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NsrR, GadE, and GadX Interplay in Repressing Expression of the Escherichia coli O157:H7 LEE Pathogenicity Island in Response to Nitric Oxide

Priscilla Branchu1‡a, Stéphanie Matrat1, Marjolaine Vareille1‡b, Annie Garrivier1, Alexandra Durand1, Sébastien Crépin2‡c, Josée Harel2, Grégory Jubelin1, Alain P. Gobert1‡

1 INRA, UR454 Microbiologie, Centre de Clermont-Ferrand-Theix, Saint-Genès-Champanelle, France, 2 Groupe de Recherche sur les Maladies Infectieuses du Porc and Centre de Recherche en Infectiologie Porcine, Université de Montréal, Saint-Hyacinthe, Quebec, Canada

Abstract

Expression of genes of the locus of enterocyte effacement (LEE) is essential for adherence of enterohemorrhagic Escherichia coli (EHEC) to intestinal epithelial cells. Gut factors that may modulate LEE gene expression may therefore influence the outcome of the infection. Because nitric oxide (NO) is a critical effector of the intestinal immune response that may induce transcriptional regulation in enterobacteria, we investigated its influence on LEE expression in EHEC O157:H7. We demonstrate that NO inhibits the expression of genes belonging to LEE1, LEE4, and LEE5 operons, and that the NO sensor nitrite-sensitive repressor (NsrR) is a positive regulator of these operons by interacting directly with the RNA polymerase complex. In the presence of NO, NsrR detaches from the LEE1/4/5 promoter regions and does not activate transcription. In parallel, two regulators of the acid resistance pathway, GadE and GadX, are induced by NO through an indirect NsrR-dependent mechanism. In this context, we show that the NO-dependent LEE1 down-regulation is due to absence of NsrR-mediated activation and to the repressor effect of GadX. Moreover, the inhibition of expression of LEE4 and LEE5 by NO is due to loss of NsrR-mediated activation, to LEE1 down-regulation and to GadE up-regulation. Lastly, we establish that chemical or cellular sources of NO inhibit the adherence of EHEC to human intestinal epithelial cells. These results highlight the critical effect of NsrR in the regulation of the LEE pathogenicity island and the potential role of NO in the limitation of colonization by EHEC.

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Introduction

Enterohemorrhagic Escherichia coli (EHEC), especially those belonging to the O157:H7 serotype, are foodborne pathogens and healthy rearing animals are the main reservoir. Human infection occurs through the ingestion of contaminated food. This primary infection yields to the development of intestinal disorders, including aqueous or bloody diarrhea. Moreover, EHEC express a cardinal and well-defined virulence factor, the Shiga-toxin (Stx) encoded by genes located in lysogenic lambdoid bacteriophages. Stx is produced in the gut lumen and crosses the epithelial barrier to reach the blood and the target organs including the kidneys. In this context, infected patients may develop life-threatening complications such as the hemolytic and uremic syndrome (HUS), the main cause of renal failure in children in developed countries [1].

EHEC genes carried by the locus of enterocyte effacement (LEE), a chromosomal pathogenicity island organized in 5 operons, encode bacterial factors implicated in the intimate adherence of these bacteria to intestinal epithelial cells [2]. These genes encode a type 3 secretion system (T3SS; LEE1, LEE2, LEE3), a translocon and a syringe (LEE4) that allows bacteria to inject effectors in epithelial cells, such as the LEE5-encoded intimin receptor Tir; moreover, other proteins not carried by the LEE can be translocated by the T3SS into enterocytes [3,4]. The injected effectors and/or protein of the translocon itself interact with the host signal transduction, leading to actin polymerization and to microvilli effacement [2], to regulation of the innate immune response [5,6], and to increased electrolyte transport [7]. Regulation of gene expression within the LEE is known to be complex and governed by a large number of influences, including environmental cues or quorum sensing, and involves several specific or global regulators [8,9]. The first gene of the LEE1 operon, ler, encodes a transcriptional regulator that positively regulates the expression of all the other operons [9–11]. However a variety of extra-transcriptional mechanisms have also been...
Results

The adhesion of EHEC to intestinal epithelial cells is reduced by NO

We first examined adhesion of the E. coli O157:H7 strain EDL933 to cultured Hct-8 intestinal epithelial cells in the presence of the NO donor NOR-4. Exposure to NOR-4 at 200 μM or 500 μM did not cause any significant difference in the growth rate of EDL933, as described [17]. However, EHEC adhesion to Hct-8 cells was dramatically inhibited when NOR-4 was added to the co-cultures (Figs. 1A and 1B). The number of EHEC fixed to the cells was significantly decreased by 41±5% and 89±2% in the presence of 200 μM and 500 μM NOR-4, respectively (Fig. 1B).

To further confirm this result, we analyzed the effect of endogenous NO released by enterocytes. Hct-8 cells were first treated for 24 h with a cytokine cocktail known to stimulate the inducible NO synthase (iNOS) expression [19], washed, and then infected with the strain EDL933 in the presence or absence of the iNOS inhibitor Nω-(1-iminoethyl)-L-lysine (L-NIL). There was less EHEC fixed to NO-producing epithelial cells than to control cells (Figs. 1A and 1C). The inhibition of EHEC adhesion to Hct-8 cells treated with cytokines was abolished by the use of L-NIL (Figs. 1A and 1C).

NO inhibits LEE1/4/5 gene expression and stimulates the Gad system

The expression of genes that represent the five operons of the LEE (Fig. 2A) was analyzed after treatment with NOR-4 for 6 h. NO was consistently generated in the bacteria culture medium and reached a plateau after 6 h (Fig. S1). The expression of ler (LEE1), espA (LEE4), tir and ee (LEE5) was down-regulated by NO, while the transcription of sepZ (LEE2) was induced by 2.4-fold (Fig. 2B). The expression of the gene exV (LEE3) was not modulated by NOR-4 (Fig. 2B). Because GadE and GadX modulates LEE expression in EHEC and EPEC, respectively, [16,20], we investigated the effect of NOR-4 on gadE and gadX transcription. As shown in Figure 2C, the expression of gadE and gadX was significantly induced by 2.4- and 2.7-fold in bacteria exposed to NOR-4, respectively. Thereby, these data prompted us to wonder whether NO-dependent down-regulation of LEE1, LEE4 and LEE5 requires GadE and/or GadX.

GadE and GadX modulate the expression of LEE genes

Since the role of GadX and GadE on LEE expression is not well defined and is strongly dependent on the growth conditions [16,20,21], we first analyzed the expression of ler, espA, and tir in EDL933 ΔgadE and ΔgadX mutants. When compared to the EDL933 strain, the mRNA levels of ler, espA and tir were increased by ~1.4-, 2.3-, and 2-fold in the ΔgadE strain, respectively (Fig. 3A); these effects were reversed when the gadE mutant was trans-complemented with the gadE gene in a low copy number plasmid vector (Fig. 3A). The gadX mutation was associated with a spontaneous increase of ler transcription and with a significant reduction of espA and tir gene expression (Fig. 3A). The transcription of ler was repressed while the expression of espA was activated and that of tir was restored to the same level as the WT in the trans-complemented strain (EDL933 ΔgadX::c; Fig. 3A). These data suggest that GadE represses the expression of LEE4 and LEE5 genes independently of Ler, and that GadX represses LEE1 but activates LEE4 and LEE5 gene expression. Interestingly, the NO-4-dependent down-regulation of ler, espA, and tir was still observed in the ΔgadE, ΔgadX and ΔgadE/ΔgadX mutants (Fig. 3A), suggesting that another factor is implicated in the inhibition of LEE1/4/5 by NO.

We next wonder whether GadE and GadX repressed the LEE independently from each other or whether GadX is epistatic to GadE as in E. coli K12 [22]. The expression of ler was similar in a ΔgadE/ΔgadX double mutant and in the EDL933 ΔgadX strain (Fig. 3A), indicating that GadX is epistatic to GadE in controlling LEE1. Conversely, espA and tir mRNA levels were increased in EDL933 ΔgadE/ΔgadX when compared to the WT strain, as in the...
ΔgadE strain, demonstrating that GadE is epistatic to GadX for the regulation of LEE4 and LEE5. Therefore we investigated whether GadE controls gadX expression. Figure 3B shows a 33% decrease in gadX mRNA levels in the ΔgadE mutant, indicating that GadE activates gadX expression. In addition, we observed 3.1-fold more gadE mRNA copies in the ΔgadE strain than in the WT strain (Fig. 3B) and gadE mRNA levels were dramatically reduced in the complemented strain (Fig. 3E), suggesting that GadX is a repressor of gadE expression. Therefore, the moderate increase in ler expression observed in the ΔgadE strain (Fig. 3A) is likely due to the lower level of GadX in this strain and not to a direct effect of GadE on ler transcription. Lastly, the activation of gadX

Figure 1. Adhesion of EDL933 to intestinal epithelial cells. Hct-8 cells, pre-treated or not with a cytokine cocktail for 24 h, were co-cultured for 6 h with the EHEC strain EDL933 ± NOR-4 or L-NIL. A: Cells and bacteria were visualized after Giemsa staining; magnification, ×63. B and C: The number of bacteria adherent per Hct-8 cell was counted on 15 microscopic fields. For B, * P<0.05, ** P<0.01 compared to the co-cultures without NOR-4; n = 6. For C, ** P<0.01 vs. cells not stimulated with cytokines; $ P<0.05 vs. cells treated with cytokines; n = 6.

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transcription by NOR-4 was suppressed in the gadE mutant, but not in the EDL933 ΔgadE-c strain (Fig. 3B), while the NO-dependent induction of gadE mRNA expression was still observed in the ΔgadX strain (Fig. 3B). These data indicate that NO activates gadX expression through GadE.

The repression of LEE1/4/5 genes is mediated by NsrR

NsrR is a transcriptional regulator that regulates gene expression in response to NO [18]. Therefore we investigated whether NsrR regulates gadE, gadX, and the LEE genes. In the absence of NO, the mRNA levels of ler, espA, and tir were 6.8, 7.1, and 14.3-fold lower in
the ΔnsrR mutant than in the WT strain, respectively (Fig. 4A). The expression of these genes was similar in the strains EDL933 and EDL933 ΔnsrR-c (Fig. 4A). Moreover, the NO-dependent regulation of these LEE genes was abrogated in EDL933 ΔnsrR and was restored in the complemented strain (Fig. 4A). Inversely, the transcription of gadE and gadX was significantly increased in the ΔnsrR mutant, but not in the complemented strain. The expression of these two genes was not affected by NOR-4 in the nsrR-deficient strain (Fig. 4B).

These data suggest that NsrR is a transcriptional activator of LEE1, LEE4, and LEE5 and a repressor of gadE, which in turn modulates gadX expression. NsrR loses its ability to regulate the expression of LEE and gad genes in the presence of NO.
The NsrR binding on the promoter regions of LEE 1/4/5 is inhibited by NO.

The investigation of GadE, GadX and/or NsrR direct binding to the gadE, gadX, and LEE promoter regions was performed by chromatin immunoprecipitation (ChIP) experiments using the EDL933 ΔgadE, ΔgadX, and ΔnsrR mutants expressing the 6-His-GadE, the 6-His-GadX, and the 6-His-NsrR fusion proteins, respectively.

We first analyzed the gadX promoter described by Hommais et al. [15], the three promoters described for gadE in E. coli K12 [22] (Fig. S2), and the gadA promoter as a positive control for GadE and GadX binding [23]. Surprisingly, we found that GadE and GadX did not bind to the gadX and gadE promoters, respectively (Figs. 5A and 5B), indicating that activation of gadX by GadE and repression of gadE by GadX occur through indirect regulations. As expected,
the binding of GadX and GadE to the gadC promoter region was observed (Figs. 5A and 5B).

Two ler promoters have been described in EHEC, the distal P1 promoter and a putative proximal P2 promoter (Fig. S2). The P1 promoter is common to EHEC and EPEC, while the P2 promoter is present only in EHEC [24–26]. Neither GadE (Fig. 5A) nor GadX (Fig. 5B) bound to either of these promoters (Figs. 5A and 5B). These data indicate that GadE and GadX do not repress ler expression directly. The LerE4 promoter has been identified in EHEC upstream of sptL [27], sptL being the second gene of the operon. In EPEC, it has been shown that Ler-mediated activation of the LEE5 operon requires sequences between positions -198 and -75 relative to the transcriptional start site [28]. Two primer pairs overlapping this region have been designed for ChIP experiments, amplifying a LEE5 distal (P1LEE5) and a LEE5 proximal (P2LEE5) region (Fig. S2). ChIP experiments showed that neither GadE nor GadX bound to the LEE4 and LEE5 promoters (Figs. 5A and 5B). Lastly, the binding of GadE and GadX to the LEE1/4/5 promoter regions was not modulated by NOR-4. These data indicate that control of LEE4 and LEE5 expression by GadE and GadX is due to indirect effects.

In contrast, NsrR bound to the distal LEE1 promoter (P1LEE1), to the LEE4 and LEE5 promoters, and to the promoter of hmpA, a well-known NsrR target gene (Fig. 5C). Furthermore, NsrR binding to these promoter regions was inhibited when the bacteria were grown in the presence of NOR-4 (Fig. 5C). We did not observed NsrR binding to the gadE and gadX promoters (Fig. 5C).

We thus performed bio-informatics analysis to identify putative NsrR-binding sites in the LEE1, LEE4 LEES, gadE, and gadX promoters in the strain EDL933. We used the homologous sequences of seven NsrR-binding sites described in E. coli K12 [29] to generate the sequence logo of the NsrR box in the strain EDL933 (Fig. 5D). We then performed bioinformatics analysis on the LEE1, LEE4 and LEE5 promoter sequences by the Gibbs Sampler Motif Software, using the matrix of the seven putative NsrR-binding sites of EDL933. In agreement with the ChIP data, bioinformatics analysis identified sequences presenting high identity with the NsrR consensus binding site in the LEE1 (P1), LEE4 and LEE5 promoters regions (Fig. 5D and Fig. S2), but not in the promoters of gadE and gadX. The analysis indicated a 23 bp putative NsrR-binding site in the promoters of LEE1 (86.9% identity) and LEE4 (78.2% identity), but only a second half-site NsrR-binding site in the LEE3 promoter (90.9% identity for the half site; Fig. 5D). In silico analyses performed using the BLAST program indicated that these putative binding sites are conserved in a number of EHEC and EPEC strains, but not in the LEE1/4/5 promoter regions (Fig. 5D and Fig. S2), but not in the LEE1/4/5 promoters (Figs. 5A and 5B). Lastly, the binding of GadE and GadX to the LEE1/4/5 promoter regions was not modulated by NOR-4. These data indicate that GadE and GadX do not repress ler expression directly. The LerE4 promoter has been identified in EHEC upstream of sptL [27], sptL being the second gene of the operon. In EPEC, it has been shown that Ler-mediated activation of the LEE5 operon requires sequences between positions -198 and -75 relative to the transcriptional start site [28]. Two primer pairs overlapping this region have been designed for ChIP experiments, amplifying a LEE5 distal (P1LEE5) and a LEE5 proximal (P2LEE5) region (Fig. S2). ChIP experiments showed that neither GadE nor GadX bound to the LEE4 and LEE5 promoters (Figs. 5A and 5B). Lastly, the binding of GadE and GadX to the LEE1/4/5 promoter regions was not modulated by NOR-4. These data indicate that control of LEE4 and LEE5 expression by GadE and GadX is due to indirect effects.

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**NsrR interacts with the RNA polymerase complex**

Since NsrR has been exclusively described as a transcriptional repressor, we investigated the molecular mechanism underlying the direct activation of LEE gene expression by NsrR. For many transcriptional activators, increase of the transcription level results from the recruitment of RNA polymerase through direct interaction between the regulatory protein and one or several subunits of the polymerase [30]. We therefore examined if NsrR can interact with σ2 and σ5 RNA polymerase subunits. To this end, His-tagged NsrR and hemagglutinin (HA)-tagged polymerase subunits σ5 (RpoA) or σ70 (RpoS) were co-expressed in bacteria. His-NsrR was purified under native conditions using a nickel affinity resin and the different fractions were analyzed by western-blot. As positive controls, RpoA and RpoS were also co-expressed with His-Crp or His-Crl, respectively, two well-known interacting partners [31,32]. All tagged proteins were properly expressed as revealed by their immunodetection in the whole extract samples (Fig. 6). As expected, HA-RpoA and HA-RpoS co-eluted with His-Crp or His-Crl, respectively. No HA-tagged protein was detected in the His eluates of the negative controls, i.e., bacteria expressing only HA-tagged proteins (Fig. 6). Importantly, HA-RpoA and HA-RpoS were also specifically recovered in the eluted fractions from the His-NsrR purifications. This finding demonstrates that NsrR can interact with the RNA polymerase complex and suggests that NsrR activates LEE gene expression through the recruitment of RNA polymerase.

**Adhesion of the regulatory mutants to HeLa cells**

In order to confirm the role of NO, NsrR, GadE, and GadX in regulating LEE expression, we investigated the attachment of the regulatory mutants to HeLa cells after 6 h of infection in the presence or absence of NOR-4. As expected, EDL933 adhered to HeLa cells and when NOR-4 was added to the co-culture the level of adhesion was dramatically reduced to that of the ΔnsrR mutant that lacks a functional T3SS (Figs. 7A and 7B). The adhesion of the ΔgadE and ΔgadX strains was higher than that of the parent strain, correlating with the repressive effect of AR regulatory proteins on LEE gene expression (Figs. 7A and 7B). Conversely, the ΔnsrR mutant was less adherent than the WT strain (Figs. 7A and 7B). The complementation of these three mutants restored the adhesion phenotype of the parental strain (Figs. 7A and 7B). Under NO exposure, adherence properties were affected for the ΔgadE and ΔgadX mutants but not for the ΔnsrR mutant (Figs. 7A and 7B), demonstrating that NsrR is the key regulator controlling the T3SS-dependent adhesion of EHEC in response to NO.

**Discussion**

In the present report, we show that NO, a critical mediator of the host innate immune response, is a potent inhibitor of LEE gene expression in EHEC O157:H7 and consequently inhibits the adhesion of these pathogens to intestinal epithelial cells. We identified NsrR as an unrecognized regulator that controls the expression of LEE genes in response to NO, and we propose a regulatory model presenting the role of NsrR, GadE and GadX in LEE expression (Fig. 8). In the absence of NO (Fig. 8A), NsrR directly activates LEE1, LEE4, and LEE5 gene expression, and indirectly represses gadE and therefore gadX expression. We also show that GadE indirectly activates gadX expression and represses LEE4 and LEE5 expression independently of Ler, while GadX inhibits gadE and LEE1 expression. When NsrR binds NO (Fig. 8B), it is released from its target DNA, leading to loss of induction of LEE1/4/5 genes and to the up-regulation of gadE and, consequently, gadX. In this context, the NO-dependent EEE1 down-regulation is due to absence of NsrR-mediated activation and to the inhibitory effect of GadX. In parallel, the inhibition of LEE4 and LEE5 gene expression is due to absence of NsrR and Ler-dependent activation and to increase of GadE level. This model assumes that repression of gadX expression by NsrR is mediated by GadE, which is consistent with the observation that the NO-dependent activation of gadX is abrogated in the ΔgadE and ΔnsrR mutants.

NsrR is a key negative regulator of the nitrosative stress in enterobacteria [18,33]. NsrR has always been described as a transcriptional repressor. In addition, its DNA-binding activity is suppressed in the presence of NO, yielding to the expression of various genes mainly involved in NO detoxification [18,33]. In non-pathogenic E. coli, NsrR also regulates expression of genes involved in metabolism, motility, protein degradation, surface attachment, stress response and transmembrane transport [29,34]. Our data indicate that NsrR is also a repressor of the genes gadE and gadX. Nonetheless, the NsrR-dependent repression of gadX is
NO Inhibits the Adhesion of EHEC to Enterocytes

A

GadE Binding (AU)

P_gadX P_1LEE1 P_2LEE1 P_1LEE4 P_1LEE5 P_2LEE5 P_gadA

B

GadX Binding (AU)

P_1gadE P_2gadE P_3gadE P_1LEE1 P_2LEE1 P_1LEE4 P_1LEE5 P_2LEE5 P_gadA

C

NsR Binding (AU)

P_1gadE P_2gadE P_3gadE P_gadX P_1LEE1 P_2LEE1 P_1LEE4 P_1LEE5 P_2LEE5 P_hmpA

D

11 bp half site

Promoter Sequence

P_1LEE1 TAAATGATT TAAATATATAG 0.64
P_LEE4 AAAAAATATTGTAACATCGT 0.95
P_1LEE5 AAAATCAGATT

p value for significance of binding.
probably mediated by GadE since the NO-dependent up-regulation of gadX is abrogated in the ΔgadE mutant. We did not find a sequence matching the NsrR consensus binding site in the gadE promoter, and ChIP experiments failed to demonstrate physical interaction between NsrR and the gadE promoter. Therefore, the effect of NsrR on gadE transcription is probably indirect and mediated by an unknown regulatory cascade controlled by NsrR.

Here we provide compelling evidence that NsrR is a direct positive regulator of LEE1, LEE4, and LEE5 operons in EHEC by binding to their own promoters. Moreover, our data also suggest that NsrR acts as a transcriptional activator by recruiting RNA polymerase to promoter regions since NsrR is able to pull-down the α and σ54 subunits of the RNA polymerase. Supporting the concept that it may also be a transcriptional activator, it has been reported that NsrR activates virulence gene expression in Salmonella Typhimurium, in particular expression of genes important for eukaryotic cell adherence, invasion and intestinal translocation, and that an nsrR mutant is impaired in invasion of HeLa cells [35]. However, in silico analysis failed to identify an NsrR consensus binding site in the promoter regions of these genes, indicating that the positive regulatory effect of NsrR is probably indirect in this pathogen [35]. Moreover, using an E. coli K12 strain harboring a multicopy plasmid that titrates out NsrR, Filenko et al. have identified by a microarray analysis 22 transcripts that could be directly or indirectly activated by NsrR [34].

The NsrR binding site is a 23 bp palindromic sequence composed of two 11 bp half sites separated by a single nucleotide, and NsrR binds to DNA as a dimer [36]. However, a number of NsrR target promoters contain only a single half site [29]. Potential NsrR consensus sequence were identified in the LEE1, LEE4 and LEE5 promoters, with a 23 pb putative NsrR-binding site in the LEE1 and LEE4 promoters, and a putative second half-site in the LEE5 promoter. It has been suggested that, when the NsrR binding site contains only a single half site, one NsrR monomer makes specific contact to the consensus half site and the other monomer forms nonspecific contact [37]. Alternatively, it has been suggested that NsrR binds as a tetramer to the complete binding motif and as a dimer when only one half site is conserved [29]. It is noteworthy that the putative NsrR binding sites

![Figure 6. NsrR interacts with RNA polymerase complex.](https://www.plospathogens.org/article/FIGURES/Figure6.png)

- **Whole Extracts**
  - HA-RpoA + + +
  - His - NsrR
  - HA-RpoA
  - His-Crp
  - His-NsrR

- **His Eluates**
  - HA-RpoA + + +
  - His - NsrR
  - HA-RpoS
  - His-Crp
  - His-NsrR/His-Crl
identified in the LEE1, LEE4 and LEE5 promoters are conserved in a number of other EHEC and EPEC strains, but not in C. rodentium, suggesting that NO also influences cell adhesion via NsrR in other E. coli attaching/effacing pathogenic human strains.

Influence of GadE on LEE gene expression remains controversial. While Tatsuno et al. described an increased expression of LEE2, LEE4, and LEE5 in a ΔgadE mutant, which is not correlated with enhancement of ler expression [20], KailasanVannaja et al. showed that GadE represses LEE expression by down-regulating ler transcription [16]. These discrepancies are proposed to be due to differences in growth medium and/or differences in the sensitivity of the assays used in each study. Interestingly, our data indicate that GadE may repress the expression of LEE4 and LEE5 via two regulatory cascades, mediated or not by Ler (Figure 8). On the one hand, we show that GadE inhibits LEE1 through GadX, because a decreased expression of gadX and an induction of LEE1 are observed in the gadE-deficient strain; this results in loss of Ler-dependent induction of LEE4/5. On the other hand, the deletion of gadX is associated with an increased expression of ler and gadE, and with an inhibition of LEE4/5, suggesting that GadE inhibits these operons independently of Ler. In accordance, the induction of espA and tir in the gadE mutant and in the ΔgadE/gadX strain demonstrates that GadX regulates LEE4/5 via the repression of gadE. However, although it has been shown in vitro that GadE can bind to the ler promoter in EHEC O157:H7 [8], we did not observe such an interaction in vivo in our experiments; this difference is probably due to the presence of binding competitors in live bacteria. Regarding GadX, we show herein that it negatively regulates ler transcription in EHEC. However, the effect of GadX on LEE1 expression is indirect since no physical interaction between GadX and the LEE1 promoter has been demonstrated. Interestingly, it has been described in

Figure 7. Regulation of adhesion of EDL933 to human epithelial cells. HeLa cells were infected with EDL933, ΔgadE, ΔgadX, ΔnrsR, or with the corresponding trans-complemented strains, in the presence or absence of NOR-4. After 6 h, cells were washed and colored with May-Grünwald Giemsa (A). The number of adherent bacteria per HeLa cell was determined from 50 cells (B).

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EPEC that LEE1 is down-regulated under conditions in which GadX is induced, namely at pH 5.5 or in contact to epithelial cells [21]; this occurs through the inhibition of the transcription of the per locus by GadX [21]. Because the perC homologue in EHEC, named pch, is involved in LEE1 induction [38], it would be interesting to now determine the role of GadX on pch expression.

The biological relevance of LEE1, LEE4, and LEE5 inhibition by NO is the decreased adhesion of E. coli O157:H7 to epithelial cells. When EHEC are ingested with the contaminated food, they first reach the stomach. It has been proposed that the acidic conditions of this ecological niche favor GadE induction and therefore limit EHEC adhesion to gastric tissues [16]. There is also abundant nonenzymatically formed NO in the gastric juice caused by acidification of nitrate and nitrite. In this context, we now propose that the NO-dependent LEE4/5 inhibition is a supplementary mechanism developed by EHEC to avoid their persistence in the stomach and to favor bacterial colonization in the colon. Moreover, we have shown in the present study that, not only a chemical source of NO, but also the reactive nitrogen species released by iNOS-expressing colonic epithelial cells inhibit the adherence of O157:H7 E. coli, and our previous work has identified NO as a potent inhibitor of Stx synthesis [17], thus, by limiting NO production, EHEC might favor their adherence to epithelial cells and therefore limit EHEC adhesion to gastric tissues [16]. There is also abundant nonenzymatically formed NO in the gastric juice caused by acidification of nitrate and nitrite. In this context, we now propose that the NO-dependent LEE4/5 inhibition is a supplementary mechanism developed by EHEC to avoid their persistence in the stomach and to favor bacterial colonization in the colon. Moreover, we have shown in the present study that, not only a chemical source of NO, but also the reactive nitrogen species released by iNOS-expressing colonic epithelial cells inhibit the adherence of O157:H7 E. coli, and our previous work has identified NO as a potent inhibitor of Stx synthesis [17], thus, by limiting NO production, EHEC might favor their adherence to epithelial cells and therefore limit EHEC adhesion to gastric tissues [16].

Materials and Methods

Bacteria, mutagenesis, and growth conditions

Strains and plasmids used in this study are listed in Table S1. The EHEC O157:H7 strain EDL933 [39] was used throughout the study. The EDL933 ΔgadE and ΔgadX mutants and the ΔgadE/ΔgadX double mutant were constructed using the one-step PCR-based method [40,41]. Mutants were verified by PCR to assess the loss of the gene and by RT-qPCR to confirm lack of expression of the gene of interest, using the primers listed in Table S2. The ΔnsrR mutant strain has been previously described [17]. For complementation analysis and ChIP experiments, the gadE, gadX, and nsrR genes were amplified with the high fidelity polymerase Pfx50 (Invitrogen) and cloned under the control of the araC promoter into a low-copy plasmid containing a 6-histidine tag (pBADHisA or pBADMycHisA; Invitrogen), or in pBAD33. The cloned genes were checked by nucleotide sequencing, and their expression was analyzed by RT-qPCR. The 6-His-NsrR-, 6-His-GadE-, and 6-His-GadX-encoding genes were expressed at the same level than the WT genes. To verify the mutation of the gadE and gadX genes, we analyzed the acid resistance of the mutant strains [42]: Acid-resistance of the ΔgadE and ΔgadX mutants dropped to 0 and 1.41% of the parent strain, respectively; acid resistance was restored in the complemented mutant strains (data not shown).

A single colony of EDL933 or isogenic mutants was grown overnight in DMEM Low glucose containing 10 mM HEPES. These cultures were diluted in fresh medium to an OD₆₀₀ = 0.03 and grown at 37°C. The medium was supplemented with ampicillin (50 µg/ml), kanamycin (50 µg/ml), chloramphenicol (25 µg/ml), L-arabinose (0.1 mM–0.5 mM), or the NO donor NOR-4 (Enzo Life Science) when required.

Bioinformatics analysis of NsrR-binding sites

The NsrR-binding sequence logo of the strain EDL933 was generated using homologous sequence of the seven NsrR-binding sites described previously by Partridge et al. in E. coli K-12 strain MG1655 [29] and the software Weblogo (http://weblogo.berkeley.edu/logo.cgi). The probabilities of occurrence matrix from the seven homologous sequences in EHEC O157:H7 strain EDL933 served as a model for the identification of a consensus sequence in the promoter regions of the target genes using the online software Gibbs Motif Sampler (http://ccmbweb.ccv.brown.edu/logo.cgi).
GadE and GadX expression was induced with 0.5 mM L-arginine in a medium buffered with 10 mM HEPES, with or without NOR-4. Overnight cultures of each strain carrying pBADHisA::nsrR plasmids, encoding 6His-GadE, 6His-GadX and 6His-NsrR, were electroporated into the respective mutants to avoid native protein interference. Overnight cultures of each strain in LB medium were diluted 1:100 in 25 ml of fresh DMEM medium buffered with 10 mM HEPES, with or without NOR-4. GadE and GadX expression was induced with 0.5 mM L-arginine and NsrR with 0.1 mM L-arginine. After 6 h of growth with shaking, ChIP was performed as described by Lambois et al. [43] with slight modifications. First, the protein-DNA complexes were cross-linked by treating bacteria with 1% formaldehyde at room temperature for 30 min. Bacteria were then washed twice with cold PBS and incubated for 30 min at 37°C in 0.7 ml of lysis buffer (10 mM Tris pH 8, 50 mM NaCl, 10 mM EDTA, and 20% sucrose) containing 10 mg/ml lysozyme (Sigma). Then, 0.7 ml of 2X IP buffer (100 mM Tris pH 8, 300 mM NaCl, 2% Igepal CA-630, 0.5% Na deoxycholate) containing 1 mM PMSF was added and samples were incubated 15 min at 37°C, cooled down on ice, sonicated, and incubated on ice for 1 min. Sonication was repeated 11 times to obtain a solution of fragmented chromatin. A 50 μl aliquot of each sample was treated with 100 μl TE containing 36 μg proteinase K for 2 hours at 37°C, incubated 8 hours at 67°C to reverse cross-linking, and the DNA was purified with the kit Qiaquick (Qiagen); this was termed as Input fraction. The rest of the fragmented chromatin was used to generate the IP fraction. After a 2 h-incubation with an anti-Histidine monoclonal antibody (Sigma), protein G sepharose 50% (40 υl) was added to each sample and incubated 1 hour at room temperature. The beads were washed twice with IP buffer, twice with 1 ml of ChIP wash buffer, twice with IP buffer, twice with 1 ml of TE buffer. The beads were resuspended in 100 μl of elution buffer (100 mM Tris HCl pH 8, 10 mM EDTA, 1% SDS), incubated 15 min at 65°C, and centrifuged at 9500 υC and 8 hours at 65°C. DNA was purified with the Qiaquick kit (Qiagen) and amplified by qPCR using the primers listed in Table S2 and depicted in Fig. S2.

The enrichment of DNA targets was calculated as follows for each protein: the promoters of interest as well as a non-specific rpoA intragenic region were amplified with specific primers (Table S2). For each DNA target, we calculated the ratio between the copy number in the IP fraction and the Input fraction; each value was then divided with the ratio obtained for the non-specific rpoA intragenic region. Then the same ratio was calculated from the enrichment of DNA targets was calculated as follows for each protein: the promoters of interest as well as a non-specific rpoA intragenic region were amplified with specific primers (Table S2). For each DNA target, we calculated the ratio between the copy number in the IP fraction and the Input fraction; each value was then divided with the ratio obtained for the non-specific rpoA intragenic region. Then the same ratio was calculated from the enrichment of DNA targets was calculated as follows for each protein: the promoters of interest as well as a non-specific rpoA intragenic region were amplified with specific primers (Table S2). For each DNA target, we calculated the ratio between the copy number in the IP fraction and the Input fraction; each value was then divided with the ratio obtained for the non-specific rpoA intragenic region. Then the same ratio was calculated from the DNA targets was calculated as follows for each protein target.

The concentration of the stable oxidized products of NO, NO3− and NO2−, was measured using the Nitrite/Nitrate Assay Kit (Cayman Chemical).

Statistics
All the data represent the mean ± SEM. Student’s t test or ANOVA with the Newman-Keuls test were used to determine significant differences between two groups or to analyze significant differences among multiple test groups, respectively.

Supporting Information
Figure S1 Kinetic of NO released by NOR-4. The concentrations of NO3− + NO2− were determined in DMEM medium containing 500 μM NOR-4. (TIF)
Figure S2 The promoter regions of gadE, gadX, LEE1, LEE4, and LEE5 in the EHEC strain EDL933. Arrows indicate the location of the primers used for the ChiP experiments. (TIIF)

Figure S3 Conservation of the putative NsrR binding site in the LEE1, LEE4 and LEE5 promoters of selected EHEC, EPEC, and C. rodentium strains. P indicates the probability that the sequence be an NsrR binding site, as determined by the online software Gibbs Motif Sampler; ns, not significant. (TIIF)

Table S1 Bacterial strains and plasmids. (DOCX)

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Table S2 List of primers. (DOCX)

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Author Contributions
Conceived and designed the experiments: PB JH GJ APG. Performed the experiments: PB SM MV AG SC GJ APG. Analyzed the data: PB GJ APG. Wrote the paper: PB GJ APG.
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