The nucleoid-associated proteins H-NS and FIS modulate the DNA supercoiling response of the pel genes, the major virulence factors in the plant pathogen bacterium Dickeya dadantii

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ABSTRACT

Dickeya dadantii is a pathogen infecting a wide range of plant species. Soft rot, the visible symptom, is mainly due to the production of pectate lyases (Pels) that can destroy the plant cell walls. Previously we found that the pel gene expression is modulated by H-NS and FIS, two nucleoid-associated proteins (NAPs) modulating the DNA topology. Here, we show that relaxation of the DNA in growing D. dadantii cells decreases the expression of pel genes. Deletion of fis aggravates, whereas that of hns alleviates the negative impact of DNA relaxation on pel expression. We further show that H-NS and FIS directly bind the pelE promoter and that the response of D. dadantii pel genes to stresses that induce DNA relaxation is modulated, although to different extents, by H-NS and FIS. We infer that FIS acts as a repressor buffering the negative impact of DNA relaxation on pel gene transcription, whereas H-NS fine-tunes the response of virulence genes precluding their expression under suboptimal conditions of supercoiling. This novel dependence of H-NS effect on DNA topology expands our understanding of the role of NAPs in regulating the global bacterial gene expression and bacterial pathogenicity.

INTRODUCTION

Intricate communication and signalling networks evolved in unicellular organisms enable the sensing of variations in both the environmental conditions and also internal metabolic states, and thus to adjust accordingly the cellular physiology. In bacteria, regulation of gene expression is exerted primarily at the level of transcription initiation (1) using numerous transcription factors (TFs) whose concentrations and activities change depending on the external and internal conditions (2). In addition, bacterial DNA topology is also highly responsive to environmental conditions and changes of DNA superhelical density modulate the distribution of the transcription apparatus on the genome. The expression of the genome is thus controlled, in response to environmental conditions, by a global regulatory network involving DNA topology and the RNA polymerase (RNAP) with associated TFs (3–5).

Global bacterial regulators are represented by a relatively small number of DNA architectural factors influencing a large numbers of genes (1,6). Chief among these are the abundant nucleoid-associated-proteins (NAPs), which are thought to influence both the chromatin structure and transcription initiation (1,3,6). TFs with much more local effects on transcription then act upon the regulatory background imposed by NAPs (1,4). This combination of specific and global regulators thus mediates the precise activation or repression by sensing changes in environmental conditions or metabolic states (1,4).

An attractive model for exploring how bacteria integrate various regulatory mechanisms to control gene expression at the transcriptional level is the regulation of virulence factors. The pel genes of Dickeya dadantii (formerly Erwinia chrysanthemi) encode pectate lyases (Pels), essential for pathogenic growth of D. dadantii. Dickeya dadantii is described as a necrotrophic, Gram-negative plant pathogen that causes disease in a wide range of plant species, including many crops of

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economic importance such as vegetables and ornamentals and also the model plant Arabidopsis (7). Soft rot, the visible symptom, is mainly due to the production of degradative enzymes, mostly Pels, that will destroy the cell wall (8). Dickeya dadantii synthesizes multiple isoforms of Pels, including five major isoenzymes (PelA, B, C, D and E) (8) and several minor isoenzymes (PelI, PelL, PelN). Plant infection requires a massive and rapid production and secretion of Pels before the plant can establish its defense reactions. The initiation of pel gene expression is, therefore, a key step in triggering the virulence of D. dadantii (9,10).

Expression of the pel genes, is under the control of a complex regulation system. Several characterized regulators (KdgR, Pir, PecS, PecT, Fur, MfbR) control the synthesis of Pels in response to various signals, such as the presence of pectin (KdgR), oxidative stress (PecS), iron starvation (Fur) and acidic shock (MfbR) (11–16). In addition, it was shown that the sugar catabolism regulator complex aAMP-CRP acts via a direct mechanism as the main activator of the pel genes (17). Furthermore, two abundant NAPs, heat-stable nucleoid-structuring protein (H-NS) and factor for inversion stimulation (FIS), influence the expression of the pel genes (18–21).

H-NS is one of the major NAPs characterized in Gram-negative bacteria and regarded as a global modulator of gene expression in response to pH, temperature, osmolality and growth phase. H-NS also influences DNA supercoiling in vivo (22,23). DNA binding by H-NS is sensitive to environmental factors. It binds to high-affinity sites and spreads along the adjacent AT-rich DNA sequences to silence transcription (24,25). H-NS is thus often referred to as a ‘universal repressor’ or ‘modulator of environmentally regulated gene expression’ (6,26). Accordingly, it has been shown that H-NS controls virulence gene expression in a variety of pathogens including Escherichia coli, Salmonella, Shigella flexneri and Vibrio cholerae (18,27–31).

FIS is another NAP that was initially characterized as a stimulator of site-specific DNA recombination. FIS is a transcriptional activator of genes and operons associated with primary metabolism, including those encoding biosynthetic enzymes and stable RNAs (32). FIS is also required for oriC-directed DNA replication and influences the topological state of DNA in the cell by repressing DNA gyrase and stimulating topoisomerase I gene expression (33,34). FIS can also influence DNA topology directly by binding to DNA. In particular it acts to preserve intermediately supercoiled forms of DNA, protecting them from extreme shifts towards the more relaxed or more negatively supercoiled ends of the topological spectrum (35,36). FIS is thus considered as a local topological homeostat (3). The level of FIS in the cell is subject to complex and multifactorial control. FIS is synthesized in large amounts during the early exponential phase of cells grown in rich medium (37), decreases soon after, and becomes nearly undetectable as cells enter the stationary phase. Recently, FIS has been implicated in the regulation of virulence gene expression in pathogenic strains of E. coli (38), in Shigella flexneri (39), in Salmonella (40,41), in Vibrio cholerae (42) and in D. dadantii (21).

Previously, we showed that in D. dadantii, H-NS displays an unusual activator phenotype on the production of Pels since its absence leads to a drastic reduction of enzyme synthesis. The reduced synthesis of Pels in the hns mutant mainly results from a negative control, exerted by H-NS, on the transcription of the strong repressor gene pecT. Inactivation of H-NS results in an overproduction of PecT, which in turn reduce the transcription of pel genes by binding to their upstream region. In addition to its action on Pels production via PecT, H-NS also represses pel gene expression by binding to extended regions on these genes regulatory regions (19). Thus, the double hns-pecT mutant produces more Pels than the pecT mutant. We also showed that FIS is involved in the temporal regulation of the pel gene expression. In a fis mutant, the induction of the Pel activity is delayed and the Pel synthesis increases during the stationary growth phase (20). Given the fact that DNA supercoiling varies with growth (3) and also under conditions encountered by pathogenic bacteria during infection (43,44), in conjunction with observations that FIS and H-NS modulate the DNA topology, we explored the possibility of interplay between these NAPs and supercoiling in the control of D. dadantii pel gene expression under conditions mimicking the hostile environments encountered during infection. We show that binding of FIS buffers the negative effects of induced DNA relaxation, whereas binding of H-NS fine-tunes the response of virulence genes repressing their transcription at suboptimal superhelical density. We thus reveal interplay between FIS, H-NS and DNA topology in optimizing the response of D. dadantii virulence genes and also show, that the binding effect of H-NS is modulated by the supercoiling state of DNA.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture conditions and DNA manipulation techniques

Bacterial strains and plasmids are described in Supplementary Table S1. Dickeya dadantii and E. coli were grown at 30°C and 37°C, respectively. The rich medium Luria broth (L.B.) was used for growth of E. coli; M63 minimal salts medium supplemented with sucrose at 0.2% (w/v) as carbon source was used as basal liquid medium for all the experiments performed with D. dadantii. When required, the antibiotics were as follows: ampicillin (Ap), 100 µg/ml; kanamycin (Km), 50 µg/ml; chloramphenicol (Cm), 25 µg/ml; and tetracycline (Tet), 20 µg/ml. Liquid cultures were grown in a shaking incubator (220 r.p.m.). Media were solidified by the addition of 1.5% agar (w/v). The acidic pH shock was performed by shifting pH from 7.0 to 4.3 for 15 min by addition of 30 mM malic acid, since malate is naturally present in the plant apoplast (12). Cells were subjected to oxidative stress, by an incubation of 15 min in the presence of 500 µM H2O2. Norfloxacin was used at sublethal concentration (100 µg/ml) to inhibit DNA gyrase in D. dadantii for 15 min. At this concentration, novobiocin has no impact on D. dadantii growth. Norfloxacin was
used at a final concentration of 20 μg/ml to inhibit DNA gyrase in *E. coli* LZ41 for 10 min. The regulatory region of *pelE* (−196 to +131 relative to the transcription initiation +1 of *pelE*) was amplified by PCR using primers RRpelELeft2court and RRpelE right (Supplementary Table S1), containing unique restriction sites, so that the resulting fragment contained EcoRI and HindIII sites at the 5′- and 3′-end, respectively. The resulting 328 bp EcoRI–HindIII restriction fragment PCR product was cloned into the pNB4 (Supplementary Table S1) to generate pEH1. In this construction the *uidA* reporter gene is expressed under the *pelE* promoter.

**Separation of plasmid topoisomers by gel electrophoresis**

The multicopy plasmid pUC18 was extracted from *D. dadantii* or *E. coli* by using the Qiagaprep Spin Miniprep kit and ~1μg of plasmid DNA was electrophoresed on 1% agarose gel containing 2.5 μg/ml chloroquine. All electrophoresis was conducted in 20 cm long agarose gel with Tris–borate EDTA (TBE) as gel running buffer. The electrophoresis was run at 2.5 V/cm for 14–16 h. Under these conditions topoisomers that are more negatively supercoiled migrate faster in the gel than more relaxed topoisomers. Analysis of topoisomers distribution was performed as described by (45). Briefly, total plasmid DNA in a chloroquine gel lane was summed and then divided into quartiles, allowing us to plot the median and interquartile range of the whole population of topoisomers.

**Preparation of topoisomers distribution**

An amount of 20 μg of pHE1 plasmid was incubated with 40 units of Vaccinia topoisomerase in the presence of different ethidium bromide concentrations (20, 30, 40 and 80 ng/μg for σ of −0.015, −0.026, −0.036 and −0.054, respectively; no ethidium bromide was added in the reaction for σ of 0) in 10 mM Tris–HCl pH 8, 100 mM NaCl and 3 mM MgCl₂ for 2 h at 37°C. The reactions were then stopped by adding EDTA and SDS at a final concentration of 40 mM and 0.15 mM, respectively. A total of 40–50 μL of proteinase K (Roche) was then added to the reaction mixtures followed by an additional incubation of 20 min at 45°C. The DNA was phenol/chloroform and chloroform extracted and precipitated with ethanol. The plasmid with σ of −0.061 was extracted from *E. coli* cells growing in exponential phase.

**Determination of σ**

For the determination of σ the topoisomer ladder of pEH1 having an overlapping distribution pattern were prepared and analysed on high-resolution agarose gels with different chloroquine concentrations and compared with a ladder of plasmids with an overlapping topoisomer distribution. The mean σ-values of plasmids were quantified by the band-counting method (46). A σ of zero was taken as the mid-point of the topoisomer distribution after complete relaxation with Vaccinia topoisomerase. σ was calculated according to the formula: σ = ΔLK/LK₀, where LK₀ for pEH1 = 5500 bp /10.4 bp/ turn = 529. The SD for all σ is ±10–15%.

**Proteins and enzyme assays**

Assay of pectate lyase was performed on tolenuized cell extracts. Pectate lyase activity was determined by the degradation of PGA to unsaturated products that absorb at 235 nm. Specific activity is expressed as micromoles of unsaturated products liberated per minute per microgram (dry weight) bacteria. Bacterial concentration was estimated by measuring turbidity at 600 nm, given that an optical density (OD) of 1.0 at 600 nm corresponds to 10⁹ *D. dadantii* bacteria per millilitre and to 0.47 mg of bacteria (dry weight) per millilitre. Fis and H-NS were purified as previously described (19,21).

**DNA isolation, primer extension and quantitative reverse transcription polymerase chain reaction (RT–PCR) analysis**

Total RNA was extracted from *D. dadantii* and *E. coli* by the hot-phenol method. For RT–PCR analysis, cDNA was synthesized, using random hexamers and Fermentas reverse DNA polymerase, and qPCR was performed using the LightCycler® faststart DNA master plus SYBR Green I kit from Roche (Roche Applied Science), as previously described (20). Target gene expression was analysed using the ‘Relative Expression Software Tool’ (REST) (47).

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**In vitro transcription**

The reactions for the *in vitro* transcription experiments were performed as previously described (20). For this purpose topoisomers of different superhelical densities of the *pel* promoter constructs pEH1 (*pelE* promoter) were used. The reaction products were solubilized in water, divided into equal parts and then submitted to primer extension with radioactively end-labeled primers uidAdeb for the reporter *uidA* mRNAs and bla3B4 for the *bla* transcript (Supplementary Table S1). The extension with primers uidAdeb and bla3B4 yields 163-bp for *pelE* and 100-bp for *bla*. The amplification products were analysed on a 6% sequencing gel. The signals obtained were detected by autoradiography on Amersham MP film and quantified using ImageMaster TotalLab version 2.01 software (GE Healthcare). *Escherichia coli* σ²⁰ RNAP was obtained from Epicentre and the protein molarity was determined based on the concentration of the batches (microgram per microlitre).

**RESULTS**

The *pel* gene expression is sensitive to alterations in the superhelical density of DNA

Our previous studies have shown that induction of Pel synthesis occurred during transition from exponential growth to the stationary phase. Since this transition is accompanied by a change in DNA superhelicity in *E. coli* (49), we hypothesized that the DNA supercoiling might form part of the regulatory repertoire employed by
D. dandantii for optimizing Pel production during growth. To test this hypothesis, we monitored Pel production in D. dandantii under conditions of DNA relaxation by novobiocin, a coumarin drug inhibiting the generation of negative supercoils by DNA gyrase (50). Pel activity was monitored both during exponential growth and also during early stationary phase after adding novobiocin to cultures at concentrations, which did not affect cellular growth (Figure 1A). In parallel we used the plasmid pUC18 transformed in D. dandantii cells to monitor the effect of drug addition on DNA topology (Figure 1B). The distribution of plasmid topoisomers was analysed by high-resolution agarose gel electrophoresis and analysed by the method of (45) allowing to assess both the differences in average linking deficit and the degree of scatter in the topoisomer population. We found that addition of novobiocin led to relaxation of plasmid DNA and decreased Pel activity at both stages of growth, suggesting that DNA relaxation reduces Pel synthesis (Figure 1).

**Figure 1.** Effect of DNA gyrase inhibition and DNA relaxation on Pel production in D. dandantii. (A) Bacteria were treated with sublethal concentration of novobiocin (100 μg/ml) and samples were removed at the indicated times and submitted to Pel activity quantification. Each value represents the mean of three experiments. Bars indicate the SD. Fold changes (FCs) are expressed as the ratio of the specific Pel activity during exposure to novobiocin, compared with that in standard growth conditions. Two different scales were used for the y-axis in order to better appreciate variations of Pel activity in the different growth phases. (B) Topoisomers of plasmid pUC18 were isolated from wild-type and separated on agarose gel containing 2.5 μg/ml chloroquine. At this concentration of chloroquine, the more negatively supercoiled topoisomers migrate faster in the gel. The experiment was performed on three separate occasions and a typical result is shown. The growth phase considered and the novobiocin treatment are indicated. Plasmid topoisomers were analysed by densitometry using image J software and the obtained results were plotted in quartiles as described by (45); here the interquartile range (25th to 75th percentile) is indicated by a filled (0 μg/ml novobiocin) or empty (100 μg/ml novobiocin) box. The picture of the entire gel is shown on the right.
To test whether this reduced enzymatic activity was due to decreased Pel mRNA levels we evaluated the impact of novobiocin treatment on the pel gene expression by quantitative real-time PCR (qRT–PCR). We selected for analyses the pelB, pelC, pelE and pelD genes encoding four out of the five major Pels, and pelL, encoding a secondary Pel. Addition of novobiocin significantly decreased the amount of all pel transcripts during both exponential growth and early stationary phase, although to different extents, with pelE mRNA showing a maximal reduction (Figure 2). This finding indicated that reduction of Pel activity on DNA relaxation was due to decreased levels of pel mRNA. Under the same conditions the amount of gyrB mRNA was increased as expected, since the gyrB promoter is activated by DNA relaxation (51), whereas the amount of hemF mRNA used as a reference gene for qRT–PCR normalization (48), did not vary significantly (Figure 2). From these data we infer that relaxation of DNA by novobiocin reduces Pel activity by decreasing the levels of pel mRNA.

Impact of DNA relaxation on pelE promoter activity

To distinguish whether the reduction of pel mRNA levels was due to decreased pel mRNA stability or diminished pel gene expression, we employed the pEH1 construct carrying a transcriptional fusion of the promoter of the pelE gene, which showed maximal reduction of mRNA levels, with uidA reporter gene. This construct was used in the genetically engineered E. coli LZ41 strain containing a modified parC allele (parC<sup>K84</sup>) encoding the A subunit of DNA relaxing topoisomerase IV. This modification of topoisomerase IV confers resistance to quinolone inhibitor norfloxacin, whereas DNA gyrase activity is inhibited, such that addition of norfloxacin induces almost full relaxation of the DNA in LZ41 strain ($\sigma = -0.015$), which cannot be achieved by novobiocin treatment in D. dadantii. Without norfloxacin treatment this strain has a near physiological superhelical density [$\sigma = -0.07$; (52,53)]. After transformation of the pEH1 construct in LZ41 cells the produced uidA transcript was quantified under conditions of norfloxacin treatment during exponential growth and early stationary phase as described above. We found that addition of norfloxacin strongly reduced the amount of the reporter uidA mRNA, whereas that of gyrB was significantly increased (Figure 3), consistent with the results obtained in D. dadantii. Similar observations were made with the pelD promoter (data no shown). These findings indicate that decreased pel gene expression after DNA relaxation results from a reduced pel gene promoter activity.

Modulation of the impact of DNA relaxation by FIS and H-NS

Previously we have shown that the pelE promoter activity is regulated by binding of two abundant NAPs,
To study the crosstalk between these NAPs and supercoiling at the pelE promoter we transformed the pEH1 reporter into LZ41 mutant strains lacking either fis or hns. Quantification of uidA transcript by qRT–PCR showed that deletion of fis substantially increased, whereas deletion of hns alleviated the negative effect of DNA relaxation on pelE promoter activity during exponential growth (Figure 3). This effect of hns mutation was still observed during early stationary phase, whereas that of fis mutation was essentially lost since no significant difference of the impact of novobiocin treatment on the amount of the uidA mRNA was observed between the WT strain and the fis mutant at this growth stage. This growth phase-dependent effect of fis mutation can be explained by the absence of both, the FIS-dependent repression of pelE in exponential phase and subsequent derepression due to dramatic decline of FIS levels at the transition to stationary phase, whereas the H-NS levels vary little (21,37).

FIS and H-NS, implicated in the modulation of DNA topology in the cell (19,20). To investigate the crosstalk between these NAPs and supercoiling at the pelE promoter we transformed the pEH1 reporter into LZ41 mutant strains lacking either fis or hns. Quantification of uidA transcript by qRT–PCR showed that deletion of fis substantially increased, whereas deletion of hns alleviated the negative effect of DNA relaxation on pelE promoter activity during exponential growth (Figure 3). This effect of hns mutation was still observed during early stationary phase, whereas that of fis mutation was essentially lost since no significant difference of the impact of novobiocin treatment on the amount of the uidA mRNA was observed between the WT strain and the fis mutant at this growth stage. This growth phase-dependent effect of fis mutation can be explained by the absence of both, the FIS-dependent repression of pelE in exponential phase and subsequent derepression due to dramatic decline of FIS levels at the transition to stationary phase, whereas the H-NS levels vary little (21,37).

To verify these effects of NAPs we next explored the D. dadantii mutants lacking the fis and hns genes. Since deletion of these genes affects overall supercoiling level in E. coli (36) we also inspected the topology of pUC18 plasmids, which did not show significant differences between the wild-type and mutant D. dadantii strains. However, addition of novobiocin showed a stronger relaxation of pUC18 plasmid DNA in the fis mutant compared to wild-type during exponential growth, while this difference was lost during early stationary phase (Figure 4A). In D. dadantii hns mutant the relaxation of DNA in response to novobiocin addition was less pronounced compared to fis mutant at both growth stages (Figure 4A). Measurements of pelE transcript levels showed that in D. dadantii fis mutant the negative effect of DNA relaxation on pelE expression was augmented, while alleviated in cells lacking hns (Figure 4B), fully consistent with observations made in E. coli LZ41. Furthermore, the effect of fis mutation was again growth phase-dependent. From these observations we infer that in D. dadantii FIS and H-NS distinctly...
modulate the effect of DNA relaxation on pelE gene transcription.

The effect of DNA superhelicity and NAPs on pelE transcription in vitro

To test this assumption directly, we first investigated the dependence of the pelE promoter activity on DNA supercoiling in a purified in vitro system using the coupled in vitro transcription/primer extension assay previously described (54). For this purpose using the pelE promoter construct pEH1 we generated topoisomers of different superhelical density (Figure 5A). Since the pelE promoter activity is negligible in the absence of the activator cAMP–CRP complex in vitro (17) we carried out experiments both with RNAP alone, and also in the presence of the activator cAMP–CRP. In both cases, the transcriptional activity of the pelE promoter, as measured by primer extension, was minimal at low superhelical densities and steeply increased from $\sigma = 0.036$ to a maximum at high superhelical density of $\sigma = 0.061$ characteristic of plasmids isolated from exponentially growing cells (Figure 5B, upper panel and 5C). In contrast, the activity of the reference bla promoter located on the
same plasmid demonstrated much less variation with changing superhelical density (Figure 5B, bottom panel). We conclude that optimal pelE promoter activity requires high negative superhelical density of the DNA in vitro.

We next carried out similar experiments by adding purified FIS and H-NS proteins to the reactions both separately and also together. To ensure high transcription rates, the reactions were performed in the presence of CRP using pEH1 both at high ($\sigma/C_0 = 0.061$) and suboptimal superhelical density ($\sigma/C_0 = 0.036$), at which the promoter activity was strongly reduced. We observed that FIS repressed pelE transcription independently of the template topology (Figure 6A, B, C and D, lanes 1–3) whereby addition of H-NS did not significantly change this inhibitory effect of FIS (lanes 6–8). However, in the absence of FIS the effect of H-NS addition showed a clear dependence on DNA topology: at high superhelical density showing maximal pelE transcription addition of H-NS had a slight stimulatory effect (Figure 6A and B, compare lanes 4 and 5), whereas at lower superhelical density of $\sigma/C_0 = -0.036$, H-NS repressed pelE transcription (Figure 6C and D, compare lanes 4 and 5). These findings, together with our in vivo observations of pelE response in fis and hns mutants, strongly suggest that the transcriptional response to DNA relaxation is directly modulated by binding of FIS and H-NS to their cognate sites in the pelE promoter region (20).

**The NAPs are involved in modulation of pel gene expression in response to stress**

DNA supercoiling varies in response to changing environmental conditions encountered by bacteria in the time course of infection (44). Previously we found that acidic shock reduced the pel gene expression in growing D. dadantii cells (12). We investigated the impact of both the acidic and oxidative stresses on DNA topology and pelE expression in exponentially wild-type and mutant strains lacking FIS and H-NS. Although to different extent, both the acidic and oxidative shocks reduced the superhelical density of the DNA in wild-type and fis mutant cells, but had no significant effect in hns mutant (Figure 7A and B). Accordingly, the inhibitory effect on pelE expression was alleviated in hns mutant (Figure 7C.
and D), whereas the response of the fis mutant was weaker than that of the wild-type but stronger than that of hns mutant. Thus again, as in previous experiments with novobiocin treatment the negative effect of both shocks on pelE expression was alleviated in hns mutant (Figure 7C and D). Taken together our findings strongly suggest that the response of pel expression to DNA relaxation induced by the acidic and oxidative shocks is mainly modulated by H-NS.

DISCUSSION
Pathogenic bacteria have to cope with a variety of adverse conditions in the host environment, such as nutrient limitation, oxidative and acidic stresses. At the same time, these signals provide the information necessary for bacteria to adjust the expression of virulence factors. Most pathogenic bacteria, including D. dadantii, have evolved sophisticated systems to sense hostile environments and trigger compensatory gene expression in order to survive within the host (12,13). Successful infection by D. dadantii requires temporal coordination of gene expression, which in human pathogens is often associated with topological changes in the bacterial DNA modulated by global regulators of gene expression, the NAPs (41,55).

In this study we investigated the role of DNA topology and the abundant NAPs, FIS and H-NS, in modulating DNA topology and expression of the pel genes in the plant pathogen D. dadantii. We observed that the activity of Pels, important virulence factors of D. dadantii, is substantially decreased when the chromosomal DNA is relaxed by inhibiting the DNA gyrase activity (Figure 1). When under the same conditions we quantified the pel gene mRNA we found reduced transcript levels (Figure 2). We next used the pelE promoter-uidA reporter fusion in E. coli and found that on DNA relaxation the promoter activity is substantially decreased (Figure 3). Consequently, when we investigated the supercoiling dependence of the pelE promoter in vitro we found that DNA relaxation strongly reduced its activity (Figure 5). Furthermore, we demonstrated that the NAPs FIS and H-NS modulate the response of pelE promoter to supercoiling both in vivo (Figure 4) and in vitro (Figure 6 and Supplementary Figure S1). Finally, we showed that FIS and H-NS modulate the DNA relaxation and the transcriptional response of pelE gene induced by stress conditions encountered by D. dadantii in the course of plant infection (Figure 7). We thus reveal interplay between FIS, H-NS and DNA supercoiling in coordinating the transcriptional response of virulence genes of D. dadantii to environmental challenge.

The role of CRP
In contrast to highly abundant NAPs shaping the chromosome structure, CRP is an abundant protein thought to function more as a classical TF (56). CRP is also one of the regulators of virulence in D. dadantii and in all of our in vitro transcription studies we necessarily included CRP because in its absence the activity of Pels (17) and the transcription of pelE promoter (Figure 5B) are nearly...
undetectable. CRP binds at a classical activator site centred at -72.5 upstream of the pelE transcriptional start (20) and obviously is required to recruit the polymerase at the promoter. However, the in vitro response of the pelE promoter to supercoiling does not depend on the presence of CRP (Figure 5B). Therefore, we do not consider the ‘all or none’ effect of CRP on pelE transcription as relevant for the response to changes of supercoiling per se. However, the binding of CRP might be relevant regarding the modulatory effects of FIS or H-NS, especially because of the partial overlap of sequences protected by binding of CRP, FIS and H-NS in the pelE promoter region (20).

Interplay of supercoiling, FIS and H-NS

FIS and H-NS are involved in homeostatic control of DNA topology in the bacterial cell (36). FIS was shown to buffer the negative effects of suboptimal superhelical density on promoter activity both locally at the level of individual stable RNA promoters (57,58) and also globally, at the level of the entire bacterial chromosome.

In principle, FIS can exert both direct and indirect effects on DNA topology and transcription (5,59). However, on transition to stationary phase the FIS concentration drops to undetectable levels (37) and we observed that in D. dadantii, as well as in E. coli, deletion of fis augments the negative effect of DNA relaxation on pelE expression during exponential growth, when the FIS protein is normally abundant (Figures 3 and 4). The in vitro effect of FIS is apparently independent of DNA superhelicity and persists even in the presence of H-NS, suggesting that FIS tightly binds at the pelE promoter. The region protected by FIS overlaps the -35 hexamer of the pelE promoter (20) and contains putative consensus FIS-binding sequences (data not shown and Supplementary Figure S2), consistent with repression of pelE transcription by promoter occlusion. Since on DNA relaxation in the fis mutant the repression of pelE is augmented this suggests a buffering role for FIS on promoter activity at suboptimal superhelical density. A notable difference to the previously observed buffering effect of FIS on rrnAP1 promoter activity (57) is that at rrnAP1 (and
other stable RNA promoters) FIS acts as an activator, whereas at the pelE promoter FIS acts as a repressor. A corollary to this argument would be that the buffering effect is independent of the directional effect of FIS on transcription, although we cannot rule out the mediating effect of other factors.

An important finding of this study is that the local transcriptional effect of H-NS on pelE promoter activity varies with DNA topology (Figure 6 and Supplementary Figure S1). In the presence of H-NS in vitro the transcriptional response of pelE promoter to suboptimal superhelical density is aggravated, while at high superhelical density the promoter activity is even slightly stimulated (Figure 6 and Supplementary Figure S1). This finding indicates that H-NS fine-tunes the sensing of superhelicity at the pelE promoter. At this promoter H-NS binds within a region spanning ~160 bp and containing three consensus H-NS-binding motifs (19) (Supplementary Figure S2 and unpublished data). Previous studies implicated a temperature-dependent change in oligomerization state of H-NS in the control of gene expression (60), and more recently two binding modes have been proposed for H-NS depending on the divalent cation concentration, one of which directly responds to pH and temperature in vitro (61). However, since in our reactions the only variable is the superhelical density of the DNA, we assume that it is the local DNA geometry, rather than protein conformation that drives the transition in the H-NS nucleoprotein complex formed at the pelE promoter. Several lines of evidence are consistent with this notion. Previous studies showed that preferential interaction of H-NS with curved DNA elements affects the plasmid linking number in vitro (62). Furthermore, investigation of the effect of H-NS on the virulence (virF) gene expression in human pathogen Shigella proposed that a temperature-dependent conformational transition of an intrinsically curved 250-bp DNA sequence between the two H-NS-binding sites facilitates cooperative binding and repression by H-NS. Importantly, this effect of temperature on H-NS repression was also dependent on the topology of the DNA template (27). Finally, cooperative binding of H-NS at the proU promoter is diminished on supercoiled plasmids compared to linear DNA (24). We therefore propose that template topology is critical for H-NS dependent repression of pelE promoter activity.

Role of FIS and H-NS in mediating the stress response

Changes in bacterial DNA supercoiling are involved in mediating the transcriptional response to various kinds of stress (43,63). Both the acidic and oxidative stresses are experienced by D. dadantii early in the course of infection within the asymptomatic phase. We observed that the acidic and oxidative shocks induce relaxation of DNA in D. dadantii (Figure 7), which was alleviated in hns mutant. Since deletion of hns gene in all our experiments alleviated both the supercoiling response to DNA relaxation and the negative effect of the latter on pelE expression we infer that H-NS is required to fine-tune the reponse of virulence genes in response to fluctuations of superhelical density. Furthermore, we did not observe any effect of fis mutation in the response to acidic shock, whereas the response to oxidative shock was alleviated but to lesser extent than by hns mutation and importantly, this alleviation was observed both at the level of supercoiling response and pelE expression (Figure 7B and D). Thus, by and large we observe close correlation between the effects of environmental conditions on supercoiling and pelE virulence gene expression. Notably, the intracellular FIS concentration is critical for both the optimum expression of the master regulator of the virulence genes ssaA in S. enterica, an organism in which the interplay between FIS and DNA supercoiling is apparent (41). FIS concentration is critical also for the activation of topoisomerase I expression under conditions of oxidative stress (34). This critical dependence on FIS concentration together with variation of FIS levels in the cell could explain the variability of responses to fis mutation we observe. Another critical parameter is the CRP concentration, as FIS and CRP bind partially overlapping sites in the pelE promoter region (20) and Supplementary Figure S2] and the repression by FIS in vitro depends on the FIS to CRP molar ratio (20). Whether the effects of changing molar ratio are further modulated by superhelical density of the template DNA remains an open question.

Our previous work providing evidence for the strong attenuated virulence of hns and fis mutants (18,21) demonstrated the importance of H-NS and FIS in the infection process of D. dadantii. How would the observed effects of FIS and H-NS aid into adaptation of D. dadantii during the asymptomatic phase? When they enter a host plant, D. dadantii cells colonize the intercellular spaces of the cortical parenchyma and migrate within the cell walls, without causing any severe injury to the cellular structure (7). During this colonization phase there is no production of plant cell wall-degrading enzymes (7), but bacteria have to adapt to the apoplastic environment, which is an acidic, low-nutrient medium. The pH range of apoplastic fluids is between 4 and 6.5, depending on the plant species (64). Furthermore, during the colonization phase, the plant perceives the bacterium as an intruder and induces a sustained ROS (reactive oxygen species) production. After the colonization phase, the bacteria may either reside latently in the plant intercellular spaces, without provoking any symptoms, or they may start the disease process. Thus, disease caused by D. dadantii is an intricate process with two successive phases, an asymptomatic phase and a symptomatic phase, that require the temporal expression of different groups of genes. DNA relaxation, H-NS and FIS might help in this temporal regulation of synthesis of the factors needed for pathogenesis. Indeed, the relaxation of DNA induced by acidic conditions of the apoplast and the resulting increased repression of H-NS on the pel gene expression could lead to a significant reduction of Pel synthesis. This could prevent an early detection by the host of plant breakdown products that signal the presence of the pathogen before it reaches a population density appropriate for successful infection (65). Later during infection, alkalization of the apoplast may be a plant response to bacterial infection. Indeed, oligogalacturonides or effectors secreted by type III secretion systems of plant pathogenic bacteria, such as...
harpins, have been shown to induce medium alkalization of plant cell cultures (66). Whichever of these mechanisms is operating in the early steps of D. dadantii infection, an alkalization of infected plant tissue has been detected even before the occurrence of maceration symptoms (67). These changes in the in planta conditions result in an increased DNA superhelical density and consequently, would decrease the H-NS repression of the pel genes. This would stimulate production of the Pels, which would, in turn, result in a transition from biotrophic to necrogenic life style. The rapid production of the plant cell wall-degrading enzymes would serve to macerate host cells and counter host defenses. Although a direct link could not be clearly established between FIS and the modulation of the synthesis of Pels by stress, it is reasonable to assume that the role of FIS would consist in buffering the fluctuations of the DNA topology as previously reported (57,58), and also observed in our experiments with novobiocin treatment.

In conclusion, this work presents the first evidence of virulence gene regulation by the DNA topology in phytopathogenic bacteria. Our data reveal important novel features, such as the modulation of the H-NS-binding effect by the supercoiling state of DNA, and enable to propose a model integrating the action of DNA topology, H-NS and FIS in modulating virulence gene expression during growth of D. dadantii in hostile conditions. Our data obtained in a plant pathogen are consistent with previously reported crosstalk between FIS and DNA supercoiling in the control of virulence in human pathogen Salmonella (41), strongly suggesting that the interplay between DNA topology and the NAPs in modulating the virulence is a general feature of the bacterial pathogens.

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online: Supplementary Figures 1 and 2, Supplementary Table 1 and Supplementary References [68–71].

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