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Short communication

Mutations in *hns* reduce the adherence of Shiga toxin-producing *E. coli* 091:H21 strain B2F1 to human colonic epithelial cells and increase the production of hemolysin

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) 091:H21 strain B2F1, an isolate from a patient with the hemolytic uremic syndrome (HUS), produces elastase-activatable Shiga toxin (Stx) type 2d and adheres well to human colonic epithelial T84 cells. This adherence phenotype occurs even though B2F1 does not contain the locus of enterocyte effacement (LEE) that encodes the primary adhesin for *E. coli* O157:H7. To attempt to identify genes involved in binding of B2F1 to T84 cells a bank of mini-Tn5 ϕ ACm^r transposon mutants of this strain was generated. Several of these mutants exhibited a reduced adherence phenotype, but none of the insertions in these mutants were within putative adhesin genes. Rather, insertional mutations within *hns* resulted in the loss of adherence. Moreover, the *hns* mutant also displayed an increase in the production of hemolysin and alkaline phosphatase and a loss of motility with no change in Stx2d-activatable expression levels. When B2F1 was cured of the large plasmid that encodes the hemolysin, the resulting strain adhered well to T84 cells. However, an *hns* mutant of the plasmid-cured B2F1 strain exhibited a reduction in adherence to T84 cells. Taken together, these results indicate that H-NS regulates the expression of several genes and some potential virulence factors in the intimin-negative B2F1 STEC strain and that the large plasmid is not required for T84 cell colonization.

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Keywords: B2F1; Adherence; Hemolysin; Non-O157

1. Introduction

Strains of non-O157 STEC have been isolated from patients with hemorrhagic colitis (HC). Some of these isolates have been associated with the hemolytic uremic syndrome (HUS) in infected persons [1–4]. Four clonally-related groups of STEC have been identified and are designated EHEC 1, EHEC 2, STEC 1, and STEC 2 [5,6]. Members of EHEC groups 1 and 2 encode the locus of enterocyte effacement (LEE) and produce intimin, an adherence factor common to both EHEC groups [6,7]. The O157:H7 strains that cause most of the major outbreaks of STEC-related illness typically belong to the EHEC 1 group. STEC 1 and 2 members typically do not encode the gene for

intimin nor do they carry the LEE pathogenicity island [6]. Serotype 091:H21 strain B2F1 [8] lacks the LEE locus, as determined by Southern blot with LEE-locus probes [9], and is categorized as an STEC 1 group member. B2F1 was originally isolated from a patient with HUS (personal communication, Dr M. Karmali). We hypothesized that B2F1, like other clinical isolates of intimin-negative STEC [10–12], has the capacity to adhere to epithelial cells by an intimin-independent mechanism. Therefore, we attempted to define factors important for the adherence of B2F1 to human intestinal epithelial cells. Although an intestinal cell adhesion for the intimin-negative 091:H21 STEC strain was not identified, three new findings were generated from this study. First, we found that the H-NS protein regulates, in a positive manner, the adherence of B2F1 to T84 human colonic epithelial cells, as well as the motility of B2F1. Second, we demonstrated that H-NS negatively regulates the plasmid-encoded hemolysin and chromosomally

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encoded alkaline phosphatase produced by B2F1. Third, a plasmid-cured strain of B2F1 was used to show that the large plasmid is not required for B2F1 to adhere to T84 cells.

2. Results and discussion

2.1. Rationale for selection of bacterial strain and eukaryotic cells

Strain B2F1, a human 091:H21 isolate that has two copies of the Stx2d-activatable [8,13] adhered to T84 cells at consistently high levels (Fig. 1A). The laboratory *E. coli* control strain DH5 α adhered poorly to T84 cells (not shown). T84 cells were used as the model cell line for adherence for two reasons. First, T84 cells are not sensitive to killing by Stx because they lack the toxin binding receptor [14]. Second, under the appropriate in vitro conditions the T84 human colonic adenocarcinoma cell line grows in a polarized manner and resembles the native intestinal colonic crypt cell from which it was derived [15]. We found that B2F1 adhered with a similar phenotype (large irregularly shaped colonies) to both polarized and non-polarized T84 cells (not shown). Therefore, we elected to use non-polarized T84 cells for most of this study for ease of maintenance and growth but confirmed all adherence results with polarized T84 cells.

2.2. Isolation of mini-Tn5phoA Cm^r adherence mutants

B2F1 was mutagenized with a mini-Tn5phoACm^r transposon to screen for mutants with insertions in potential adhesins. The mini-Tn5phoACm^r used in this study was generated by replacing the kanamycin resistance gene in

mini-Tn5phoA [16] with a chloramphenicol resistance gene (not shown). Chloramphenicol was used as the selective marker in the mutagenesis rather than kanamycin because B2F1 mutates to kanamycin resistance at a high frequency. We isolated 22,555 chloramphenicol resistant transconjugates of B2F1. Approximately 1% of the chloramphenicol resistant transconjugates were blue when grown on Luria–Bertani (LB) agar with the phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (XP). The blue mutants that contained putative active alkaline phosphatase fusions to exported proteins were screened for the capacity to bind to confluent T84 cells. Briefly, wild type and mutant strains were allowed to associate with T84 cells in plastic chamber slides for a total of 3 h at 37 °C in 5% CO₂. Samples were washed 10 times with fresh Dulbecco's PBS supplemented with Ca²⁺/Mg²⁺ fixed and stained with Leukostat (modified Wright's stain from Fisher Scientific, New Hampshire), and screened qualitatively by phase contrast microscopy (assay modified from) [17,18]. Quantitative analysis was done in a similar manner except trypsin was added to the 8-well chamber slides after the 3 h incubation period. The bacterial-eukaryotic cell suspension was then serially diluted and plated on LB agar to obtain viable colony counts. Each sample was done in triplicate. The geometric mean adherent CFU was calculated for each strain tested. Of the 253 mutants screened, two, 34.3 and 34.7, had a reduced adherence phenotype (Fig. 1B shows 34.7). Quantitative analysis of a 3 h adherence assay on polarized T84 cells revealed that approximately 10⁵ CFU/ml of strains 34.3 or 34.7 were recovered from each well of the chamber slide as compared to 10⁷ CFU/ml of wild-type B2F1.

Southern hybridization analysis of *Hind*III-restricted genomic DNA from the mutants (34.7 and 34.3) with reduced adherence showed that each mutant appeared to contain a single insertion in an approximately 6 kb DNA fragment (data not shown). Furthermore, double-restriction enzyme Southern blot analysis of the two mutants revealed that the insertion was in the same DNA fragment (data not shown). Therefore, only 34.7 was analyzed further.

2.3. Sequence analysis of the mini-Tn5phoACm^r disrupted gene of mutant 34.7

The 6 kb *Hind*III DNA fragment that contained the mini-Tn5phoACm^r from mutant 34.7 was cloned by classical methods [19] into pBR322 and sequenced. Analysis of that sequence revealed that the transposon had inserted near the start of the B2F1 *hns* gene. However, the sequence analysis also showed that *hns* and *phoA* were not in the same reading frame, a finding that indicated that neither a functional H-NS nor a functional alkaline phosphatase fusion protein could be produced in 34.7. Therefore, we concluded that H-NS regulates the expression of the native alkaline phosphatase in B2F1, since 34.7 produced blue colonies on media with XP. Other investigators have reported similar findings when H-NS was inactivated [20].

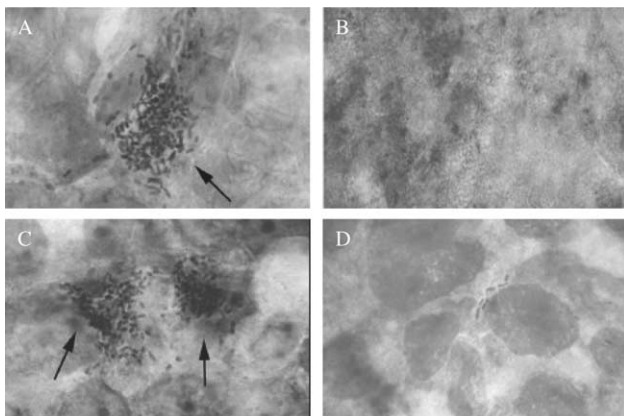


Fig. 1. Phase contrast microscopy shows the reduced capacity of strains with mutations in *hns* to adhere to T84 cells as compared to wild-type. The parental wild-type strain B2F1 is shown in panel A and its isogenic plasmid-cured derivative, S11, is shown in panel C; both strains exhibit a wild-type adherence phenotype on T84 cells. In contrast, *hns* mutants 34.7 (panel B) and S11358.19 (panel D) are deficient in adherence. Arrows indicate clusters of adherent bacteria.

2.4. Phenotypic analysis and complementation of mutant 34.7 with native H-NS

Inactivation of H-NS affects the expression of several unlinked and unrelated genes encoded in *E. coli* and *Salmonella typhimurium* [21,22]. Therefore, several characteristics of 34.7 that are potentially relevant to STEC virulence were examined. Mutant 34.7 grew at rates similar to B2F1 and expressed Stx2d-activatable at wild-type levels (not shown), but unlike the parental B2F1 strain, mutant 34.7 was nonmotile (Table 1). However, the non-motile phenotype of 34.7 was most likely not responsible for the failure of 34.7 to adhere to T84 cells, since other mini-Tn5*phoACm*^r non-motile mutants did adhere to T84 cells (not shown). Strain 34.7 also produced large zones of hemolysis on agar that contained 5% washed sheep red blood cells (SRBCs) in contrast to the small turbid zones of hemolysis produced by wild-type B2F1, Table 1. The hemolysin operon is located on the large ~90 kb plasmid carried by the vast majority of EHEC and STEC clinical isolates [23]. However, hemolytic activity is generally not detectable in the cell-free filtrates of wild-type STEC [23], an observation that we confirmed with B2F1 (not shown). In contrast, hemolytic activity was detectable in culture supernatants of 34.7 and was quantifiable by a hemolytic tube assay [24], Table 1. Furthermore, the amount of hemolysin in bacterial culture supernatants and whole cell lysates of 34.7 was greater than that produced by the parent (Table 1), as determined by Western blot analysis with antibody raised against α -hemolysin [24]. Thus, these Western blot data taken together with the hemolytic phenotype of 34.7 noted on SRBC agar and in tube assays indicate that hemolysin production is deregulated in 34.7. Similarly, H-NS in complex with Hha, a protein that belongs to a relatively new family of modulators, controls the expression of the thermoregulated α -hemolysin operon encoded by uropathogenic *Escherichia coli* [25].

The adherence capacity of mutant 34.7 was restored to wild-type levels when *hns* was introduced into 34.7 on a moderate copy number plasmid while transformation with the vector alone failed to complement the adherence defect of 34.7 (data not shown). This finding confirmed that the *hns* mutation in 34.7 was responsible for the defect in adherence. Similarly, the wild-type hemolytic phenotype was restored in mutant 34.7 complemented with wild-type *hns*, as demonstrated by small turbid zones of hemolysis around the colonies on SRBC agar (not shown, see Table 1 for a summary of phenotypic data).

2.5. Construction of a *hns* insertion mutation in a plasmid-cured derivative of B2F1 and assessment of the capacity of that mutant to adhere to T84 cells

We next sought to separate the reduced adherence phenotype of 34.7 from the hyper-hemolytic phenotype exhibited in that mutant by constructing a *hns* mutation in a B2F1 derivative lacking hemolysin. First, a hemolysin negative derivative of B2F1 was generated by curing the large plasmid from a mutant of B2F1 that carried a mini-Tn5*phoACm*^r insertion on the large plasmid. The mutant was cured by repeated passaging of the mutant in LB containing ampicillin/cycloserine. Of 11 chloramphenicol sensitive derivatives of the mutant, one, strain S11, was found not to hybridize to the probe for the large plasmid [26] and did not have a visible large plasmid present in plasmid preparations (not shown). As expected, S11 still adhered to T84 cells with a wild-type phenotype (Fig. 1C) and was not hemolytic on SRBC agar (Table 1). Second, an *hns* mutation was introduced into the non-hemolytic S11 by integration of a mobilizable suicide plasmid that contained a portion of the *hns* coding sequence into the wild-type *hns* in S11. (Note: repeated attempts to transfer the null mutation from 34.7 to S11 by allelic exchange or P1 transduction failed.)

Table 1
Genotypic and phenotypic characteristics of B2F1, S11 and their isogenic *hns* mutants

Strain	Relevant genotype	Phenotype				
		Alkaline phosphatase ^a	Adherence ^b	Hemolysis (titer) ^c	Hemolysin ^d	Motility ^e
B2F1	Wt	-/+	+	-/(0)	-	+++
34.7	B2F1 <i>hns</i> :: mini-Tn5 <i>phoACm</i> ^r	++	-	++(8)	+	-
34.7 (pHNS)	B2F1 <i>hns</i> :: mini-Tn5 <i>phoACm</i> ^r (pHNS)	-	+	-/(ND) ^f	ND	+
S11	B2F1 (large plasmid cured)	-/+	+	-(ND)	-	++
S11358.19	S11 <i>hns</i> :: pMS358	+	-	ND (ND)	ND	+
S11358.19 (pHNS)	S11 <i>hns</i> :: pMS358 (pHNS)	-	+	ND (ND)	ND	+

^a Alkaline phosphatase was determined as blue colony color after growth on LB plates with XP.

^b Adherence was measured on T84 cells as described in the text.

^c Hemolysis was detected as a clearing of red blood cells around colonies grown on tryptose blood agar with 10 mM CaCl₂ and 5% SRBCs. The hemolytic titer was determined as the reciprocal dilution where lysis of sheep erythrocytes was evident after 2 h at 37 °C.

^d Hemolysin was detected by Western blot with antibody to α hemolysin [24].

^e Motility was assayed after growth in semi-solid agar plates at 30 °C overnight.

^f ND-not determined.

Successful insertional inactivation of a gene by this method has been previously reported [27].

The construction of the *hns* mutation in S11 is described briefly as follows. First, a 358 bp portion of *hns* from S11 was amplified by the polymerase chain reaction (PCR). Second, the truncated *hns* PCR product was ligated into a temperature-sensitive suicide plasmid, pSTAMP [28], to generate plasmid pMS358. Third, plasmid pMS358 was electroporated into S11 and transformants that were ampicillin resistant were recovered at 30 °C. Fourth, stable colonies of S11 (pMS358) were incubated overnight at 44 °C on agar containing ampicillin to select for colonies with pMS358 integrated into *hns* encoded on the chromosome of S11. Putative cointegrates were analyzed by PCR and Southern blot analysis to confirm that integration of the plasmid into *hns* had occurred (not shown). Insertion of the plasmid into wild-type *hns* generated two incomplete copies of *hns* only one of which retained an intact promoter region. The H-NS produced from the copy of *hns* with an intact promoter was predicted to lack six amino acid residues at its carboxy-terminal end.

The in vitro growth rate of the S11 *hns* mutant, S11358.19, was similar to that of wild-type S11 (not shown). However, as predicted, S11358.19 showed reduced adherence to T84 cells compared to S11 (Fig. 1D). Furthermore, a quantitative adherence assay demonstrated that 10⁵ CFU/ml of S11358.19 were recovered as compared to 10⁶ CFU/ml of its parental strain S11 and B2F1. (The reason for the lower overall adherence of the wild-type B2F1 in this set of assays as compared to the assays described above is unknown, however, the adherence of B2F1 and S11 was consistently 10-fold higher than that of the *hns* mutant of S11.) These results demonstrate that the absence of H-NS function, not the presence of increased STEC hemolysin, was responsible for the reduction in the adherence of mutant 34.7 to T84 cells. Mutant S11358.19 was also blue on XP agar and exhibited reduced motility, whereas the *hns* mutant of B2F1 was non-motile, Table 1. This result suggested that the activity of H-NS in mutant S11358.19 was not completely ablated. When S11358.19 was transformed with a low copy number plasmid that encoded full-length H-NS protein, the adherence and alkaline phosphatase phenotypes were restored to wild-type levels (not shown). However, full motility was not restored to S11358.19 (pHNS), see Table 1. One explanation for this finding is that the truncated H-NS polypeptide produced by the mutant S11358.19 interacted with wild-type H-NS supplied in trans, which, in turn, resulted in a decrease in H-NS activity. Such a dominant negative phenotype is plausible since H-NS is thought to act as an oligomer or homodimer [22,29].

In summary, *hns* mutations in B2F1 or its plasmid-cured derivative S11 reduced the capacity of those mutants to adhere to T84 cells and resulted in a reduction in motility and an elevation in alkaline phosphatase activity. In B2F1 the *hns* mutation also increased the plasmid-encoded

hemolytic activity. Regulation of STEC-hemolysin expression in STEC or O157:H7 has not been previously described. Furthermore, genes encoded by the large plasmid have not been reported to be controlled by chromosomal gene products in STEC. In the enteropathogenic *E. coli* (EPEC) regulation of attachment and effacement genes by Ler, which is homologous to H-NS of *Salmonella*, has been reported [30]. Bustamante and colleagues showed that Ler activates transcription of LEE genes by competing for binding with H-NS and that in the absence of Ler H-NS represses the expression of LEE genes important in AE lesion formation [31]. For O157:H7 EHEC strains, *hns* has been suggested to be a repressor of the *espA* operon [32], the products of which are involved in early steps of adherence. Although Ler is also present in *E. coli* O157:H7, intimin-negative STEC do not encode either the LEE pathogenicity island or the Ler gene. Furthermore, our report suggests that H-NS may play a somewhat different role in the regulation of B2F1 adherence. The eventual identification of gene(s) that encode STEC adhesins in LEE-negative strains will facilitate our understanding of the mechanism by which H-NS regulates adherence.

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