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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* (formely *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 cAMP-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, osmolarity 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 H_2O_2. Novobiocin 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 initition +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 ∼1 μg 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 Vaccina 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 $MgCl_2$ 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 U 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: $\sigma = \Delta LK/LK_0$, where LK_0 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 toluenized 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 10^9 *D. dadantii* bacteria per milliltre and to 0.47 mg of bacteria (dry weight) per millilitre. Fis and H-NS were purified as previously described (19,21).

RNA 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^R 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). *hemF* and *lpxC* (48) were selected as the reference gene for real-time RT–PCR to provide an accurate normalization in *D. dadantii; lpxC* and *rpoB* were retained as reference genes for normalization in *E. coli*.

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* σ^{70} 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 occured 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. dadantii* 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. dadantii* 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).

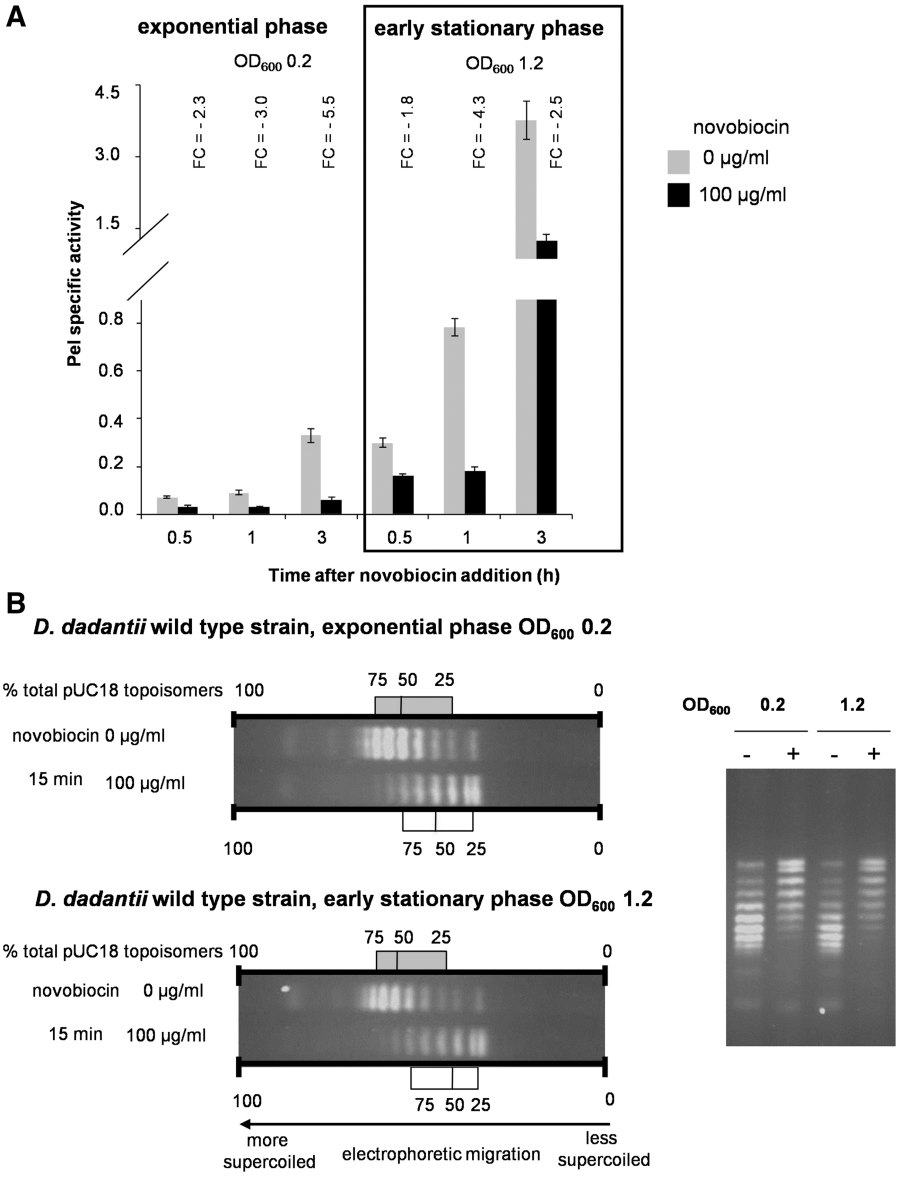

Figure 1. Effect of DNA gyrase inhibition and DNA relaxation on Pel production in *D. dadantii*. (**A**) Bacteria were treated with sublethal concentration of novobiocin (100 µg/ml) and samples were removed at the indicated times and submitted to Pel activity quantification. Each value represents the 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 ($parC^{K84}$) 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,

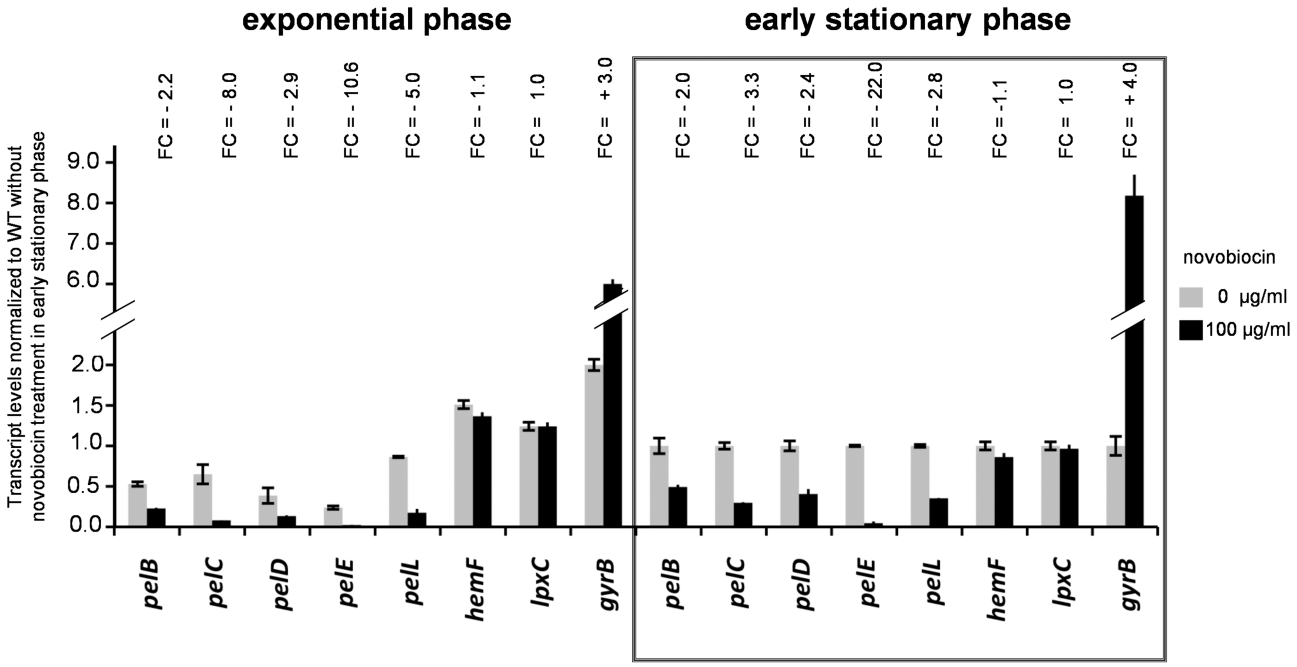

Figure 2. Impact of DNA gyrase inhibition and DNA relaxation on *pel* gene expression in *D. dadantii*. Bacteria were treated with sublethal concentration of novobiocin (100 µg/ml) and samples were removed 15 min post-treatment for chromosomal gene expression analysis using qRT–PCR. The oligonucleotides used are indicated in Supplementary Table S1. The *lpxC and hemF* genes were used as references for normalization, and the *gyrB* gene was used as control for novobiocin treatment. Each value represents the mean of three experiments. Bars indicate the SD. FCs are expressed as the ratio of the specific gene-expression level during exposure to novobiocin, compared with that in standard growth conditions, normalized to the level of expression of the *lpxC* and *hemF* genes. The transcript levels for each gene obtained without novobiocin treatment in the early stationary phase was arbitrary taken as 1. Results obtained with novobiocin treatment are different from those without treatment with $P < 0.05$ in a one sample *t*-test, except for the reference genes *hemF* and *lpxC*. Two different scales were used for the *y*-axis in order to better appreciate variations for the different genes.

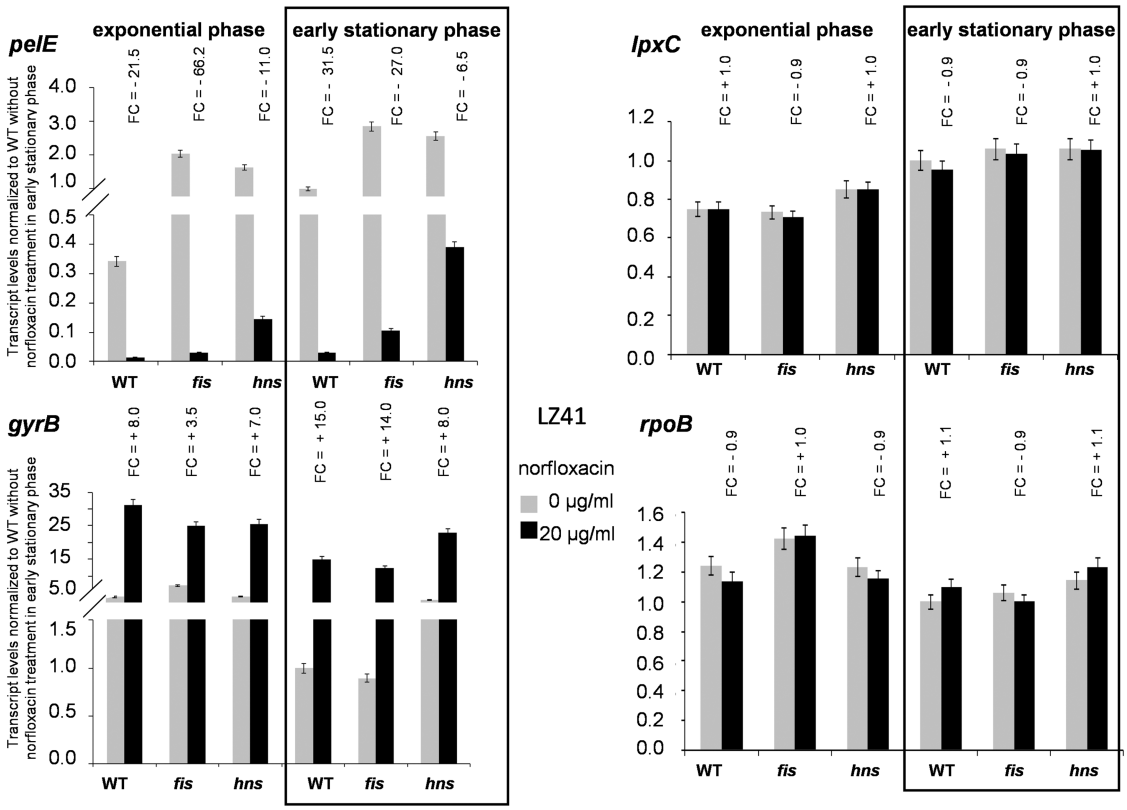

Figure 3. H-NS and FIS modulate the effect of DNA relaxation on *pelE* promoter activity. The genetically engineered *E. coli* LZ41 strain (Supplementary Table S1) and its *hns* and *fis* mutant derivatives containing plasmid pEH1 (*pelE* promoter-*uidA* fusion) were treated with norfloxacin (20 µg/ml) for 10 min before samples were removed for *uidA* transcript quantification using qRT–PCR as indicated in Figure 2. The strains used and the retained growth phases are indicated. The *lpxC and rpoB* genes were used as references for normalization and *gyrB* gene as control for norfloxacin treatment. Each value represents the mean of three experiments. Bars indicate the SD. FCs are expressed as the ratio of the specific gene-expression level during exposure to norfloxacin, compared with that under standard growth conditions, normalized to the expression level of the *lpxC* and *rpoB* genes. Results obtained with norfloxacin treatments are different from those without treatment with P-values < 0.05 in a one sample t-test. Two different scales were used for the y-axis in order to better appreciate expression variations in the different genetic backgrounds. The transcript levels of the *pelE* expression obtained in the WT strain without novobiocin treatment in the early stationary phase was arbitrary taken as 1.

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 augmented, 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

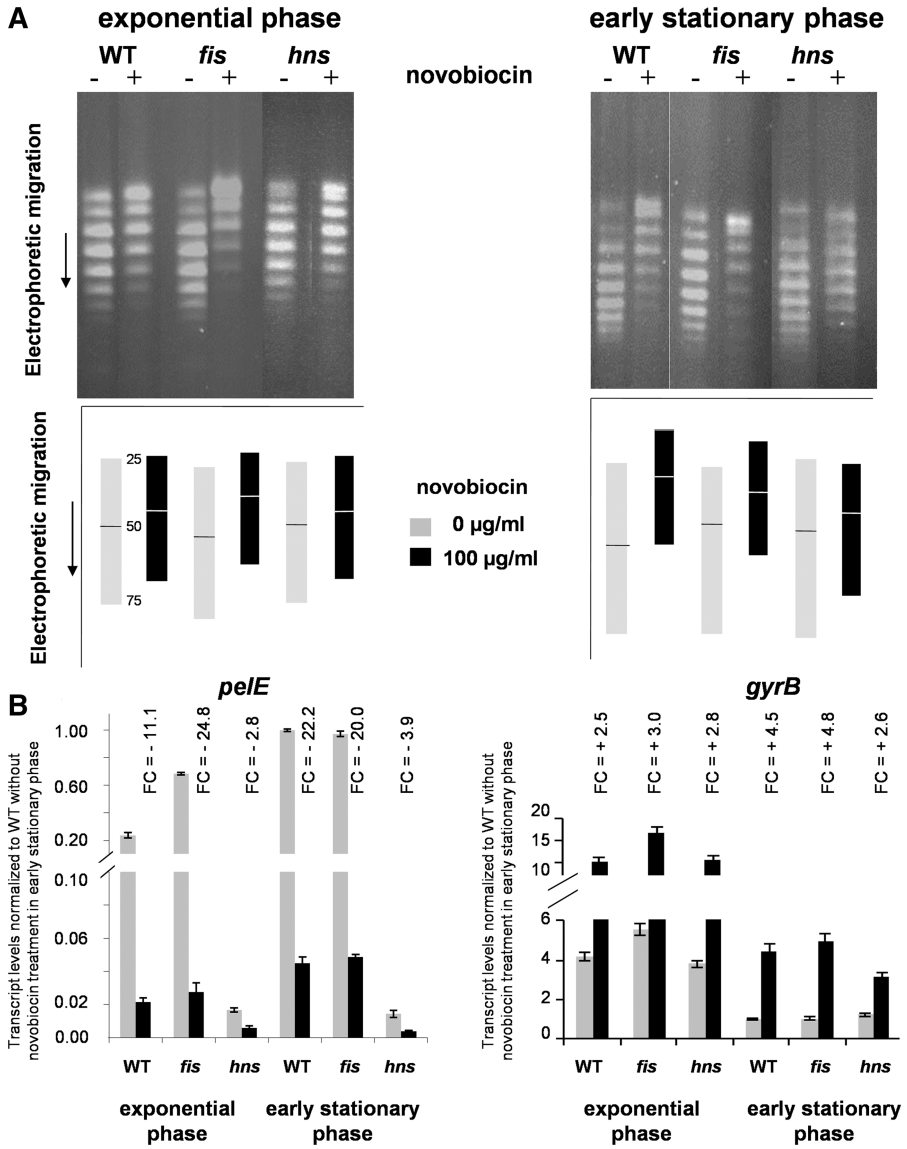

Figure 4. FIS and H-NS modulate the supercoiling sentivity of the *pelE* gene in *D. dadanti*. (**A**) Novobiocin treatment and pUC18 plasmid topoisomers analysis were performed as described for Figure 1. (**B**) qRT–PCR experiments measuring the *pelE* and *gyrB* transcript levels were performed as described in Figure 2. The strains used and the analysed growth phases are indicated. Each value represents the mean of three independent experiments. Bars indicate the SD. Results obtained with novobiocin treatments are different from that without treatment with *P*-values < 0.05 in a one sample *t*-test. FCs are expressed as a ratio of the specific gene-expression level during exposure to novobiocin compared with that in the medium without novobiocin, normalized to the level of expression of the reference genes *lpxC and hemF*. Two different scales were used for the *y*-axis in order to better appreciate expression variations in the different genetic backgrounds. The transcript levels of *pelE* gene obtained in the WT strain without novobiocin treatment in the early stationary phase was arbitrary taken as 1.

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 σ −0.036 to a maximum at high superhelical density of σ −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

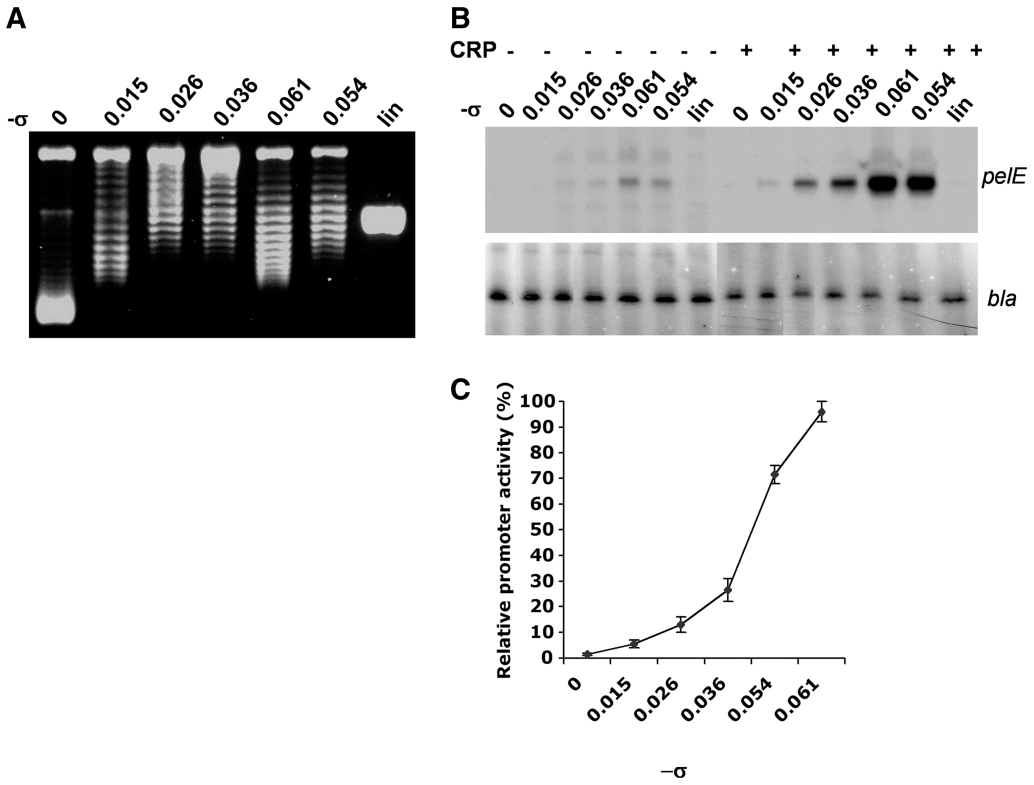

Figure 5. Dependence of *pelE* transcription on superhelical density of DNA *in vitro*. (**A**) High-resolution agarose gel electrophoresis of pEH1 preparations used for *in vitro* transcription; the negative superhelical density of plasmid preparations ($-\sigma$-values) are indicated. The showed gel contains 2 μg/ml chloroquine; lin corresponds to linearized plasmid, 0 corresponds to circular plasmid fully relaxed by Vaccinia topoisomerase in the absence of ethidium bromide. Under the conditions of electrophoresis the fully relaxed plasmid, as well as the next most relaxed plasmid ($-\sigma = 0.015$) migrate as positively supercoiled species, whereas the plasmids with high negative superhelicity ($-\sigma = 0.054$ and $-\sigma = 0.061$) migrate as negatively supercoiled species. The plasmid populations intermediate between these (e.g. $-\sigma = 0.026$) migrate as mixtures of the negatively and positively supercoiled species. (**B**) Quantitative primer extension of the *pelE* mRNA (top) generated by *in vitro* transcription from pEH1 plasmids of different superhelical densities. Half of the same mRNA preparations were used to mesure *bla* transcription as an internal control (bottom). The concentrations of RNAP and CRP used were 50 and 20 nM, respectively. (**C**) Graphic representation of the dependence of *pelE* transcription on superhelical density *in vitro*. The data shown are those obtained in the presence of RNAP and CRP. All values are normalized to the amount of transcript obtained from plasmid DNA of $\sigma = -0.061$. Each value represents the mean of two independent experiments. Bars indicate the SD.

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 (σ −0.061) and suboptimal superhelical density (σ −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 σ −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

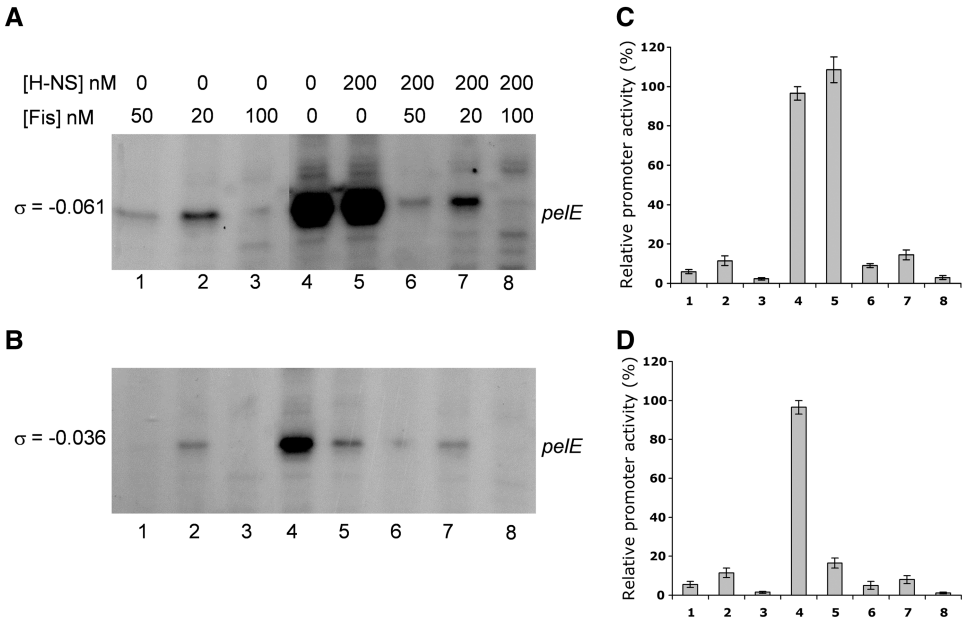

Figure 6. *PelE* expression regulation by H-NS is modulated by the supercoiling state of DNA. (**A**) Quantitative primer extension of *pelE* mRNA generated by *in vitro* transcription from plasmid of high superhelical density ($\sigma = -0.061$) showing maximal transcription (Figure 5). (**B**) Quantitative primer extension of *pelE* mRNA generated by *in vitro* transcription from plasmid at suboptimal superhilical density ($\sigma = -0.036$). All the experiments were performed in presence of 50 nM RNAP and 20 nM CRP; the concentrations of FIS and H-NS used are indicated above the part A. (**C**) Quantification of the data obtained in two independent experiments with two technical replicates at maximal superhelical density as shown in (A). (**D**) Quantification of the data obtained in two independent experiments with two technical replicates at suboptimal superhelical density as shown in (B). The values are normalized to the amount of transcript obtained in the absence of H-NS and FIS (lane 4). Bars indicate the SD.

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

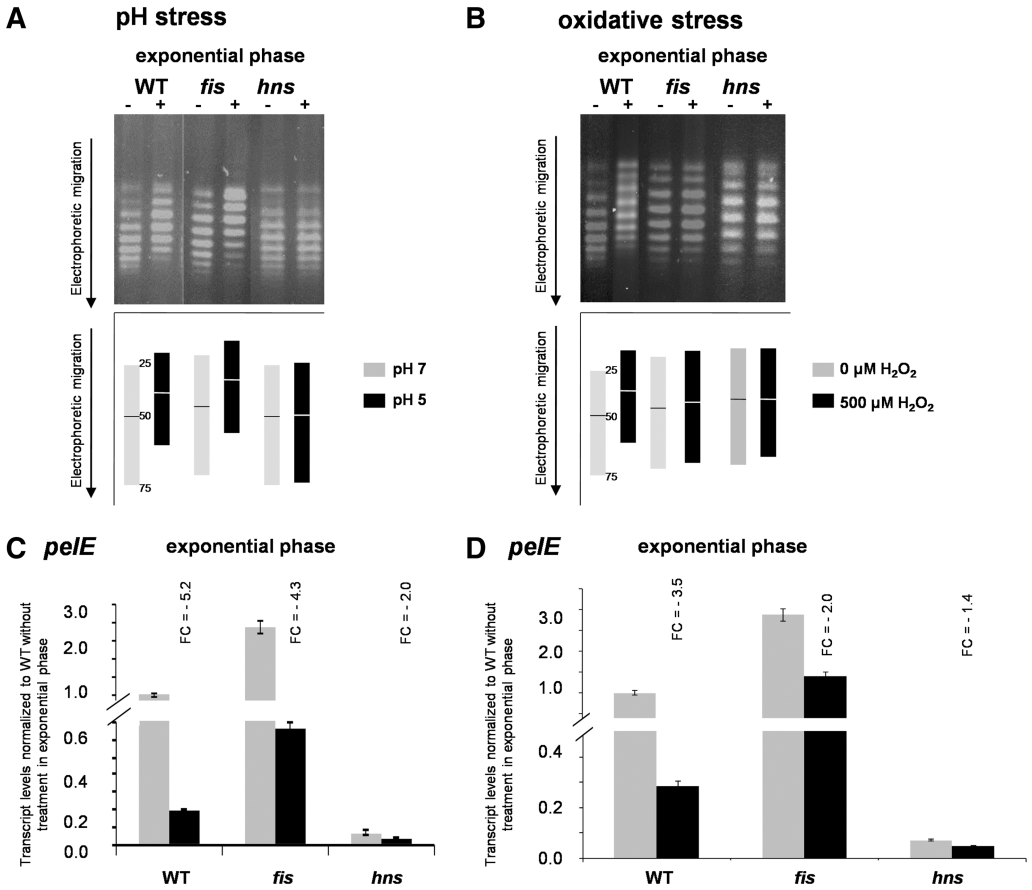

Figure 7. Effect of acidic and oxidative stresses on DNA relaxation and on *pelE* gene expression. Acidic shock and oxidative stress were induced during exponential growth phase by malic acid and hydrogen peroxide, respectively (see 'Materials and Methods' section). (**A**) Topoisomers of plasmid pUC18 were isolated from wild-type strain and its *hns* and *fis* mutant derivatives submitted to acidic shock. (**B**) The same as (A) after oxidative stress. Topoisomers were separated on agarose gels containing 2.5 µg/ml chloroquine. The obtained results were plotted in quartiles as described by (45). (**C**) Effect of acidic shock and (**D**), oxidative stress on *pelE* gene expression; qPCR experiments and representation of the data are as described in Figure 2. FC are expressed as a ratio of the specific gene-expression level during stress conditions compared with that in standard growth conditions, normalized to the level of expression of the reference genes *lpxC and hemF*. Two different scales were used for the *y*-axis in order to better appreciate expression variations in the different genetic backgrounds. The transcript levels of *pelE* gene obtained in the WT strain without novobiocin treatment was arbitrary taken as 1.

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 vivo* (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 during 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 reponse 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 *ssrA* 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 apoplast environment, which is an acidic, low-nutrient medium. The pH 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, alkalinization 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 alkalinization of plant cell cultures (66). Whichever of these mechanisms is operating in the early steps of *D. dadantii* infection, an alkalinization 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 genes 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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Conflict of interest statement. None declared.

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