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Original article

Regulation of *pel* genes, major virulence factors in the plant pathogen bacterium *Dickeya dadantii*, is mediated by cooperative binding of the nucleoid-associated protein H-NS

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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 production of pectate lyases (Pels) that can destroy plant cell walls. Previously, we found that nucleoid-associated protein (NAP) H-NS is a key regulator of *pel* gene expression. The primary binding sites of this NAP have been determined here by footprinting experiments on the *pelD* gene, encoding an essential virulence factor. Quantitative analysis of DNAse I footprints and surface plasmon resonance imagery experiments further revealed that high-affinity binding sites initiate cooperative binding to establish the nucleoprotein structure required for gene expression silencing. Mutations in the primary binding sites resulted in reduction or loss of repression by H-NS. Overall, these data suggest that H-NS represses *pelD*, and by inference, other *pel* genes, by a cooperative binding mechanism, through oligomerization of H-NS molecules.

Keywords: Phytopathogenic bacteria; H-NS/ Virulence genes; Nucleoid-associated rrotein; *Erwinia chrysanthemi*

1. Introduction

H-NS, a heat-stable nucleoid-structuring protein, is one of the major NAPs characterized in Gram-negative bacteria. Genes under H-NS control in enteric bacteria are often responsive to abrupt environmental changes. H-NS is thus regarded as a global modulator of gene expression in response to pH, temperature, osmolarity and growth phase. H-NS operates through a variety of mechanisms, including preventing open complex formation by RNA polymerase and trapping open complexes [24,11]. H-NS also influences DNA supercoiling in vivo and in vitro [8,17]. DNA binding by H-NS is sensitive to environmental factors. H-NS binding to DNA in vitro is facilitated by structural characteristics of DNA, such as curved AT-rich regions [18]. Recently, a binding motif was identified in the H-NS high-affinity sites. H-NS binds to high-affinity sites and spreads along the adjacent AT-rich DNA sequences to silence transcription [18,4]. H-NS is thus often referred to as a "universal repressor" or "modulator of

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environmentally regulated gene expression" [1,8]. These AT-rich sequences are often horizontal gene transfer (HGT) elements. Thus H-NS is a xenogenic silencer [30]. 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* [7,10,33,40].

We have previously shown, in the plant pathogenic enterobacterium *Dickeya dadantii* (previously *Erwinia chrysanthemi*), that H-NS modulates production of essential known virulence factors, including degradative extracellular enzymes. Accordingly, the *D. dadantii hns* mutant shows strong attenuated virulence [27,28]. *D. dadantii* is described as a necrotrophic Gram-negative bacterium that causes disease in a wide range of plant species [35,21]. It is listed in the top 10 of the most important bacterial plant pathogens [22]. Soft rot, the visible symptom, is mainly due to the production of degradative enzymes, mostly pectate lyases (Pels) degrading pectin, an essential component of the plant cell wall [35]. *D. dadantii* synthesizes multiple isoforms of Pels, including five major isoenzymes (PelA, B, C, D and E) [12]. Plant infection requires massive rapid production and secretion of Pels before the plant can establish its defensive reactions. The initiation of *pel* gene expression is therefore a key step in triggering the virulence of *D. dadantii* [9,16,37]. Among the *pel* genes, *pelD* expression is most strongly affected by pectin derivatives, which makes it a key element in the induction of *D. dadantii* virulence [13,3].

Expression of *pel* genes is under the control of a complex regulatory system. Several characterized regulators (KdgR, Pir, PecS, PecT, Fur, MfbR, VfmE) control the synthesis of Pels in response to various signals, such as the presence of pectin (KdgR), oxidative stress (PecS), iron starvation (Fur), acidic shock (MfbR) and unknown quorum sensing molecules (VfmE) [25,26,32,36]. 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 [34,29]. Furthermore, in addition to H-NS, another abundant NAP, a factor for inversion stimulation (FIS), influences the expression of the *pel* genes [14,19,20,27].

We showed that, in *D. dadantii*, H-NS displays an unusual activator phenotype on production of Pels, since its absence leads to a drastic reduction in enzyme synthesis. The reduced synthesis of Pels in the *hns* mutant mainly results from negative control exerted by H-NS upon transcription of the strong repressor gene *pecT*. Inactivation of H-NS results in overproduction of PecT which, in turn, reduces transcription of *pel* genes by binding to their upstream region. In addition to its effect on Pels production via PecT, H-NS also represses *pel* gene expression by binding to extended regions comprising the regulatory sequences of these genes [28]. Thus, the double *hns-pecT* mutant produces more Pels than the *pecT* mutant. Recently, we also found that expression of the *pel* genes is strongly decreased when the chromosomal DNA is relaxed by antibiotic treatment or environmental (oxidative and acid) stresses. Furthermore, we showed that H-NS modulates the response of *pel* promoters to supercoiling both in vivo and in vitro. Indeed, H-NS mostly represses *pel* gene expression on DNA templates with low superhelical density [31]. However, the precise mechanism of H-NS binding, and thus, H-NS mediated repression, at the *pel* gene promoters has not yet been fully elucidated. Here we address the mechanism of specific DNA recognition by the H-NS protein at the promoter of the *pelD* gene, encoding an essential virulence factor in *D. dadantii*.

2. Materials and methods

2.1. Bacterial strains, growth conditions and chemicals

Escherichia coli K-12 DH5α was used for cloning procedures. *E. coli* strain CSH50 (F- λ-*ara Δ(lac-pro) rpsL thi fimE*::*IS1*) and its *hns* derivative were used for quantification of promoter expression. The wild-type *D. dadantii* 3937 strain was used for this study. Strains were routinely grown in LB at 37 °C or 30 °C with aeration. Plasmids were maintained by the addition of ampicillin (100 μg ml^{-1}).

2.2. Construction of promoter-*uidA* fusion

Plasmid pTL4, a derivative of pNB4 [2] containing the full *pelD* promoter (*pelD*1, −81 to +132 relative to the transcription initiation +1 of *pelD*) [19], was used for quantification of *pelD* promoter expression. The derivative fragments, carrying mutations in the different binding sites of H-NS (sites 1, 2, 3 and 1 + 2 + 3) (Table 1) and containing *Eco*RI and *Hind*III sites at the 5′ and 3′-end, were obtained by direct synthesis (GeneCust). These 313 bp *Eco*RI-*Hin*dIII fragments were inserted into the pNB4 plasmid containing the *uidA* promoterless gene [2], digested by the same enzymes to generate pZO1, pZO2, pZO3 and pZO4 (Fig. 5), modified in H-NS binding sites 3, 2, 1 and in all three sites, respectively. In these constructs, the *uidA* reporter gene is expressed under the *pelD* promoter.

2.3. β-glucuronidase activity assay

The assay of β-glucuronidase was performed on toluenized cell extracts. β-glucuronidase activity was measured by monitoring degradation of *p*-nitrophenyl-ß-D-glucuronide into *p*-

Table 1
Mutations at H-NS high-affinity binding sites. H-NS high-affinity sites (1, 2 and 3) on *pelD* contain DNA sequence displaying greater similarity with the H-NS canonical consensus [18]. Mutations within the H-NS canonical consensus sequence were introduced and are displayed in the last column.

H-NS binding site	H-NS consensus score	H-NS consensus sequence	WT site	*hns modified site*
1 (−30 → −21)	(5.28-)	TCGATAAATT	AAACAATCGA	AGCCCGTCGA
2 (−18 → −9)	(4.79+)	TCGATAAATT	ACGCTTAAAA	ACGCCGGGCA
3 (+18 → 27)	(5.69 +)	TCGATAAATT	GCGTTTCATT	GCGTCGGGCT

nitrophenol, which absorbs at 405 nm [2]. Specific activity is expressed as μmol of unsaturated products released min^{-1} mg^{-1} (dry weight) bacteria. To measure the dry weight of cells, 1 ml of bacterial culture with turbidity at 600 nm (OD_{600}) of 1 was centrifuged and the supernatant was eliminated. The obtained pellet of bacterial cells was desiccated to remove any additional moisture (i.e. by incubation at ~60 °C), and then weighed using a classical scale. We found that an optical density at $0D_{600}$ nm of 1.0 corresponds to 10^9 bacteria ml^{-1} and to 0.47 mg of bacteria (dry weight) ml^{-1} [36].

D. dadantii H-NS protein was purified according to published protocols [39].

2.4. In vitro DNA/protein interaction

DNase I footprinting were performed as previously described [28]. The *pelD* regulatory region was recovered from plasmid pWN2481 [19]. To label the coding fragment, 10 μg of plasmid DNA was digested by EcoRI-HindIII, followed by filling in the HindIII end in the presence of (α-^{32}P) dCTP (3000 Ci $mmol^{-1}$, GE HealthCare) and the Klenow fragment of DNA polymerase. To label the non-coding fragment, a similar amount of pWN2481 was digested by EcoRI-ApaI; then, the EcoRI end was labelled in the presence of (α-^{32}P) dATP (3000 Ci $mmol^{-1}$, GE HealthCare) as described above. The labelled DNA fragments were purified, after electrophoresis, on agarose gels using the Qiagen gel extraction kit. About 2 nM (at least 100,000 c.p.m.) of labelled probe were used for DNase I footprinting experiments.

2.5. Surface plasmon resonance imaging (SPRi) analysis

The SPRi approach consists of immobilizing ligands to a surface and then observing changes in the refractive index at the surface as molecules bind. SPRi measurements were carried out using an SPRi–Plex™(GenOptics) as described previously [5]. Briefly, 1 μM of 5′-end thiol-labelled DNA fragments of the *pelD* promoter region was immobilized to the prism surfaces by direct spotting. Once dried, the surface was mounted in the *SPRi–Plex*™ apparatus. After short washing with 10 μl injection of 1 M NaCl followed by continuous flow (50 μl/min) with binding buffer, DNA spots were visually identified by the relative change in percent reflectivity and delineated in the accompanying software as circles on the image. Various concentrations of H-NS protein were then injected across the surface in binding buffer at 50 μl/min. Images were recorded at a rate of 2 Hz, and the rate of change of percentage reflectivity across the area of each predetermined spot was used to calculate the kinetics of change in binding at the surface.

3. Results and discussion

3.1. Analysis of H-NS binding on the pelD promoter using DNase I footprinting

The choice of *pelD* for investigation is based on the fact that this gene encodes an essential virulence factor for *D. dadantii*, and previous studies showed that *pelD* was one of the *pel* genes most strongly regulated by H-NS [28]. In a previous study using DNase I footprinting assays, we demonstrated that H-NS binds to an extended region of at least 200 bp on the *pelD* promoter [28]. However, the conditions used in those experiments, and particularly, the narrow concentration range of the H-NS protein used, were not suitable for accurately identifying putative high-affinity or nucleation site(s). Here we analyzed the affinity of binding of H-NS to both strands of the *pelD* promoter by using H-NS over a wide range of protein concentrations (Fig. 1).

Although protection by H-NS was observed on both strands, the non-coding strand was chosen for quantification because of the higher resolution obtained on this strand with low H-NS concentrations. Several H-NS binding sites, extending from position −176 to +87, were observed. Two bands at positions +22 and −28 were especially responsive to H-NS binding, since they disappeared in the presence of relatively low concentrations of H-NS. Further analysis of the *pelD* promoter region encompassing these two positions (between positions −35 and +30) revealed the presence of three sequence motives of 10 nucleotides that closely match the high-affinity H-NS binding sequences found in the *proU* gene of *E. coli*, 5′-TCGATATATT-3′ [18,4]. In a consistent manner, the sequence motif of the *pelD* promoter displaying the highest similarity with the *E. coli* high-affinity binding motif of H-NS is located around position +22 (site 3), followed by the −28 binding site (site 1). In addition to these high-affinity sites, one additional sequence motif (site 2) is observed near the *pelD* promoter region (Fig. 2). As the intensity of the band at position −28 is relatively low, we selected the +22 position for subsequent analyses. The degree of protection by H-NS at this position was then quantified as a function of total protein concentration (expressed as dimer concentration). The resulting binding isotherms are shown in Fig. 3.

At a concentration of 30–45 nM, there was a burst of binding at the −22 site, illustrated by a sudden disappearance of the corresponding DNA segment. This indicates a stoichiometric interaction of H-NS with the *pelD* DNA segment encompassing position −22, and thus argues for a highly cooperative mechanism with an estimated apparent global equilibrium dissociation constant (*Kd*) of around 35 nM (Fig. 3a). At the same time, quantification of the reactivity of the protection located at position −15 (Fig. 3b), for which a gradual increase was observed, revealed non-cooperative binding of H-NS at this position, with an estimated apparent global *Kd* of 195 nM. Thus, it is suggested that the nucleation of H-NS binding on the *pelD* promoter occurs at sites encompassing bases at positions −28 and +22. It is particularly interesting that these two high-affinity binding sites for H-NS are located on either side of the transcription initiation site (Table 1). Thus nucleation of binding at these sites, followed by cooperative spreading of H-NS binding, may prevent RNA polymerase from initiating transcription at the *pelD* promoter.

Hyperreactive sites were present at several positions in the analyzed region of the promoter, particularly between the two high-affinity binding sites. A DNase I hyperactive site reflects local distortion of the DNA structure, induced by H-NS binding

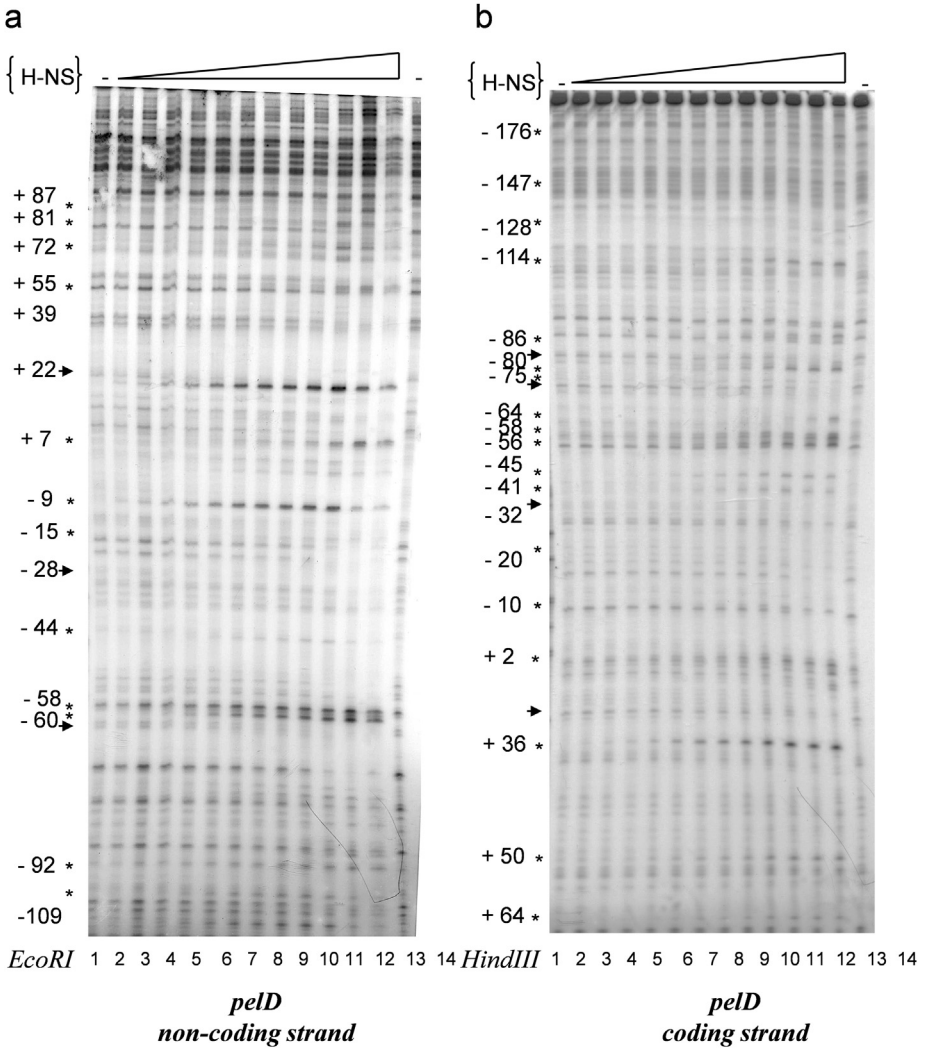

Fig. 1. DNase I footprinting analysis of H-NS binding to the *pelD* promoter. Reactions were performed on both strands for *pelD*. Concentrations of H-NS are 20, 30, 45, 60, 90, 120, 150, 175, 250, 375, 500 and 2000 nM for lanes 2–13, respectively. A hyphen (-) at the top denotes reactions without H-NS. Sequences are numbered with respect to the transcriptional start site (+1) of the corresponding genes. Asterisks indicate nuclease-hypersensitive sites induced by binding of H-NS; arrows indicate; highly protected bands. End-labelled restriction sites are indicated at the bottom.

Fig. 2. Sequence of the *pelD* promoter. Arrow indicates the transcription initiation site +1, previously determined [29]. Blue and red asterisks indicate nuclease-hypersensitive sites induced by binding of H-NS to the bottom and top strand, respectively. The two nucleotides in bold at positions −28 and +22 are highly responsive to H-NS binding, as revealed by DNAse I footprinting in Fig. 1. H-NS high-affinity binding sites are indicated by standard lines. Sequences framed in gray have significant similarity to the H-NS binding motif [18]; the score obtained by PRODORIC (virtual footprint) is shown below; + and − indicate coding and non-coding strand, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

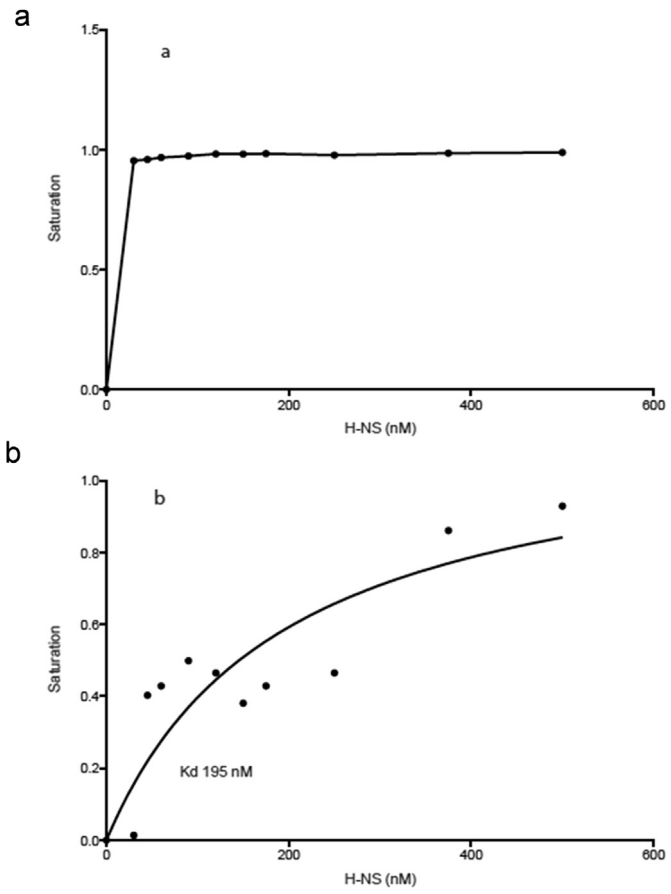

Fig. 3. Binding curves based on DNase I footprinting of the non-coding strand of the *pelD* promoter region centered on the −22 binding site (a) and centered on the −15 binding site (b). Bands on a gel were quantified using a phosphorimager scanner and fitted using Origin software. The degree of saturation is expressed as a function of total H-NS concentration.

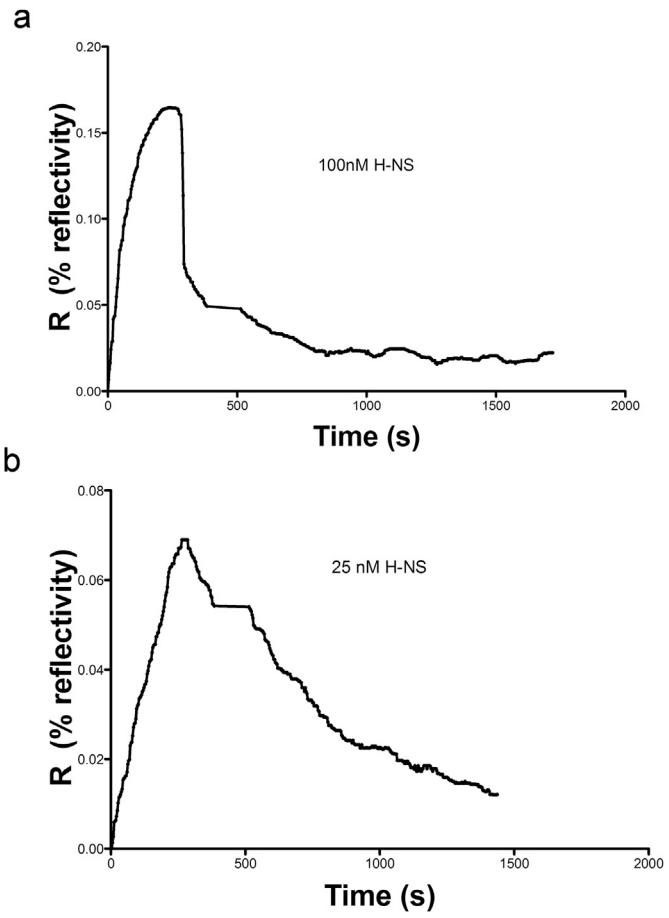

Fig. 4. SPRi for H-NS binding to the *pelD* promoter fragment immobilized on an SPRi prism surface. SPRi sensorgrams showing changes in % reflectivity as a function of H-NS (100 nM) and H-NS (25 nM) binding to the immobilized *pelD* promoter fragment on an SPRi surface. The association phase is fitted by a single exponential model $Y_t = Y_{\max}(1 - \exp^{(-k_{obs}t)})$, where Y_t is the % reflectivity at time t; Y_{max} is the amplitude of the phase, and the observed rate constant $k_{obs} = k_a[C] + k_d$, $k_a k_d$ is the association rate constant. k_d is the dissociation rate constant calculated from a simple exponential fit of the dissociation phase using $Y_{(t)} = Y_{\max} \exp^{(-k_d t))}$ and [*C*] is the concentration of H-NS. The K_d value is then simply the ratio of k_d/k_a.

in adjacent regions, that might affect binding of transcriptional regulators or of the RNA polymerase. Interestingly, these sites are clustered within the −30 to +28 region (positions −15, −9, +7, +20 on Fig. 1), suggesting that distortion of the extended RNA polymerase binding region might contribute to H-NS silencing of *pelD* promoter expression. A similar mechanism has recently been proposed for the *Salmonella enterica* pathogenicity island 6 [6]. This observation is in contrast to those made concerning the *proU* and *LEE5* promoters, where H-NS binds at sites downstream of the promoter (centered at positions +25 and +130) and upstream of the promoter (−138 and −110), respectively [4,38].

3.2. SPRi of H-NS binding to immobilized pelD promoter sequences

DNase I footprint experiments were unable to measure affinity at the high-affinity sites. We therefore immobilized the *pelD* promoter fragments on an SPRi prism surface, as described in Materials and methods, and H-NS at 25 or 100 nM was flown across the surface at 25 °C. As seen in Fig. 4, since H-NS was retained by the immobilized DNA, the signal increased throughout the injection phase, and subsequent continuous flow with the buffer removed dissociating protein from the surface. These curves were fitted to simple binding isotherms, as described in the legends to Fig. 4, giving on and off rates that were used to calculate the relative Kd values of 4 nM at 25 nM H-NS and 45 nM at 100 nM H-NS. Although the fits did not take into account cooperativity, they illustrate the 10-fold difference between values obtained at the two concentrations and confirm that, at low H-NS concentrations (25 nM), the protein binds to relatively high-affinity sites (presumably to sites around positions −28 and +22), whereas, at higher concentrations (100 nM), by spreading, the protein occupies lower-affinity binding sites, resulting in a lower global affinity. These results are therefore in agreement with the DNase I titration experiments.

3.3. Impact of H-NS high-affinity binding sites on pelD expression

To ascertain the mechanism of *pelD* gene silencing by H-NS through synergistic or cooperative binding, we constructed

a series of plasmids containing the same segment of the *pelD* promoter (−181 to +132) used for DNase I footprinting experiments, fused to the β-glucuronidase gene. Mutations at H-NS binding sites 1, 2 and 3 or at all three positions were introduced into these constructs. Due to plasmid instability observed in *D. dadantii* and interference induced by the effect of H-NS on PecT expression, these experiments were performed in *E. coli* CSH50 [23]. Indeed, it has been previously shown that most of the global regulatory systems, particularly that of H-NS, are conserved between *E. coli* and *D. dadantii* [15,27]. Given the fact that the H-NS sites selected are located (H-NS sites 1 and 2) in the vicinity of the σ^{70}RNA polymerase binding sites on *pelD*, or overlap (H-NS site3) with the binding site of the KdgR repressor (−4 to +53) [19], the impact of mutations was evaluated by the ratio of activities obtained in the *hns* mutant and the parental strain. As previously observed [28], at least a 3.5-fold increase in β-glucuronidase activity was observed in the *hns* mutant with the WT-promoter. The elimination of either H-NS binding site 1 or 3 resulted in loss of repression by H-NS, while elimination of the site at position 2 led to a 1.8-fold increase in *pelD* promoter expression. Moreover, the triple mutation showed the same result as mutations at binding site 1 or 3. Thus, the similar losses of repression in the absence of either binding site 1 or 3 compared to the wild-type construct suggested interdependency or cooperativity between the operators (Fig. 5). We therefore propose that these two sites constitute nucleation sites for H-NS binding on the *pelD* promoter, although it is possible that there remain other unidentified sequences which also contribute to nucleation of binding and spreading. For example, the site centered at position −14 might play a role in oligomerization of H-NS between the two nucleation sites.

The pertinent question is whether the mechanism described for *pelD* regulation by H-NS also applies to other *pel* genes. H-NS binding sites displaying a high similarity score with the *E. coli* high-affinity binding motif were previously identified in similar locations in the *pelE* regulatory region [31], suggesting by inference that H-NS may use an analogous promoter-silencing mechanism by cooperative binding to regulate expression of *pelD and pelE* genes in *D. dadantii*. On the assumption that gene silencing by H-NS in all cases involves cooperative binding and oligomerization, another relevant question is whether the difference in spatial organization of nucleation sites observed for *pelD*, *proU* and *LEE5* promoters serves any additional purpose. We hypothesize that, on an evolutionary timescale, the distinct organization of nucleation sites might be dictated by the strategy of minimizing interference with the organization of binding sites for other regulatory proteins involved in control of these virulence genes.

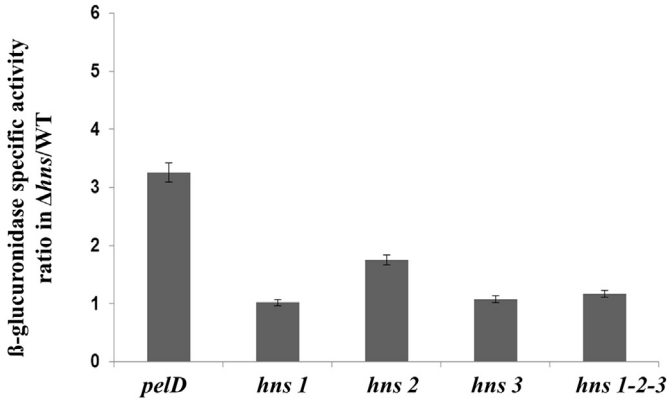

Fig. 5. GUS activity of wild-type and mutated *pelD* promoters in wild-type and *hns* strains. The *pelD* WT promoter and the various promoter constructs containing the *uidA* fusion were cloned into the promoter probe pNB4 vector. β-Glucuronidase-specific activity (GUS) was monitored in the mid-exponential growth phase for wild-type and *hns* strains. Each value represents the mean of three independent experiments. Bars indicate the standard deviation.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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