria to quickly adapt EPS synthesis to changes in environmental conditions. The presence of EPS is particularly important for the survival of bacteria in dry conditions. In addition to PecS, H-NS is the second repressor controlling *celZ* expression. It remains to be determined whether H-NS exerts its control on the *celZ* gene by a direct action or by a regulatory cascade.

In contrast, inactivation of the *hns* gene results in a strong decrease in pectate lyase production, suggesting an unusual activator function for H-NS. Only a few examples of positive regulation by H-NS have been reported in *E. coli*, and it is generally admitted that in these cases it results from indirect mechanisms (Atlung and Ingmer 1997). The observation that when *pel* genes are cloned in *E. coli* their expression is increased 2-fold in an *E. coli hns* mutant compared with the parental strain (James and Hugouvieux-Cotte-Pattat 1996) suggests that the positive control exerted by H-NS on *pel* gene expression results from an indirect mechanism in *E. chrysanthemi*, involving regulator(s) that is (are) absent in *E. coli*.

Fig. 4. Representative examples of symptoms induced by *Erwinia chrysanthemi* 3937 and its H-NS⁻ derivative on **A**, chicory leaves and **B**, *Saintpaulia ionantha*. About 10^7 cells were injected into chicory leaves, which were then incubated in a moist chamber at 30°C for 24 h. Similar results were obtained on five different leaves. For *S. ionantha* infection, one leaf per plant was inoculated with 3×10^7 bacteria. Evolution of the pathogenesis was scored as follows: 1 = no maceration symptom; 2 = maceration localized at the inoculated area; 3 = maceration covering the inoculated leaf; 4 = maceration invading the petiole and other leaves.

It is possible that the H-NS action on pectate lyase synthesis recruits a repressor such as KdgR, PecS, PecT or is mediated via the RpoS–RsmA system. Indeed, recent data obtained in *E. carotovora*, a bacteria taxonomically related to *E. chrysanthemi*, indicate that RpoS activates the synthesis of the RsmA protein, which controls the expression of many genes, including *pel* genes (Mukherjee et al. 1998). In *E. coli*, H-NS controls RpoS synthesis (Atlung and Ingmer 1997). If these mechanisms are conserved in *E. chrysanthemi*, they could explain the effect of H-NS on pectate lyase synthesis. It is possible, however, that the H-NS action on *pel* gene expression may involve another regulator not yet identified. Further detailed work is required to determine the exact mechanism of H-NS regulation on *pel* gene expression. Finally, H-NS is involved in the regulation of pectate lyase synthesis by temperature, as previously established for the virulence factors of *S. typhimurium* and *S. flexneri* (Atlung and Ingmer 1997). By regulating the pectate lyase synthesis in response to at least two signals rather than simply temperature or growth phase diminishes the likelihood that the *pel* genes will be expressed in inappropriate conditions.

In summary, we have shown that the global functions of the *E. chrysanthemi* H-NS protein are similar to those of H-NS in *E. coli*. Our data, however, reveal important novel features such as the degree of plant virulence is reduced in an H-NS-deficient strain. This reduced virulence probably results from the decreased pectate lyase levels, one of the most important

virulence factors, but also could be a result of the motility loss and growth alteration of this strain. Further work will clarify the precise mechanisms by which H-NS controls the virulence genes expression, particularly those of *pel* genes, and should integrate this global regulator in the regulatory network controlling the synthesis of pectate lyases.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids are described in Table 1. *E. chrysanthemi* and *E. coli* cells were grown at 30 and 37°C, respectively, in Luria-Bertani (LB) medium or M63 minimal medium (Miller 1972) supplemented with carbon source (0.2%, except for polygalacturonate and pectin at 0.4%) and, when required, with 40 µg of amino acids per milliliter and antibiotics at the following concentrations per milliliter: ampicillin, 25 or 100 µg; kanamycin and chloramphenicol, 50 µg; and tetracycline 20 µg. Fermentation tests were performed on MacConkey agar plates containing 0.2% salicin. To test motility, equal quantities of bacteria were loaded into holes in the middle of 0.4% LB agar plates. Plates were checked between 12 and 24 h after inoculation. Motility was determined by measuring the diameter of the colony.

Genetic techniques.

Transductions with phage phiEC2 were performed as described (Réisibois et al. 1984). Marker exchange recombination

Table 1. Bacterial strains and plasmids

Strain	Genotype or description	Source or reference
<i>Escherichia coli</i>		
NM522	Δ(<i>lac-proAB</i>) <i>thi hsd5 supE</i> [<i>F'</i> <i>proAB</i> + <i>lacI^f</i> <i>lacZ</i> ΔM15]	Stratagene, La Jolla, CA, U.S.A.
TP500	<i>F-leuB6 serB1203 thi-1 zch506::Tn10 zdd230::Tn9</i>	Lejeune and Danchin 1990
TP504	<i>F-leuB6 serB1203 thi-1 zch506::Tn10 zdd230::Tn9 Δ(drc-drs-irk-hns)</i>	
<i>Erwinia chrysanthemi</i>		
3937	Wild-type strain isolated from <i>Saintpaulia ionantha</i>	Laboratory collection
A350	<i>lmrT^c lacZ2</i>	Hugouvieux-Cotte-Pattat and Robert-Baudouy 1985
A3379	<i>lmrT^c lacZ2 hns::Kan</i>	This work
A3380	<i>hns::Kan</i>	This work
A2575	<i>lmrT^c lacZ2 eps::Tn5-B21</i>	Condemine et al. 1999
A3382	<i>lmrT^c lacZ2 eps::Tn5-B21 hns::Kan</i>	This work
A3387	<i>lmrT^c lacZ2 pelE::Mulac Cm</i>	Laboratory collection
A3420	<i>lmrT^c lacZ2 pelE::Mulac Cm hns::Kan</i>	This work
A3779	<i>lmrT^c lacZ2 proV::uidA Cm</i>	This work
A3794	<i>lmrT^c lacZ2 proV::uidA Cm hns::Kan</i>	This work
Plasmids		
pBR322	Ap ^r Tc ^r	Laboratory collection
pSR1662	pBR322 with 2.4-kb <i>NruI</i> – <i>PvuII</i> fragment harboring the <i>E. chrysanthemi celZ</i> gene	Laboratory collection
pWN2144	pBS with 1.3-kb <i>PvuI</i> – <i>SnaBI</i> fragment harboring the <i>E. chrysanthemi kdgR</i> gene	Laboratory collection
pWN3094	pBR322 with 4.2-kb <i>Sau3A</i> fragment harboring the <i>E. chrysanthemi hns</i> gene	This work
pWN3095	pBR322 with 2.4-kb <i>ClaI</i> – <i>SphI</i> fragment harboring the <i>E. chrysanthemi hns</i> gene	This work
pWN3096	pBR322 with 1.1-kb <i>ClaI</i> – <i>HindIII</i> fragment harboring the <i>E. chrysanthemi hns</i> gene	This work
pRK767	Mobilizable vector, Tc ^r , RK2 derivative	Gill and Warren 1988
pWN3097	pRK767 derivative with 1.1-kb <i>ClaI</i> – <i>HindIII</i> fragment harboring the <i>E. chrysanthemi hns</i> gene	This work
pSR2489	pUC18 with an 8.8-kb <i>Sau3A</i> fragment harboring the <i>E. chrysanthemi proU</i> operon	This work
Phage		
PhiEC2	General transducing phage of <i>E. chrysanthemi</i>	Réisibois et al. 1984

^a Genotype symbols are according to Bachmann (1990). *lmrT^c* = transport system encoded by the gene *lmrT*, which mediates entry of lactose, melibiose, and raffinose into the cells, is constitutively expressed. *lacZ* indicates that the 3' end of this gene is truncated.

was obtained after growth in a low-phosphate-concentration medium as described (Roeder and Collmer 1985). The *hns::Kan^r* marker exchange was performed in a merodiploid strain harboring the native *hns* gene on a low-copy number plasmid pRK767. After successful integration of the *hns::Kan^r* construction into the chromosome, the pRK-*hns* plasmid was eliminated by successive cultures without antibiotic.

The *uidA-Cm* cassette (Bardonnet and Blanco 1992) includes a promoterless *uidA* gene with a conserved Shine-Dalgarno sequence. Insertion of this cassette in a gene in the correct orientation generates a transcriptional fusion.

Plasmid pSR2489 containing the *proU* operon was linearized with *MamI*, which is located in the coding *ProV* region, and ligated with the 3.8-kb *uidA-Cm* cassette obtained by *SmaI* digestion of pUIDC1. Ampicillin- and chloramphenicol-resistant clones were selected and checked for their production of β -glucuronidase. Restriction analysis of the plasmids confirmed the correct localization and orientation of the *uidA-Cm* cassette. One plasmid giving rise to a *proV::uidA* fusion was then introduced into *E. chrysanthemi*. Marker exchange recombination between the chromosomal allele and the plasmidborne mutated allele was selected after successive culture in low-phosphate medium in the presence of chloramphenicol. The A3779 strain containing the *proV::uidA* fusion was retained to study *ProV* regulation.

Plate tests and enzyme assays.

Clones producing pectate lyases were detected on medium containing polygalacturonate. After growth, plates were flooded with a solution of copper acetate (10%), which forms a blue complex with the polymer, leaving clear haloes around clones producing pectate lyases. Detection of cellulase activity was performed with Congo red as described (Teather and Wood 1982). Assays of pectate lyases, β -glucuronidase, and β -galactosidase were performed on toluenized cell extracts. Pectate lyase activity was assayed as described (Moran et al. 1968). β -Glucuronidase activity was measured by following the degradation of *p*-nitrophenyl- β -D-glucuronide as described (Bardonnet and Blanco 1992). β -Galactosidase activity was measured by following the degradation of *o*-nitrophenyl- β -D-galactoside as described (Miller 1972).

Recombinant DNA and RNA techniques.

Preparations of *E. chrysanthemi* chromosomal DNA, plasmid DNA, restriction digestions, ligations, DNA electrophoresis, transformations, and electroporations were carried out as described by Ausubel et al. (1987). Nucleotide sequence analysis was performed by the chain-termination method with a double-stranded DNA template and the use of a T7 sequencing kit (Pharmacia, Uppsala, Sweden). The resulting data were analyzed by the MAC Molly TETRA program (SoftGene, Berlin, Germany). The generation of the *E. chrysanthemi* gene library was performed as described (Reverchon et al. 1997).

The procedure for RNA isolation has been described previously (Reverchon et al. 1997). The concentration of RNA in different samples was detected spectrophotometrically, normalized for each sample, and verified on a reference gel with rRNA as the loading control. For Northern blot analysis, total RNAs were separated on formamide-formaldehyde denaturing 1% agarose gels (Ausubel et al. 1997). The 1,185-bp

MamI-*Bst*1107I fragment from pSR1662 was used as the *celZ* probe and the 800-bp *PstI*-*XhoI* of pWN2144 was used as the *kdgR* probe.

Analytical procedures.

Isoelectrofocusing was performed with a polyacrylamide gel containing a pharmalyte gradient (Pharmacia) from pH3 to pH10. An overlay with a PGA-CaCl₂-agarose gel and staining with 0.05% ruthenium red allowed for the specific detection of pectate lyase activity.

Pathogenicity tests.

Chicory leaf infections were performed as described by Reverchon et al. (1997). Five leaves were inoculated with each strain and incubated at 30°C in a dew chamber at 100% relative humidity. Disease severity was determined 2 days after inoculation. To determine bacterial multiplication, rotted tissue were collected and homogenized in M63 medium. Colony forming units were determined by plating appropriate dilutions on agar plates. Pathogenicity on 24 potted *S. ionantha* plants was assayed as described by Sauvage and Expert (1994) with minor modifications; overnight bacterial cultures grown in M63 medium containing glycerol as the carbon source were diluted to reach an optical density at 600 nm of 0.3. The inoculum was contained in 100 μ l of the resulting suspension (approximately 3×10^7 bacteria). Progression of *proV* symptoms was scored daily for 6 days.

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