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# Active Cytotoxic Necrotizing Factor 1 Associated with Outer Membrane Vesicles from Uropathogenic *Escherichia coli*

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**Cytotoxic necrotizing factor type 1 (CNF1) is one of the virulence factors produced by uropathogenic *Escherichia coli* (UPEC). How this toxin is translocated from the bacterial cytoplasm to the surrounding environment is not well understood. Our data suggest that CNF1 may be regarded as a secreted protein, since it could be detected in culture supernatants. Furthermore, we found that CNF1 was tightly associated to outer membrane vesicles, suggesting that such vesicles play a role in the secretion of this protein. Interestingly, vesicle samples containing CNF1 could exert the effects known for this protein on HeLa cell cultures, showing that CNF1 is transported by vesicles in its active form. Taken together, our results strongly suggest that outer membrane vesicles could be a means for the bacteria to deliver CNF1 to the environment and to the infected tissue. In addition, our results indicate that the histone-like nucleoid structuring protein H-NS has a role in the downregulation of CNF1 production and that it affects the outer membrane vesicle release in UPEC strain J96.**

Uropathogenic *Escherichia coli* (UPEC) strains are responsible for at least 80% of human urinary tract infections (UTIs), which include urethritis, cystitis, and pyelonephritis (21). UTI is among the most common bacterial infections in humans and has been shown to be an independent risk factor for both bladder cancer and renal cell carcinoma (30). To achieve pathogenicity, the UPEC strains produce many virulence factors such as adhesins (P and type 1 pili), hemolysin, aerobactin siderophore system, a capsule, and cytotoxic necrotizing factor type 1 (CNF1). CNF1 belongs to a group of bacterial necrotic substances which include the dermonecrotic toxin of *Bordetella* spp. (14), the virulence plasmid-encoded CNF2 of *E. coli* (14, 29), and the recently described CNFy from *Yersinia pseudotuberculosis* (24). Unlike CNFy, whose mechanism of action is specific to RhoA (13), CNF1 affects three proteins of the Rho family of the small GTPases: RhoA, Rac1, and Cdc42. CNF1 deamidates Gln63 of RhoA (or the corresponding Gln61 of Rac1 and Cdc42), leading to the loss of both intrinsic and GAP-stimulated GTPase activities but without affecting the GTP-binding activity (14). This modification renders these GTPases constitutively active, which commonly results in formation of cellular actin stress fibers, filopodia and lamellipodia, with an overall effect of blocking the cytokinesis, leading to giant multinucleated cells (7, 23, 25). CNF1 is a 115-kDa A-B toxin, in which a catalytic A domain is located in the C-terminal region of about 300 amino acids and where the cell binding B-domain is in the N-terminal region (amino acids 53 to 190) (6, 22). No typical signal peptide is found in the CNF1 sequence, and the secretion pathway of this toxin remains unclear. In this work we show that CNF1 is secreted and that it is associated with membrane vesicles. Furthermore, we present evidence that CNF1 is down regulated by H-NS (histone-like nucleoid structuring protein).

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## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** As UPEC clinical strains, we used *E. coli* J96 (17) and an isolate (RZ422) from the collection of the "Institut für Molekulare Infektionsbiologie der Universität Würzburg" obtained from patients suffering from either cystitis or pyelonephritis (39). *E. coli* LE392 was transformed using either a pISS391 cosmid or empty vector pHC79 (20), resulting in the K12/*cnf1*$^+$ strain and LE392/pHC79, respectively. The J96 mutants used in these studies were the Δ*hns* derivative BEU801 (33) and the Δ*cnf1* derivative USU128, which carries the Δ*cnf1–120* allele (32).

Bacterial cells were allowed to grow at 37°C on LA (LB medium supplemented with agar) plates or in LB medium. The same media were used for BEU801 and the LE392 transformants supplemented with 15 μg/ml tetracycline and 50 μg/ml carbenicillin, respectively.

**PCR detection of the *cnf1* gene.** The oligonucleotide primers used to confirm the presence of the intact or mutant CNF1 gene in UPEC strains were 5′-TATTAATCTTCACAGAGGAG-3′ (forward primer) and 5′-GGCCAATAAATAATTTCCCGAATC-3′ (reverse primer); the PCR conditions have been previously described (32).

**Cell fractionation and culture supernatants.** Cytoplasmic, periplasmic, and membrane fractions were obtained following procedures described previously (38).

The supernatants from overnight cultures were passed through a 0.45-μm-pore-size Millipore filter (to remove bacterial cells) and centrifuged at 100,000 × *g* for 3 h to spin down any floating material. Proteins soluble in the supernatants were obtained from 500 μl of these samples by trichloroacetic acid (TCA) precipitation. The precipitated proteins were resuspended in Tris-HCl buffer.

**Isolation of OMVs.** Outer membrane vesicles (OMVs) were isolated as previously described (36). Briefly, bacterial cultures were centrifuged at 5,000 × *g* for 30 min at 4°C, and the supernatants were filtered through a 0.45-μm-pore-size polyvinylidene difluoride membrane. The filtrates were submitted to ultracentrifugation (100,000 × *g* for 3 h) at 4°C in a 45Ti Rotor (Beckman Instruments, Inc.). The vesicle samples were obtained by suspending the resulting pellets in 20 mM Tris-HCl (pH 8.0). Before analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the vesicle preparations were precipitated with 10% TCA in order to free the samples from non-protein interfering substances.

**Western blot analysis.** Samples were subjected to SDS-PAGE using various acrylamide percentages and were transferred to a polyvinylidene difluoride membrane using a semidry transfer system from Bio-Rad. The membrane was blocked overnight in 5% milk powder in phosphate-buffered saline (PBS)-Tween 20 at 4°C. The primary antibodies were rabbit polyclonal antiserum raised against CNF1 (6), the TolC protein (35), the H-NS protein (18), the Lep protein (4), the DsbA protein (1), the OmpA protein (11), and the Crp protein (this laboratory, unpublished data), respectively. Detection using horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Amersham Biosciences) was carried out following the instructions provided with an ECL$^+$ kit (Amersham Biosciences). Chemiluminescent bands were imaged by autoradiography or by using a Fluor-S MultiImager (Bio-Rad).

**Dissociation assay.** A dissociation assay was carried out as described by Horstman and Kuehn (15), with few modifications. Briefly, 20 μl of the Δ*hns* J96 strain BEU801 vesicle preparation was treated in 1 M NaCl, 0.8 M urea, 1% SDS, or 20 mM Tris-HCl, pH 8.0, on ice for 1 h in a total volume of 1 ml. Samples were centrifuged for 1 h, and the pellets were resuspended in 10 μl of Tris-HCl buffer. The resulting supernatants were TCA precipitated and resolubilized in the same amount of Tris-HCl buffer. Both pellets and supernatants were analyzed.

**Electron microscopy.** The purified vesicle samples were visualized after negative staining using 0.5% uranyl acetate in a JEM 2000EX electron microscope (JEOL, Akishima, Tokyo, Japan) at 100 kV.

**Atomic force microscopy (AFM).** Bacterial cultures were grown overnight on LA plates at 37°C. Bacteria were washed and resuspended in 100 μl of ultrapure water, and 10 μl of the suspension was placed onto freshly cleaved mica (Goodfellow Cambridge Ltd., Cambridge, United Kingdom). The cells were incubated for 5 min at room temperature and rinsed with water. After the specimens were dried, they were placed into a desiccator for at least 2 h. Samples were imaged in air with a Nanoscope IIIa AFM (Digital Instruments, Santa Barbara, CA) by using the tapping mode with standard silicon cantilevers oscillating at resonant frequency (270 to 305 kHz).

**Cell lines and growth conditions.** HeLa cells were seeded (about $5 \times 10^4$ cells per well) onto cover glasses (diameter, 12 mm) placed in a 24-well tissue culture plate (Sarstedt) in minimum essential medium (Sigma) supplemented with 10% fetal bovine serum and eventually 15 μg/ml of chloramphenicol, at 37°C in a humid environment containing 5% $CO_2$.

**HeLa cell treatment with CNF1 or vesicle samples.** Purified CNF1 was used at a concentration of 2 nM. In tests using OMVs, we used 100 μl of each vesicle preparation. The pure CNF1 or OMVs were added at cell seeding, and the culture medium was replaced with fresh medium after 20 to 24 h and 48 h of incubation, respectively. The cells were then monitored for up to 72 h under these conditions. At different time points, cells were subjected to phase-contrast microscopy or stained.

**DAPI staining.** Cells were fixed with 3% paraformaldehyde for 10 min at room temperature, washed twice with PBS, and stained with a 1 μg/ml DAPI (4′,6′-diamidino-2-phenylindole hydrochloride) solution for 10 min. After two washes the cells were subjected to fluorescence microscopy.

**Actin staining.** The cells were stained for actin filaments as previously described (34), with some modifications. Briefly, the cells were fixed with 3% paraformaldehyde for 10 min at room temperature, washed twice with PBS, and permeabilized with 0.5% Triton X-100 for 3 min. After two washes with PBS, the actin microfilaments were stained with Alexa Fluor 568 phalloidin (Invitrogen/Molecular Probes) for 20 min at room temperature and washed thoroughly to remove the excess of fluorescent dye. The specimens were then mounted in a medium containing Cityfluor (Cityfluor Ltd., London, United Kingdom), and the cells were subjected to fluorescence microscopy on the following day.

**Quantitations and statistics.** Western blotting data were quantified using the volume analysis tool of the QuantityOne software (Bio-Rad). The protein amounts were determined using a bovine serum albumin standard curve in a Bio-Rad protein assay kit. Multinucleation was evaluated as the percentage of multinucleated cells from the total number of cells observed (at least 28 cells taken in different areas of the coverslips in each case) under light microscopy. All data are presented as the means ± standard deviations from values obtained in independent experiments.

## RESULTS

**CNF1 expression and subcellular localization in wild-type and Δ*hns* mutant UPEC strains.** It is well known that CNF1 is composed of a cell binding domain (N-terminal), a membrane-spanning domain, and a catalytic C-terminal domain (3), while no typical signal peptide has been found in the sequence. We decided to determine the CNF1 subcellular localization by fractionation of UPEC strain J96. For these experiments the J96 Δ*hns* mutant (strain BEU801) was studied as well, since our preliminary studies (see also below) indicated that whole-cell lysates of this derivative contained more CNF1 compared to wild-type J96. When the cell fractions of these strains were analyzed by Western immunoblotting, CNF1 was found in the cytoplasmic and periplasmic fractions (Fig. 1A). The cytoplasmic fraction of the Δ*hns* mutant contained more CNF1, which is in good accordance with the CNF1 content of the whole-cell samples. A similar difference in the amounts of CNF1 was detected also in the periplasmic fractions (Fig. 1A). These results suggested that the protein could cross the bacterial inner membrane. We used three well-known *E. coli* proteins as markers with respect to the cytosolic protein fraction and the periplasmic fraction, respectively. The cyclic AMP receptor protein (Crp) is a cytosolic protein acting as a transcriptional regulator (8), and the histone-like nucleoid-associated protein H-NS is an abundant peptide that binds to DNA and regulates the expression of several genes in *E. coli*. The DsbA (disulfide oxidoreductase) protein is a major component of the system for disulfide bond formation in the periplasm (2). Western blotting using antibodies directed against the cytoplasmic markers Crp and H-NS indicated that the appearance of CNF1 in the periplamic fraction was not a consequence of contamination by the cytosol due to, e.g., bacterial cell lysis. As expected, DsbA, which is known to reside in the periplasm, was found in large amounts in these fractions (Fig. 1A). Furthermore, Western blot analysis revealed the presence of CNF1 in outer membrane (OM) fractions from both J96 wild-type and Δ*hns* mutant strains (Fig. 1B). Leader peptidase (Lep), used in these studies as an inner membrane (IM) marker, was present only in the IM fractions (Fig. 1B), and the common *E. coli* porins were found in the OM fractions (data not shown).

In order to determine whether the CNF1 protein was released from the bacterial cells, supernatants from UPEC cultures were filtered and centrifuged as described in Materials and Methods. A relatively strong CNF1 band could be detected in the samples from strains BEU801 (J96 Δ*hns*) and LE392/pISS391. A corresponding band but with lower intensity was present in the case of the supernatant from the J96 wild type as well, while no CNF1 was detected in the RZ422 sample (Fig. 1C). Based on data analysis of the CNF1 bands shown in Fig. 1C using the volume analysis tool of the QuantityOne software (Bio-Rad), it was estimated that only a minority (<5%) of the CNF1 protein is secreted from the bacterial cells.

As indicated above, when analyzing the total cell lysates for CNF1 by Western immunoblotting, we found that the Δ*hns* mutant expressed about 10 times more CNF1 than the wild type, which suggested a downregulation of CNF1 production by H-NS in wild-type J96 (Fig. 2A and B). We used the *E. coli* Δ*cnf1* strain as a negative control and the OM protein TolC as a reference when quantifying the relative amount of CNF1 in the J96 derivatives.

**CNF1 is associated with OMVs.** Since recent findings suggest that protein toxins in some cases may be released via OMVs (36), we decided to determine whether CNF1 could be detected in association with such vesicles.

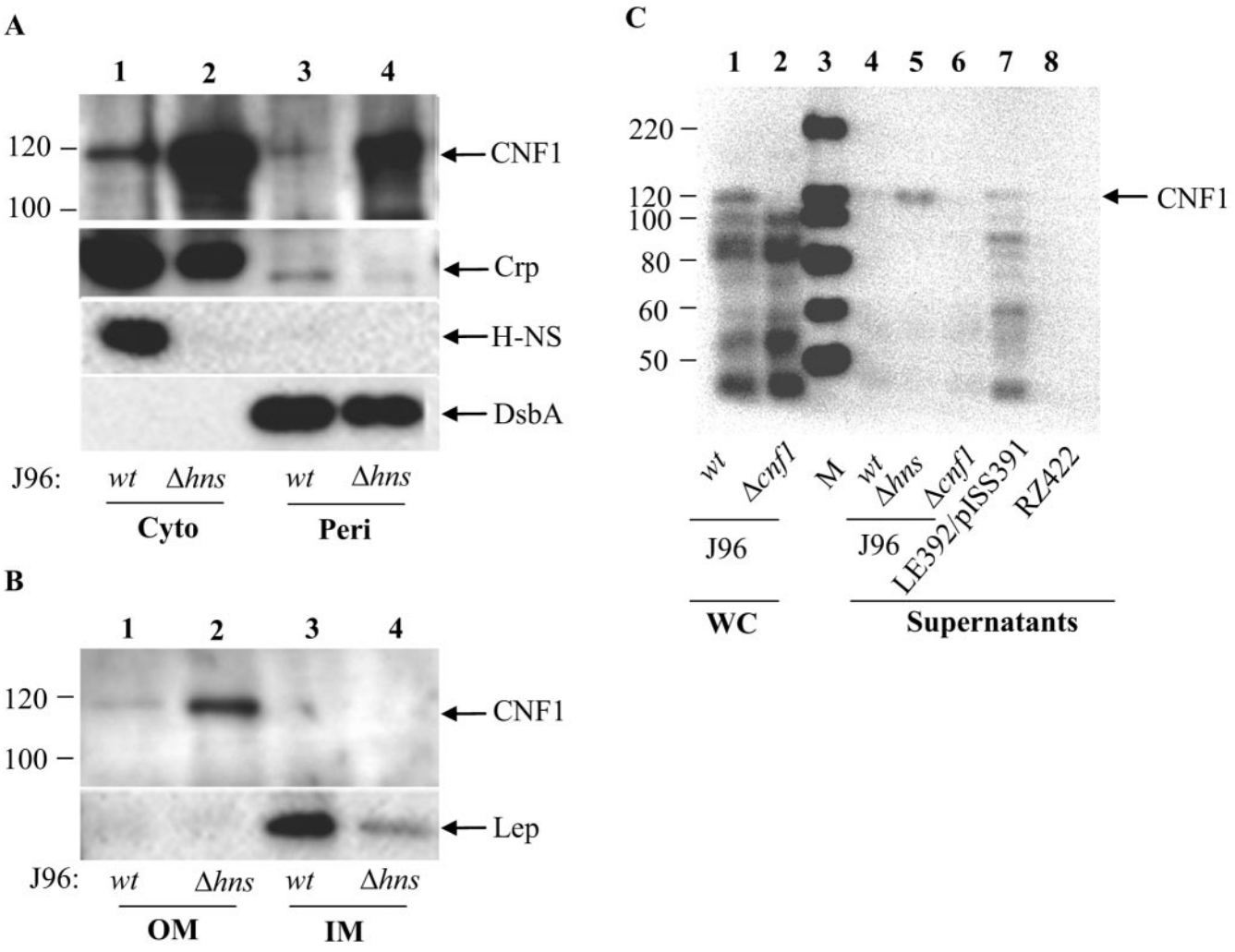


FIG. 1. CNF1 subcellular localization and secretion in *E. coli* J96. (A) Western blot analysis of the cytoplasmic (Cyto) and periplasmic (Peri) fractions from J96 (*wt* and Δ*hns*). The numbers at the left indicate the sizes of molecular standards (MagicMark XP; Invitrogen Life Technologies) on a 6.5% polyacrylamide gel. Along the right side are shown the proteins (CNF1, Crp, H-NS, and DsbA) detected by specific antisera. (B) Western blot analysis of the OM and IM fractions from J96 (*wt* and Δ*hns*). The numbers at the left indicate the sizes of molecular standards (MagicMark XP) on a 13.5% polyacrylamide gel. Along the right side are shown the proteins (CNF1 and Lep) detected by specific antisera. (C) Western blot detection of CNF1 in the bacterial culture supernatants. Lanes 1 and 2 are whole-cell lysates (WC) from J96 (*wt*) and USU128 (J96 Δ*cnf1*), positive and negative controls for CNF1, respectively. The numbers at the left indicate the sizes of the recombinant proteins of the MagicMark XP Western protein standard (Invitrogen Life Technologies) loaded in lane 3. The supernatants were obtained from J96 (*wt*, lane 4), BEU801 (J96 Δ*hns*, lane 5), USU128 (J96 Δ*cnf1*, lane 6), LE392/pISS391 (lane 7), and RZ422 (lane 8) broth cultures. In each case, 500 μl of supernatant after filtration and centrifugation was precipitated with 10% TCA. The precipitate was dissolved in 10 μl of 20 mM Tris-HCl (pH 8.0), mixed with 4× sample buffer and boiled before loading. wt, wild type.

Studies of the bacteria using AFM revealed that the Δ*hns* cells had more vesicles surrounding the bacteria than wild-type strain J96 after growth on plates (Fig. 3A and B). Furthermore, the electron micrographs of the purified vesicle samples from these two strains after growth in LB medium confirmed that the Δ*hns* mutant derivative released more OMVs than the wild type (Fig. 3D and E). The total protein concentration of vesicles from the Δ*hns* mutant was almost 10-fold that of its wild-type counterpart (Fig. 3C), and this could be observed on gels when either Coomassie blue staining or silver staining was used. Presumably, much of the difference was due to the presence of more pili proteins since the Δ*hns* mutant is hyperpiliated, but in part we consider the difference due to more OMVs. In support of this, a Western blot analysis revealed that OmpA, the OM protein used as a marker for OMVs (36), was about twofold more abundant in the OMV preparation from J96 Δ*hns* in comparison with that from the wild-type J96 strain (Fig. 4A). There was no significant difference in the quantity of vesicles released when the J96 wild-type and the Δ*cnf1* mutant (USU128) strains were compared (Fig. 3D and F), and the OmpA band intensities were similar in these two strains (Fig. 4A). These observations suggest that absence of the H-NS protein alters the quantity of vesicles released from J96. The presence of OmpA protein in the vesicle samples as analyzed by Coomassie-stained SDS-PAGE gels and Western immunoblotting (Fig. 4A and data not shown)

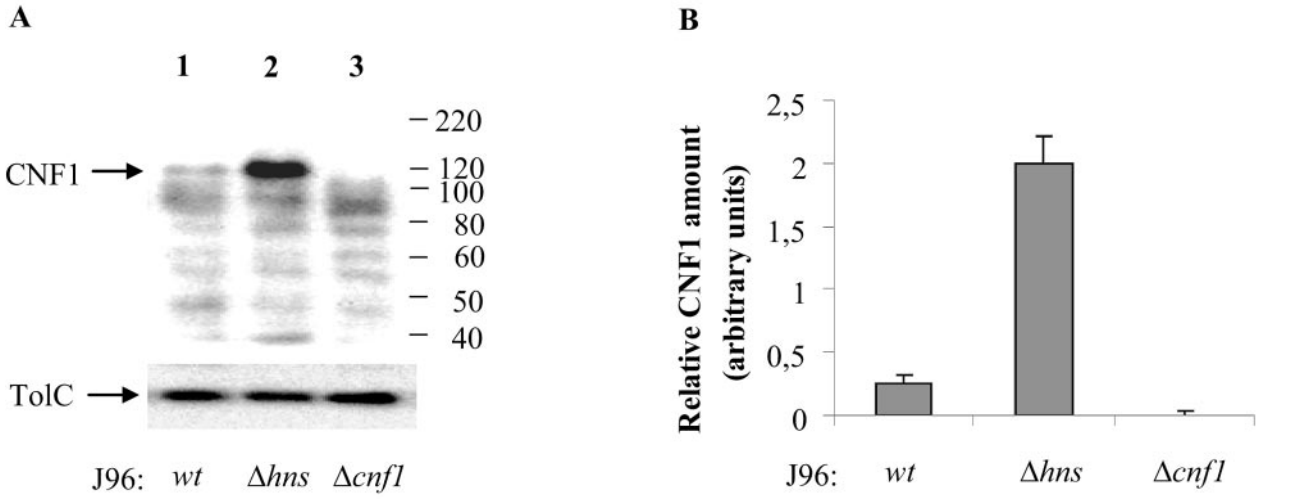


FIG. 2. Western blot analysis of whole-cell lysates with anti-CNF1 antibody (A) Samples were obtained from J96 (lane 1), J96 Δ*hns* mutant (lane 2), and J96 Δ*cnf1* mutant (lane 3) strains. The numbers at the right indicate the sizes of molecular standards (MagicMark XP, Invitrogen Life Technologies) on an 8% polyacrylamide gel. (B) Relative CNF1 amount was calculated from the estimated CNF1 quantity divided by that of TolC in each sample. wt, wild type.

confirmed that the preparations are OMVs (15, 36, 37). The vesicle preparations were precipitated with 10% TCA in order to free the samples from non-protein interfering substances.

To reveal the eventual presence of CNF1 in the OMVs, Western blotting with anti-CNF1 antibodies was performed. As shown in Fig. 4A, a weak but recognizable immunoreactive band corresponding to CNF1 was detected in the vesicle samples of the UTI clinical isolate J96 (Fig. 4A, lanes 3 and 8). In analogy with previous results, vesicles from Δ*hns* mutant bacteria showed a stronger CNF1 band (Fig. 4A, lane 4). In the case of another clinical extraintestinal pathogenic *E. coli* iso-

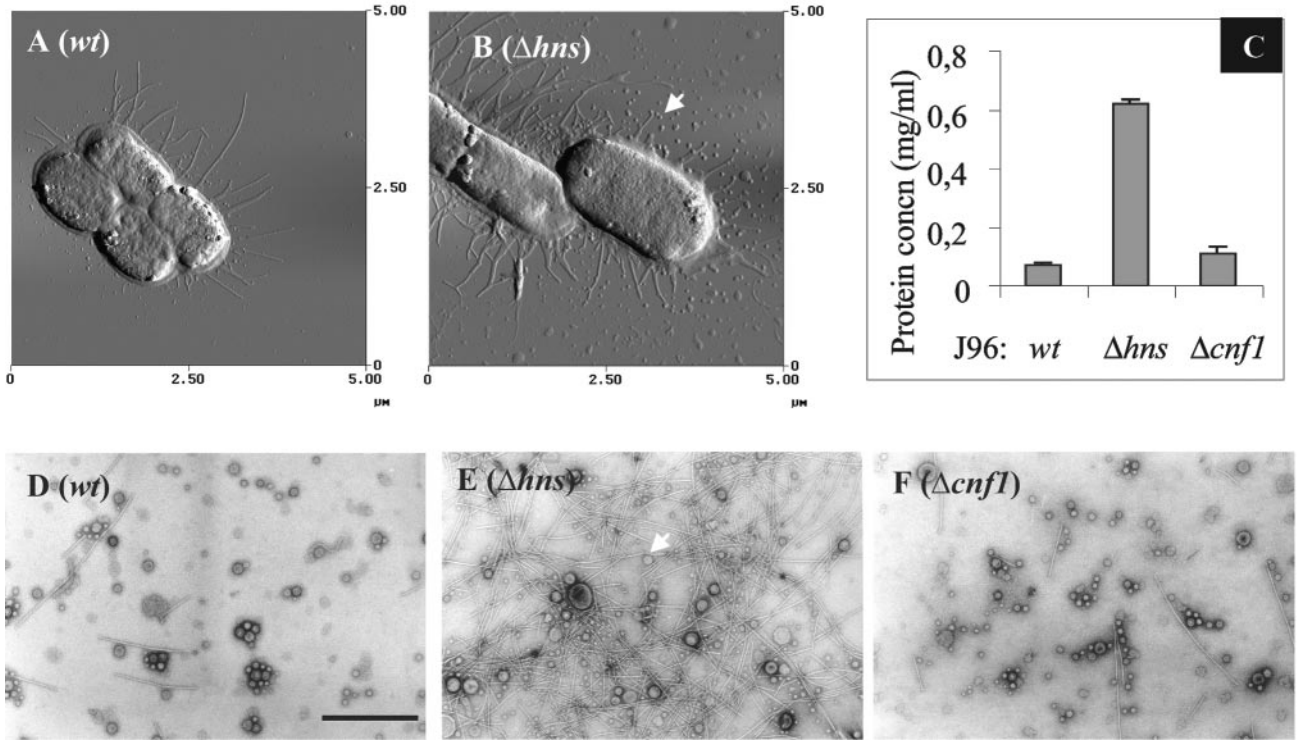


FIG. 3. Comparison of OMVs released by J96 and its Δ*hns* and Δ*cnf1* mutants. (A and B) AFM images of J96 wild-type and Δ*hns* mutant bacteria, respectively. (C) Protein quantification in the vesicle samples from respective strains. The electron micrographs of the purified vesicles are shown in panels D (J96), E (Δ*hns*, BEU801), and F (Δ*cnf1*, USU128). Bar, 500 nm. The arrows show examples of the released OMVs. wt, wild type.

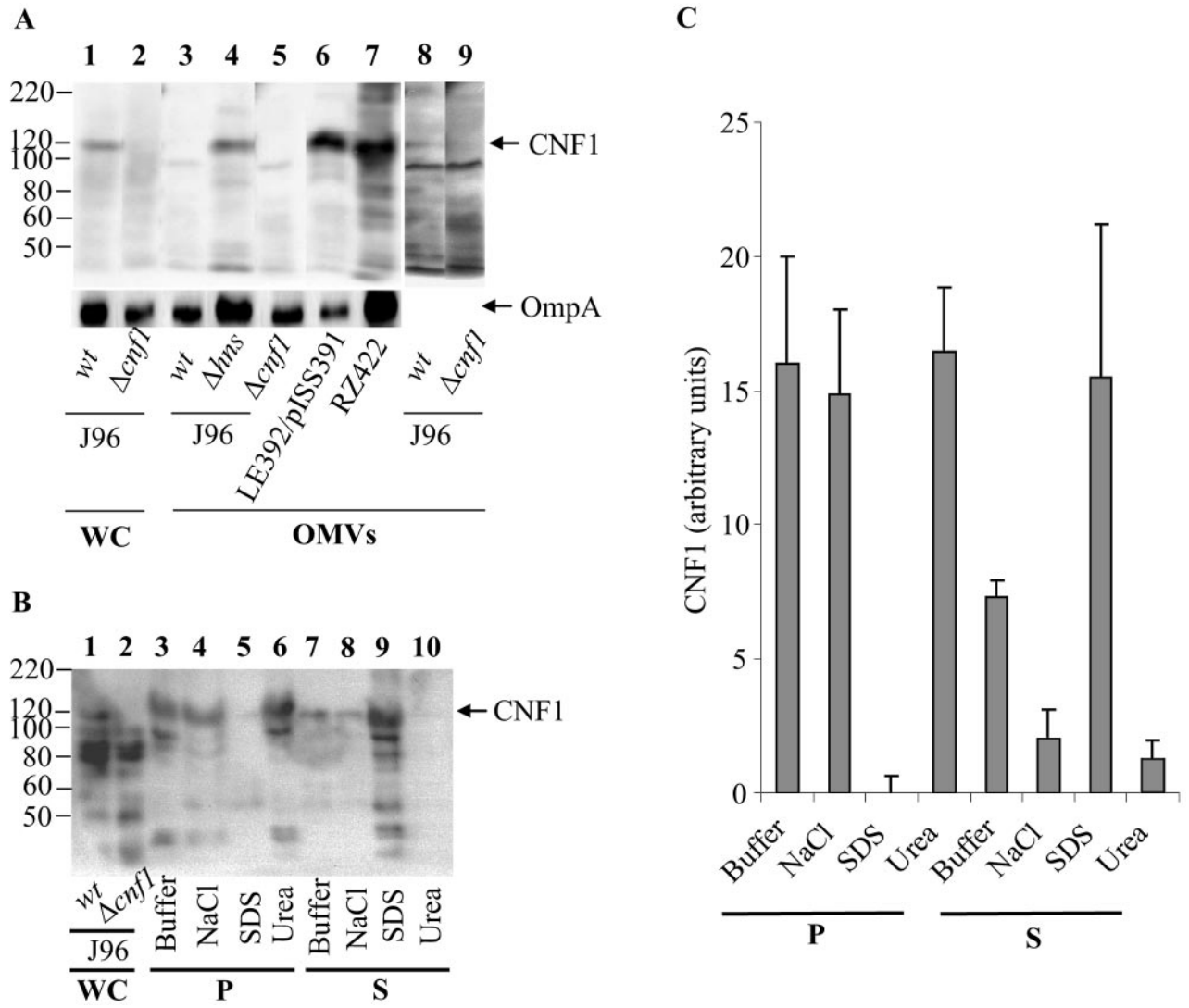


FIG. 4. CNF1 is associated with OMVs. (A) Whole-cell lysates (WC) from J96 (lane 1) and USU128 (lane 2) were used as positive and negative controls for CNF1, respectively. OMVs analyzed by Western blotting were from J96 (*wt*, lanes 3 and 8), BEU801 (Δ*hns*, lane 4), USU128 (Δ*cnf1*, lanes 5 and 9), LE392/pISS391 (K12/*cnf1*$^+$, lane 6), and RZ422 (clinical isolate, lane 7). Lanes 8 and 9 show results from enhanced detection by autoradiography in the case of OMVs from *wt* and Δ*cnf1* mutant J96, respectively, since the bands were too weak to be seen otherwise (lanes 3 and 5). The lower panel shows the OmpA band in the various samples as detected by Western blot analysis. The quantification of the levels of OmpA (by using the Fluor-S MultiImager and QuantityOne software) gave the following relative values for the five different OMV samples (the wild-type sample in lane 3 was arbitrarily set to 1): 1.0, 2.04, 1.17, 0.65, and 2.78, respectively, for lanes 3 to 7. (B) Lanes 1 and 2 were the same as shown in panel A. OMVs from the J96 Δ*hns* mutant were treated with Tris-HCl buffer (lanes 3 and 7), NaCl (lanes 4 and 8), SDS (lanes 5 and 9), and urea (lanes 6 and 10). After treatment, the samples were centrifuged, and the pellet (P) as well as the supernatants (S) was analyzed by Western blotting. The numbers at the left in panels A and B indicate the sizes of molecular standards. (C) Quantitative analysis of the dissociation assay. The CNF1 amounts (arbitrary units) were obtained by employing the volume analysis tool of the QuantityOne software (Bio-Rad), and the values are means ± standard deviations from two independent sets of data as shown in panel B. wt, wild type.

late, strain RZ422, the band attributed to CNF1 was even stronger (Fig. 4A, lane 7). In order to further confirm the presence of CNF1 in the vesicle samples, we used an *E. coli* K-12 strain carrying a cosmid that contains the CNF1 determinant, strain LE392/pISS391. The whole-cell lysate of this strain showed a relatively high CNF1 level, even with this low-copy-number cosmid (data not shown). The vesicle sample from LE392/pISS391 showed a band indicating that CNF1 was relatively abundant in the vesicle fraction (Fig. 4A, lane 6). Taken together, these data show evidence of an association of CNF1 with membrane vesicles in *E. coli*.

In order to access the type of association between CNF1 protein and OMVs, we performed a dissociation assay as described in Materials and Methods. The vesicle preparations were treated with 20 mM Tris-HCl, pH 8.0, 1 M NaCl, 0.8 M urea, or 1% SDS. In these experiments, the J96 Δ*hns* mutant was analyzed since the vesicle preparations from this strain showed relatively large amounts of CNF1. As shown in Fig. 4B

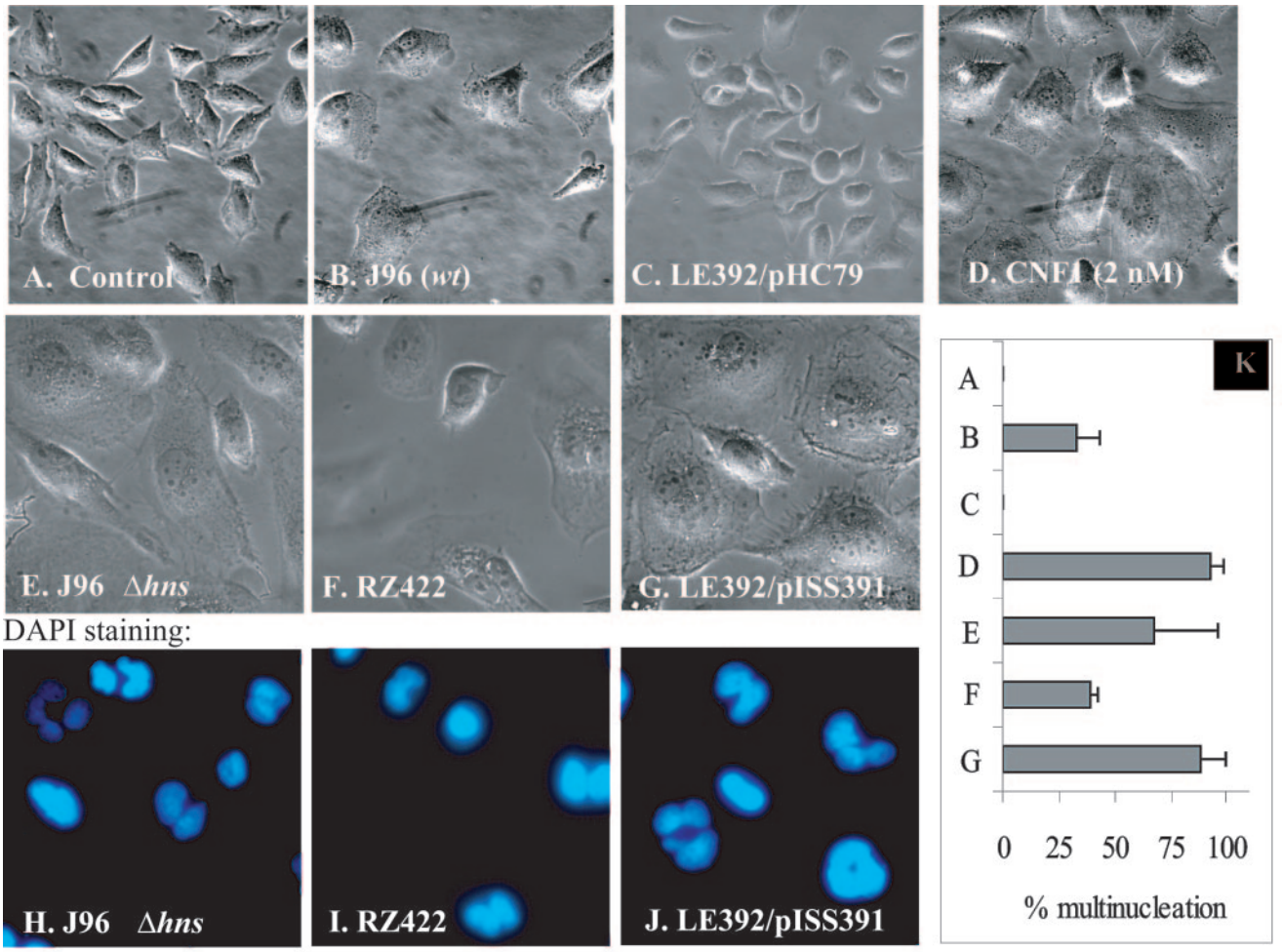


FIG. 5. CNF1-containing vesicle preparations induce enlargement and multinucleation in HeLa cells. The vesicle samples analyzed were from the J96 (B), J96 Δ*hns* mutant (E and H), RZ422 (F and I) and LE392/pISS391 (G and J) strains, and the amounts of total protein in these vesicle preparations were about 7 μg, 60 μg, 10 μg, and 10 μg, respectively (which correspond to 100 μl of each vesicle sample). The same volume (100 μl) of the OMV preparation from LE392/pHC79 was used in these assays (C). Control HeLa cells without any treatment are shown in panel A, whereas panel D shows HeLa cells treated with purified CNF1 (2 nM). Cells were incubated for 48 h, and the magnification was the same for each sample. Panels A to G are phase-contrast micrographs, whereas panels H to J are fluorescence micrographs of cells after DAPI staining. Panel K shows the percentage of multinucleated cells as means ± standard deviations from observation of cells represented in the different samples as shown above: panel A, $n = 86$ cells; panel B, $n = 32$ cells; panel C, $n = 53$ cells; panel D, $n = 28$ cells; panel E, $n = 67$ cells; panel F, $n = 56$ cells; and panel G, $n = 58$. wt, wild type.

the CNF1 protein was recovered with vesicles in the pellet after treatment with urea, salt (NaCl), or buffer. Upon SDS solubilization of the vesicles, however, there was no detection of the CNF1 protein in the pellet. The CNF1 protein was instead liberated to the supernatant in this case. The CNF1 protein was not found in the supernatant after urea treatment, while it could be detected after treatment with NaCl, but not at a greater extent than with the buffer (Fig. 4C). These data suggest that CNF1 is tightly associated with the OMVs since it was resistant to salt and urea disruptions and was liberated only after SDS solubilization.

**CNF1 in the vesicle is in its active form.** Since we could detect CNF1 in vesicle samples from some bacterial strains, the question arose as to whether the vesicle-associated CNF1 protein was active. To address this, we checked if the vesicle samples containing CNF1 could exert the effects known for this protein on HeLa cell cultures.

In these experiments, HeLa cells were treated with the vesicle samples obtained from the RZ422, J96, J96 Δ*hns*, J96 Δ*cnf1*, LE392/pHC79, and LE392/pISS391 strains and purified CNF1 (as positive control). The CNF1-containing vesicle preparations induced enlargement (phase-contrast microscopy) and multinucleation (fluorescence microscopy after DAPI staining) of HeLa cells, and the effects were clearly observed after 48 h of incubation (Fig. 5). The J96 Δ*hns* mutant caused a more pronounced effect than the wild type, suggesting a correlation between the CNF1 amount and the changes occurring in HeLa cells. After 72 h of incubation, more multinucleated HeLa cells could be observed when the vesicle samples from J96 were used. At this time, many cells treated with pure CNF1 or vesicle preparations with high CNF1 content were more or less dead. Within the first 24 h, no clear difference was recorded for all the samples, and the cells seemed to be quite intact. On the other hand, the effect of the pure CNF1 at 2 nM was greater

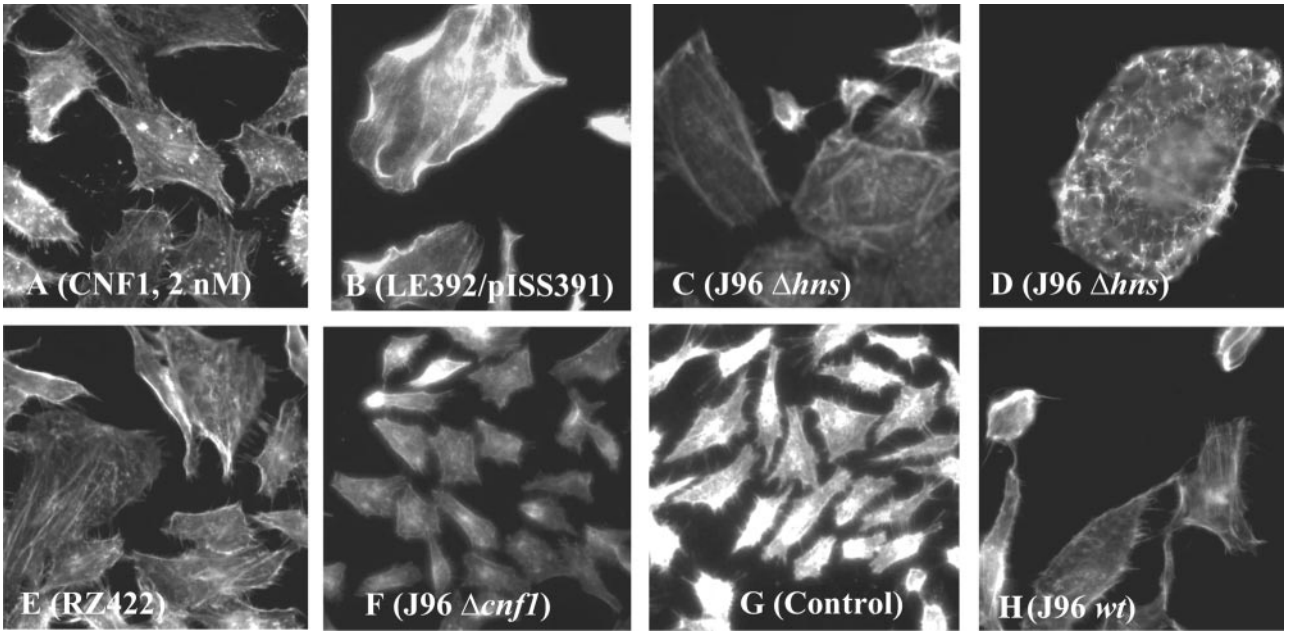


FIG. 6. Stress fiber formation by CNF1 containing vesicles in HeLa cells after 48 h. Fluorescence micrographs of cells treated by 2 nM CNF1 (A) or 100 μl of OMV samples from LE392/pISS391 (B), J96 Δ*hns* mutant (C and D), RZ422 (E), J96 Δ*cnf1* mutant (F), and J96 *wt* (H) strains. Untreated HeLa cells are shown in panel G. The magnification was the same in all cases, and the cell represented in the panel D is the occasionally observed "ultra-giant cell" after treatment with the J96 Δ*hns* mutant. wt, wild type.

than the effects of any of the vesicle samples studied, except in the case of the K12/*cnf1*$^+$ (LE392/pISS391) strain, when quantified as the percentage of multinucleated cells for the various samples (Fig. 5K). HeLa cells treated with vesicle preparations from the LE392/pHC79 or the J96 Δ*cnf1* strain were neither enlarged nor multinucleated (Fig. 5C and data not shown).

As CNF1 is well known to modify the small GTPases of the Rho family and therefore leads to formation of stress fibers, we investigated the effect of the purified vesicles on F-actin. During the first 20 h, there was no obvious induction of stress fiber formation by vesicles from any of the strains. However, after 48 h we could observe these fibers in HeLa cells that were treated with vesicle samples that contained CNF1 (Fig. 6). As in the case of the enlargement and the multinucleation effects, the appearance of stress fibers was variable according to the strain, and it seemed to be due to CNF1 in a concentration-dependent fashion. With the Δ*hns* mutant vesicle sample, we could occasionally observe some very large, "ultra-giant" cells (Fig. 6D) that were larger than any of the giant cells induced by other strains or vesicle samples or by the pure protein (2 nM CNF1).

## DISCUSSION

We here show that the CNF1 toxin from uropathogenic *E. coli* is released from the bacteria by a pathway that includes OMVs.

While we found that CNF1 in the bacterial cells is mainly localized in the cytoplasm of UEPC strain J96 and its Δ*hns* derivative BEU801, detectable amounts of this protein were also present in the periplasmic fractions. This suggests that the CNF1 protein is able to cross the cytoplasmic membrane despite the fact that no signal peptide is present in its sequence and, therefore, is not considered to be secreted by a Sec-dependent type 2 mechanism (3). Interestingly, we could detect the CNF1 protein in the supernatants from strains J96, J96 Δ*hns*, and LE392/pISS391 in broth cultures. These data suggest that CNF1 may be regarded as a secreted protein. Since it recently has been shown that some bacterial toxins could be secreted via OMVs (36), we analyzed such OMVs from some UPEC strains and could indeed detect the presence of CNF1 in these OMV samples. The association of the CNF1 protein with OMVs was shown to be rather tight since the protein was still recovered together with vesicles after ionic and urea disruption and could be liberated from the vesicles only upon SDS solubilization. From these results we may consider that CNF1 might be localized inside the lumen of the vesicles, hence providing a protected means of transport for the toxin. CNF1 might also be partially anchored to the vesicle membrane as our data suggest its presence in the OM fraction (Fig. 1B). Whether the membrane-spanning domain of CNF1 plays a role in its association with vesicles remains to be elucidated. We could also detect CNF1 in vesicle samples from other clinical isolates of the O6 serotype, among which *E. coli* RZ422 produced slightly more CNF1 than the J96 (serotype O4) wild type. In the case of an enterotoxigenic *E. coli* (ETEC), it has recently been shown that vesicles serve as specifically targeted transport vehicles that mediate entry of active enterotoxin and other bacterial envelope components into host cells, therefore suggesting a role in virulence for ETEC vesicles (19). Considering our findings, we suggest that OMVs from UPEC strains can play such a role as well.

Our preliminary data suggested a downregulation of CNF1 expression by the H-NS protein in UPEC strain J96. This is the first time that CNF1 was shown to be under regulation by this nucleoid protein. H-NS is known as a pleiotropic regulator that

causes repression or silencing of transcription at some promoters (5, 10). The observation that the Δ*hns* mutant of strain J96 released more vesicles and overexpressed CNF1 made this strain a good tool for us to localize the CNF1 in cell fractions and to study the protein in general in a UPEC background.

Recently, it has been reported that the Tol-Pal system of *E. coli*, required to maintain the OM integrity, plays a role in vesicle production. Indeed, the periplasmic overproduction of TolR domains, as well as Tol ligands, was shown to increase vesicle release in *E. coli* by destabilizing the bacterial cell envelope (12). Whether *hns* is involved in this system or another pathway remains to be elucidated. In the case of an ETEC strain, it was found that vesicle production is affected by H-NS as a threefold increase in OMVs released from the Δ*hns* mutant was observed (16). The authors attributed this to the pleiotropic effects of the *hns* mutation. Other studies have reported H-NS to be involved in downregulation of toxins in different gram-negative bacteria including some proteins in the ToxR regulatory cascade in *Vibrio cholerae* (27), hemolysin in *Serratia marcescens* (9), and products of the *spv* virulence locus in *Salmonella enterica* serovar Typhimurium (28) and of two structural genes (*mxiC* and *icsB*) in *Shigella flexneri* (31).

When HeLa cells were treated with CNF1-containing vesicle samples, enlargement and multinucleation were clearly observed only after 2 days of incubation. This delay in the toxin effect might be partly explained by the cell division process. It is well known that fast growing mammalian cells usually enter mitosis only 24 h after the previous division (about 12 h of rest in the $G_1$ phase, 6 h to synthesize the genetic material, and 6 h to prepare for the division in the $G_2$ phase) (26). Since enlargement and multinucleation are thought to be a result of an inhibition of the cytokinesis, it is not surprising to have no visible effects of the CNF1 on the first day after treatment. In the presence of CNF1, the cells, which are allowed to grow without division and undergo cytoskeleton rearrangements by the toxin, become giant and multinucleated. Of course, another possible explanation of the delay could be that the cascade of reactions that is started by CNF1 for the induction of the cell modifications is slow enough to display the final effects only later.

Altogether, our data indicate that active CNF1 is associated with OMVs in UPEC. As for other toxins, this could be a means for the bacteria to deliver CNF1 to the environment and to the infected tissue.

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