The Hha Protein as a Modulator of Expression of Virulence Factors in *Escherichia coli*

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We constructed *hha* derivatives from both a clinical uropathogenic *Escherichia coli* isolate (strain FVL4) and a wild *E. coli* strain causing bovine diarrhea (strain CCB21) and analyzed the effect of the *hha* allele on the expression of the different virulence factors exhibited by these strains. Expression of hemolysin and of the Vir antigen was altered in *hha* mutants. Whereas production of hemolysin by strain FVL4 was repressed both at a low temperature and at high osmolarity, the *hha* allele accounted for a significant increase of hemolysin production under these conditions. Also, the low temperature-sensitive expression of the Vir adhesin was modified in *hha* mutants, which were able to express this adhesin at a low temperature. Expression of other virulence factors, such as cytotoxic necrotizing factor type 1 and 2 toxins, remained unmodified in *hha* derivatives of strains FVL4 and CCB21.

The Hha protein of *Escherichia coli* has been identified when analyzing hemolysin expression in *E. coli*. The well-characterized hemolysin determinant of plasmid pHly152 (19, 32) includes, in addition to a four-gene cluster (*hlyCABD*), a sequence (*hlyR*) located about 1.5 kbp upstream of the *hly* operon that is needed for hemolysin expression (38). Clones harboring the *hlyCABD* cluster but lacking the *hlyR* sequence express hemolysin very poorly (17), but high levels of hemolysin expression are obtained when such clones harbor the *hha* mutation (16). The product of the *hha* gene, the Hha protein (31), shows strong homology to the YmoA protein of *Yersinia enterocolitica*, a temperature-dependent modulator of the expression of virulence factors in this microorganism (12, 15). The *hha* gene is able to at least partially complement the phenotype of *ymoA* mutants (28), and also the *ymoA* gene, albeit in a high-gene-dosage system, is able to complement the phenotype of *hha* mutants (2). Both proteins have been considered representatives of a new class of modulators of gene expression in enterobacteria (15, 28). The phenotypes and properties of both *hha* and *ymoA* mutants resemble those of *hns* mutants (11, 12, 18, 21, 23, 26), and it has also been suggested that Hha and YmoA are histone-like proteins (11, 12).

As has been shown for other virulence factors, hemolysin expression is modulated by environmental factors such as osmolarity, temperature, and anaerobiosis (30). Concerning osmolarity, the highest levels of hemolysin expression are obtained when cells grow in salt-free medium. In fact, the *hlyR* sequence appears to be essential for hemolysin expression only when cells grow in a high-osmolarity medium. In salt-free medium, hemolysin expression in cells containing the *hlyCABD* cluster and the *hlyR* sequence is very similar to that in cells that contain the *hly* cluster but lack *hlyR* (11). In high-osmolarity medium, hemolysin expression is repressed, such repression being extreme when the *hlyR* sequence is absent. The *hha* mutation compensates for such a drastic reduction in hemoly-

sin expression in cells grown in high-osmolarity medium, both when the *hly* determinant includes *hlyR* (16) and when it does not (11). It is apparent that the Hha protein participates in the repression of hemolysin encoded by the hemolysin determinant of plasmid pHly152 in response to an increase of the osmolarity. In this study we extend our observations about the role of Hha as a modulator of the expression of hemolysin itself and also of other virulence factors in *E. coli*. We have constructed *hha* derivatives from both human and bovine *E. coli* isolates and analyzed the effect of the *hha* allele on the expression of some of the virulence factors expressed by these strains.

A set of different *E. coli* isolates of both clinical and bovine origin were transduced with a P1 lysate obtained from *E. coli* Hha-3 (*hha::Tn5*) (16). It was possible to obtain kanamycin-resistant clones from two of the strains tested (CCB21 and FVL4). *E. coli* FVL4 (O75:K1:H7 Hly⁺, cytotoxic necrotizing factor type 1 positive [CNF1⁺]) was isolated as causing urinary tract infections in human patients (8), and *E. coli* CCB21 (O54:K?:H21 Vir⁺ CNF2⁺, heat-labile enterotoxin II [LT-II] positive) was isolated as causing diarrhea in calves (7). Both *E. coli* strains displayed a wide variety of putative virulence factors, including hemolysin (strain FVL4), Vir adhesin (strain CCB21), CNF1 (strain FVL4), and CNF2 and LT-II (strain CCB21). The presence of CNF1 and CNF2 genes in these strains has been confirmed by the PCR technique (10). Also, the LT-II gene has been amplified in strain CCB21 by using specific primers (3a). We used for this study transductants obtained from both strains, termed FVL4H and CCB21H. Insertion of Tn5 in the *hha* gene of strains FVL4H and CCB21H was confirmed by PCR analysis, as has been performed for strain Hha-3 (1). When chromosomal DNA isolated from the transductants was amplified by using primers complementary to one of the ends of the *hha* gene and to the O end of Tn5, amplification fragments identical to those obtained with chromosomal DNA from strain Hha-3 were obtained (data not shown). To correlate alterations in the regulation of the expression of the virulence factors exhibited by strains FVL4H and CCB21H to the *hha::Tn5* allele, both strains were also transformed with plasmid pCB1H (2), a low-copy-number plasmid that contains the wild-type *hha* gene

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FIG. 1. Effect of the growth temperature on hemolysin production by strains FVL4, FVL4H, and FVL4H(pCB1H). The HlyA polypeptide was detected by Western blot analysis. Molecular mass markers (in kilodaltons) are shown to the left.

cloned with its own promoter in plasmid pLG338-30 (13). Alterations in regulation of gene expression that could be complemented by plasmid pCB1H were considered to be related to the *hha*::Tn5 allele.

It has been previously shown that *hha* mutants increase hemolysin expression when cells harboring the hemolytic plasmid pHly152 grow in high-osmolarity medium (16). It has also been shown that hemolysin expression is modulated by osmolarity as well as by temperature (30). Considering the strong homology between YmoA and Hha proteins and the fact that YmoA is a temperature-dependent modulator of the expression of different virulence factors in *Y. enterocolitica*, we tested if the Hha protein also participates in temperature-dependent modulation of gene expression. We first used the hemolytic strain FVL4 to test the effect of the *hha* mutation on the temperature- and osmolarity-dependent expression of hemolysin. To do this, cultures of strains FVL4, FVL4H, and FVL4H(pCB1H) were grown either at 37°C in salt-free Luria-Bertani (LB) medium and in LB medium containing 0.5 M NaCl or in LB medium at 20 and at 37°C. Hemolysin expression was monitored both by the determination of the hemolytic activity in culture supernatants and by Western blot (immunoblot) analysis. Samples were collected at an optical density at 600 nm of 0.5 for the determination of the hemolytic activity as previously described (16). Briefly, culture supernatants were mixed with washed bovine erythrocytes and incubated at 37°C. The release of hemoglobin was measured by the increase in A_{543} . For the detection of the HlyA polypeptide by Western blot analysis, 10-ml samples were collected from cultures entering the stationary phase (optical density at 600 nm = 1.0). Upon centrifugation, supernatants were 200-fold concentrated by acetone precipitation and used for the detection of hemolysin as previously described (11), using rabbit polyclonal antiserum raised against HlyA. Whereas, as described, low temperature and high osmolarity repressed hemolysin expression in strain FVL4, its *hha* derivative significantly increased hemolysin production when grown either at 20°C (Fig. 1; Table 1) or in a high-osmolarity medium (Fig. 2; Table 1). Plasmid pCB1H complemented the effect of the *hha* allele on hemolysin ex-

TABLE 1. Effect of growth temperature and osmolarity on the external hemolytic activity of culture supernatants from strains FVL4, FVL4H, and FVL4H(pCB1H)

Strain	Hemolytic activity (U) ^a in LB medium			
	At 37°C	At 20°C	Salt free	Containing 0.5 M NaCl
FVL4	220	5	300	29
FVL4H	280	76	400	194
FVL4H(pCB1H)	200	3	330	13

^a Hemolytic units = $1,000 \times (A_{543}/\text{optical density at 600 nm unit} \cdot \text{min} \cdot \text{ml})$.

pression, thus confirming that the Tn5 insertion in the *hha* gene is responsible for the deregulation of hemolysin expression depending upon osmolarity and temperature.

The Vir plasmid harbored by strain CCB21 codes for the CNF2 toxin and for a surface antigen which mediates in vitro adherence to calf epithelial tissues (29, 33, 35). Recently, it has been shown that fimbriae encoded by the Vir plasmid belong to the F17 family (27, 34). Vir⁺ cells produce fimbriae at 37°C but not at a low temperature (18°C) (29). We tested if the *hha* mutation influenced the expression of the Vir fimbriae. Expression of the adhesin was monitored both by slide agglutination using specifically adsorbed antiserum (6) and by the ability of cells to cause mannose-resistant hemagglutination, which was determined as previously described (5). Production of the adhesin was assessed in strains CCB21, CCB21H, and CCB21H(pCB1H) grown at high and low temperatures and also in high- and low-osmolarity media. As previously reported (29), growth at a low temperature resulted in repression of the expression of the Vir adhesin (Table 2). On the other hand, the *hha* derivative of strain CCB21 expressed the adhesin at both 37 and 20°C. Expression of the Vir adhesin in strain CCB21H(pCB1H) was, as in the parental CCB21 strain, repressed at 20°C. These results clearly show that the Hha protein participates in the temperature-dependent modulation of the expression of the Vir antigens. Concerning osmolarity, cells growing at 37°C under either high- or low-osmolarity conditions did not significantly modify the expression of the adhesin.

FIG. 2. Effect of osmolarity on hemolysin production by strains FVL4, FVL4H, and FVL4H(pCB1H). The HlyA polypeptide was detected by Western blot analysis. Molecular mass markers (in kilodaltons) are shown to the left.

TABLE 2. Temperature- and osmolarity-dependent expression of the Vir antigen in strains CCB21, CCB21H, and CCB21H(pCB1H)

Culture medium	Growth temp (°C)	Agglutination with Vir antigen-specific antiserum/MRHA ^a		
		CCB21	CCB21H	CCB21H (pCB1H)
CFA ^b	37	+++ / +++	+++ / +++	+++ / +++
Minca-Is	37	+++ / +++	+++ / +++	+++ / +++
LB (NaCl free)	37	+++ / +++	+++ / +++	+++ / +++
LB	37	+++ / +++	+++ / +++	+++ / +++
LB (0.5 M NaCl)	37	+++ / +++	+++ / +++	+++ / +++
CFA	20	-/-	+++ / +++	-/-
Minca-Is	20	-/-	++ / ++	-/-
LB	20	-/-	++ / ++	-/-

^a MRHA, mannose-resistant hemagglutination with calf erythrocytes; +++, strong reaction; ++, medium reaction; -, no reaction with anti-Vir antigen antibody or no hemagglutination in the presence of mannose.

^b CFA, colonization factor antigen medium.

During growth under these conditions, the *hha* allele did not affect its expression (Table 2).

We also investigated if, in addition to modulating hemolysin expression, the *hha* mutation affects the expression of other toxins produced by strains CCB21 and FVL4. Again, toxin production was assayed both under low- and high-osmolarity conditions and at low and high growth temperatures. Toxins assayed were CNF1 (strain FVL4) and LT-II and CNF2 (strain CCB21). With respect to CNF1 and CNF2 necrotoxins, detected upon mitomycin treatment as previously described (4, 5), different osmolarity and temperature conditions caused small differences in toxin production, but the *hha* allele did not significantly modify this (Table 3). With respect to LT-II, assayed as previously described (9), strain CCB21 produced it at similar low levels under all conditions assayed. Strain CCB21H failed to produce LT-II, irrespective of the growth medium or of the incubation temperature. Nevertheless, transformation of plasmid pCB1H in strain CCB21H did not result in the derepression of LT-II production (data not shown).

We show here that the Hha protein, in addition to participating in the temperature- and osmolarity-dependent regulation of hemolysin expression, participates in the modulation of other virulence factors. With respect to hemolysin itself, we extended our previous observations with the hemolysin determinant of plasmid pHly152 to the determinant of strain FVL4. The Hha protein plays a role in both osmolarity- and temperature-dependent modulation of hemolysin expression. High-osmolarity conditions repress hemolysin production (30). The

Hha protein must participate in such a repression, as suggested by the fact that *hha* mutants increase the production of the toxin under such conditions. It must be pointed out that, when grown in a high-osmolarity medium, the *hha* derivative of strain FVL4 does not express hemolysin at levels similar to those of the parental strain growing in low-osmolarity medium. This can be interpreted either as the Hha protein coparticipating with other factors in the osmolarity-mediated repression of hemolysin expression or as, the Hha protein being the unique factor causing repression, viable *hha* cells harboring compensatory mutations that partially alleviate the deregulatory effects caused by the lack of the Hha protein. In any case, it is worth mentioning here that the effect of the *hha* mutation on osmolarity-mediated regulation of hemolysin expression resembles that of *osmZ* (*hns*) on the osmolarity-mediated repression of the *proU* promoter, that is, increasing of *proU* expression under low-osmolarity conditions, but not at the same level as that obtained under full expression (high-osmolarity conditions) (20). Considering repression of hemolysin at 20°C, the effect of the *hha* mutation is similar to that observed with respect to osmolarity: *hha* mutants increase hemolysin expression at 20°C, but not to the same extent as that when growing at 37°C. Thermoregulation and osmoregulation of hemolysin by the Hha protein strikingly resemble what has been shown for H-NS with other virulence determinants (14, 24, 26, 36).

A very interesting effect of the *hha* mutation is the deregulation of the temperature-mediated repression of the adhesin encoded by the Vir plasmid. Phenotypically, this effect is the same as that observed with *hns* mutants and the expression of the Pap pilus adhesin. *E. coli drdX* (*hns*) mutants were characterized as derepressing the *pap* operon at a low temperature (18). Recently, it has been shown that *E. coli hns* mutants also express at low temperatures the colonization factor antigen I fimbriae (24). Interestingly, two different proteins such as H-NS and Hha appear to participate in the same microorganism in regulatory processes that respond to the same environmental signals.

The synthesis of CNF1 and CNF2 toxins was only slightly modified by osmolarity and temperature, the *hha* allele not influencing it. With respect to the effect of the *hha* mutation on LT-II synthesis, the results obtained in this work are not conclusive. Whereas strain CCB21 constitutively expresses LT-II at low levels, its *hha* derivative does not. Nevertheless, plasmid pCB1H failed to complement the effect of the *hha* mutation, thus suggesting that repression of LT-II production in strain CCB21 in fact is not directly related to the *hha* allele. In addition, the low production of LT-II by the wild strain CCB21 makes it difficult to obtain quantitative results to precisely compare toxin production between the different strains. Further genetic analysis is required to precisely measure and compare LT-II expression in the different strains. In any case, if the *hha* mutation in fact accounts for repression of LT-II synthesis, these phenotypic properties of *hha* mutants would also resemble those of *hns*: whereas the expression of some genes is enhanced, the expression of others is repressed (3, 20–22, 25, 37).

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TABLE 3. Effect of the *hha* mutation on the production of CNF1 and CNF2 toxins by strains FVL4 and CCB21, respectively

Culture medium	Growth temp (°C)	Dilution ^a for:			
		CNF1		CNF2	
		FVL4	FVL4H	CCB21	CCB21H
TSB ^b	37	1/128	1/128	1/256	1/256
LB (NaCl free)	37	1/128	1/128	1/256	1/256
LB (0.5 M NaCl)	37	1/128	1/128	1/128	1/128
TSB	20	1/64	1/64	1/128	1/128
LB (NaCl free)	20	1/64	1/64	1/64	1/64
LB (0.5 M NaCl)	20	1/64	1/64	1/16	1/16

^a Highest dilution of filtrates of cultures treated with mitomycin that produced the cytopathic effect exerted by CNF1 and CNF2.

^b TSB, tryptic soy broth.

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