Proteomics Characterization of Outer Membrane Vesicles from the Extraintestinal Pathogenic *Escherichia coli* Δ*tolR* IHE3034 Mutant*

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Extraintestinal pathogenic *Escherichia coli* are the cause of a diverse spectrum of invasive infections in humans and animals, leading to urinary tract infections, meningitis, or septicemia. In this study, we focused our attention on the identification of the outer membrane proteins of the pathogen in consideration of their important biological role and of their use as potential targets for prophylactic and therapeutic interventions. To this aim, we generated a Δ*tolR* mutant of the pathogenic IHE3034 strain that spontaneously released a large quantity of outer membrane vesicles in the culture supernatant. The vesicles were analyzed by two-dimensional electrophoresis coupled to mass spectrometry. The analysis led to the identification of 100 proteins, most of which are localized to the outer membrane and periplasmic compartments. Interestingly based on the genome sequences available in the current public database, seven of the identified proteins appear to be specific for pathogenic *E. coli* and enteric bacteria and therefore are potential targets for vaccine and drug development. Finally we demonstrated that the cytolethal distending toxin, a toxin exclusively produced by pathogenic bacteria, is released in association with the vesicles, supporting the recently proposed role of bacterial vesicles in toxin delivery to host cells. Overall, our data demonstrated that outer membrane vesicles represent an ideal tool to study Gram-negative periplasm and outer membrane compartments and to shed light on new mechanisms of bacterial pathogenesis. ***Molecular & Cellular Proteomics 7:473–485, 2008.***

Extraintestinal pathogenic *Escherichia coli* (ExPEC)[1] are the most common enteric Gram-negative species causing a large variety of extraintestinal infections, including urinary tract infections, pneumonia, meningitis, osteomyelitis, and soft tissue infections (1–3). Furthermore ExPEC is the leading cause of severe sepsis, accounting for several thousands of deaths every year in the United States alone (4, 5).

Because of its aggressiveness and because of the alarming incidence of antibiotic resistance encountered in several recent disease isolates, ExPEC has become the object of intense research aimed at the development of an efficacious prophylactic therapy. Different approaches have been undertaken in this direction (6), but none of them have so far led to a product sufficiently satisfactory for human use. A variety of whole-cell vaccine formulations, administered via vaginal or oral routes, have been assessed in animal models and human clinical trials in Europe (7–15). These vaccines hold some promise but require further evaluation, particularly in consideration of the fact that whole cell-based vaccines are likely to elicit undesired immune responses against antigens shared with commensal *E. coli* strains. Capsular polysaccharide vaccines have been shown to confer protection in animal models. However, because *E. coli* polysaccharide exhibits high variability (*E. coli* has been classified in more than 80 capsular serotypes (16)), polysaccharide-based vaccines are unlikely to confer a sufficiently broad protection. Finally different toxins, such as the α-hemolysin (HlyA) (17–19), the cytotoxic necrotizing factor-1 (20), and the cytolytic distending toxin (21, 22), have been identified in several ExPEC isolates and have been shown to contribute to pathogenesis, but their role as potential vaccines remains to be demonstrated. In summary, alternative strategies for vaccine candidate identification need to be undertaken.

Because protection against ExPEC infections appears to be largely mediated by antibody responses both in the animal models and in humans (23–26), the detailed characterization

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[1] The abbreviations used are: ExPEC, extraintestinal pathogenic *E. coli*; APEC, avian pathogenic *E. coli*; CDT, cytolethal distending toxin; IPEC, intestinal pathogenic *E. coli*; LT, heat-labile enterotoxin; OMV, outer membrane vesicle; km^r, kanamycin resistance; LB, Luria-Bertani; 2D, two-dimensional.

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of the outer membrane-associated proteins, the family of proteins which mostly contribute to humoral immunity, would be an important step forward in the identification of new vaccine candidates. Several proteomics approaches have been proposed for membrane protein characterization. Overall, these studies have led to a better understanding of bacterial membrane organization and topology (for a review, see Ref. 27). However, the majority of these approaches suffer from the drawback of being somewhat poorly selective in that serious contaminations with proteins from the cytoplasmic compartment have been reported.

Recently we have proposed a novel approach for the characterization of Gram-negative outer membrane proteins that is based on the selection of mutant strains releasing outer membrane vesicles (OMVs) in the culture supernatant (28, 29). Gram-negative bacteria are known to release OMVs when grown in liquid culture, and vesicle production is considered to be a physiological process by which bacteria exert different functions (for recent reviews, see Kuehn and Kesty (30) and Mashburn-Warren and Whiteley (31)). However, the amount of OMVs released in the liquid culture is usually quite minute, and this had prevented their detailed biochemical characterization. In an attempt to increase the amount of OMV production in *Neisseria meningitidis*, we screened a panel of mutants and found that inactivation of the *gna33* gene encoding a lytic transglycosylase, an enzyme involved in the integrity of the outer membrane, resulted in massive production of OMVs (29). When thoroughly characterized by mono- and two-dimensional electrophoresis coupled to mass spectrometry, OMVs turned out to be almost exclusively composed by proteins belonging to the outer membrane and periplasmic compartments (28). Remarkably the OMVs purified from the culture supernatant of the mutant strain elicited a robust protective immune response in experimental animals as judged by the high bactericidal activity of sera from immunized animals against strains of different hypervirulent lineages.

Production of OMVs by non-pathogenic *E. coli* strains mutated in proteins that interact with the murein layer and that form complexes cross-linking the inner and outer membranes was described a few years ago (32–35). In particular, OMV-producing mutants were generated by inactivating genes coding for the Braun's (murein) lipoprotein (Lpp) and the proteins TolA, TolB, TolQ, TolR, and Pal belonging to the Tol-Pal system. However, no information on the protein content of these OMVs has been presented so far.

With the aim of better understanding the membrane protein organization of *E. coli* and of identifying proteins to be included in vaccine formulations capable of preventing ExPEC infections, in this study we describe the inactivation of the *tolR* gene in the pathogenic *E. coli* strain IHE3034 and the detailed characterization of the protein content of the purified OMVs. Here we show that the vesicles are constituted by at least 100 proteins, the large majority of which belong to the category of outer membrane and periplasmic proteins. Interestingly among the identified proteins, the three-subunit cytolethal distending toxin (CDT) was present. The toxin was exclusively found associated to the vesicles, suggesting that OMVs may represent the natural vehicle through which CDT is delivered to the target cells. This mechanism is consistent with what has been published recently on the OMV-mediated release of the heat-labile enterotoxin (LT) and the cytotoxic necrotizing factor-1 (22, 36). To the best of our knowledge, this is the first detailed proteomics characterization of *E. coli* outer membrane and periplasmic proteins.

EXPERIMENTAL PROCEDURES

Construction of ΔtolR IHE3034 Deletion Mutant—The Δ*tolR* mutant was produced by replacing *tolR* coding sequence with a kanamycin resistance (km^r) cassette (37). To this aim, we used a three-step PCR protocol to fuse the *tolR* upstream and downstream regions to the km^r gene. Briefly the 528-bp upstream and 466-bp downstream regions of the *tolR* gene were amplified from IHE3034 genomic DNA with the primer pairs UpF (TCTGGAATCGAACTCTCTCG)/UpR-kan (ATTTTGAGACACAACGTGGCTTTCATGGCTTACCCCTTGTTG) and DownF-kan (TTCACGAGGCAGACCTCATAAACATCTGCGTTTCCCTTG)/DownR (TTGCTTCTGCTTTAACTCGG), respectively. In parallel, the km^r cassette was amplified from plasmid pUC4K (38) using the primers kan-F (ATGAGCCATATTCAACGGGAAAC) and kan-R (TTAGAAAAACTCATCGAGCATCAAA). Finally the three amplified fragments were fused together by mixing 100 ng of each in a PCR containing the UpF/DownR primers. The linear fragment (1 μg), in which the km^r gene was flanked by the *tolR* upstream and downstream regions, was used to transform the recombination-prone IHE3034 *E. coli* strain (50 μl at 10^{10} cells/ml, made electrocompetent by three washing steps in ice-cold 10% glycerol), and Δ*tolR* mutants were selected by plating transformed bacteria on Luria-Bertani (LB) plates containing 25 μg/ml kanamycin.

Recombination-prone IHE3034 cells were produced by using the highly proficient homologous recombination system (*red* operon) (39). Briefly electrocompetent bacterial cells (50 μl at 10^{10} cells/ml) were transformed with 5 μg of plasmid pAJD434 by electroporation (5.9 ms at 2.5 kV). Bacteria were then grown for 1 h at 30 °C in 1 ml of SOC broth (39) and then plated on LB plates containing trimethoprim (100 μg/ml). One transformed colony was grown in LB (5 ml) with trimethoprim (100 μg/ml) at 30 °C until $A_{600} = 0.2$. Expression of the *red* genes carried by pAJD434 was induced by adding 0.2% L-arabinose to the medium for 3 h.

The gene deletion of the *tolR* gene was confirmed by PCR genomic DNA amplification using primers specifically annealing to *tolR* (TolR_ko_-7 (ACGTACTGCTGGTGCTGTTG) and TolR_ko_-8 (AGAAAGACCGTTTTCGGGTT)) and to kanamycin resistance gene (Kan-int-For (TCGCGATAATGTCGGGCAATCAG) and Kan-int-Rev (GAGGCAGTTCCATAGGATGGCAAG)). The deletion was confirmed also using the primers tolR-F (CGGACCCGTATTCTTAAC) and tolR-R (GCCTTCGCTTTAGCATCT) annealing further upstream and downstream from the 5′- and 3′-flanking regions, respectively.

Southern Blot Analysis—Genomic DNA was prepared from overnight liquid culture of IHE3034 and its isogenic *tolR* mutant using the NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). 5 μg of each DNA were digested overnight with AvaII restriction enzyme at 37 °C and loaded on a 0.7% agarose gel with appropriate DNA size markers. A 622-bp DNA probe partially overlapping the kanamycin resistance gene was prepared by PCR from pUC4K vector (38) with the primers Kan-int-For and Kan-int-Rev. Southern blot was performed with the ECL Direct Nucleic Acid La-

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beling and Detection Systems kit (GE Healthcare) as described by the manufacturer.

Bacterial Strain Growth Conditions—IHE3034 ExPEC strain (serotype O18:K1:H7) was isolated in 1976 from a case of human neonatal meningitis (40), and CFT073 strain (serotype O6:K2:H1) was isolated from a case of acute pyelonephritis (41). The wild type strains and the respective isogenic Δ*tolR* mutants were grown on an LB agar plate at 37 °C or LB medium, in a rotary shaker, to reach $A_{600} = 0.4$. From liquid cultures, bacteria were collected by 10-min centrifugation at 4000 × *g*.

OMV Preparation by Ultracentrifugation—Culture media of wild type and Δ*tolR* strains were filtered through a 0.22-μm-pore size filter (Millipore, Bedford, MA). The filtrates were subjected to high speed centrifugation (200,000 × *g* for 90 min), and the pellets containing the OMVs were washed with PBS and finally resuspended with PBS.

Negative Staining Electron Microscopy—OMVs were fixed overnight in 2.5% glutaraldehyde in PBS and then washed and resuspended in the same buffer. A drop of suspension was placed on Formvar/carbon-coated grids, and OMVs were adsorbed for 5 min. Grids were then washed with distilled water and blotted with a filter paper. For negative staining, grids were treated with 2% uranyl acetate for 1 min, air-dried, and viewed with a Jeol JEM 1200 EXII electron microscope operating at 80 kV.

Denaturing Monodimensional Electrophoresis—OMVs were denatured for 3 min at 95 °C in SDS-PAGE sample buffer containing 2% (w/v) SDS. 20 μg of proteins were loaded onto 4–12% (w/v) polyacrylamide gels (Bio-Rad). Gels were run in MOPS buffer (Bio-Rad) and were stained with Coomassie Blue R-250.

Two-dimensional Electrophoresis—Two hundred micrograms of OMVs were separated by two-dimensional electrophoresis as described in Ferrari *et al.* (28). Briefly proteins were separated in the first dimension on a non-linear pH 3–10 gradient and in the second dimension on a linear 9–16% polyacrylamide gradient. Gels were stained with colloidal Coomassie Blue-G250 (42).

In-gel Protein Digestion and MALDI-TOF Mass Spectrometry Analysis—Protein spots were excised from the gels, washed with 50 mM ammonium bicarbonate (Fluka Chemie AG, Buchs, Switzerland), acetonitrile (J. T. Baker Inc.) (50:50, v/v), washed once with pure acetonitrile, and air-dried. Dried spots were digested for 2 h at 37 °C in 12 μl of 0.012 μg/μl sequencing grade modified trypsin (Promega, Madison, WI) in 5 mM ammonium bicarbonate. After digestion, 0.6 μl was loaded on a matrix PAC target (Prespotted AnchorChip 96, set for proteomics, Bruker Daltonics, Bremen, Germany) and air-dried. Spots were washed with 0.6 μl of a solution of 70% ethanol, 0.1% trifluoroacetic acid. Mass spectra were acquired on an Ultraflex MALDI-TOF-TOF mass spectrometer (Bruker Daltonics) in reflectron, positive mode in the mass range of 900–3500 Da. Spectra were externally calibrated by using a combination of standards prespotted on the target (Bruker Daltonics). MS spectra were analyzed with flexAnalysis (flexAnalysis version 2.4, Bruker Daltonics). Monoisotopic peaks were annotated with flexAnalysis default parameters and manually revised. Protein identification was carried from the generated peak list using the Mascot program (Mascot server version 2.2.01, Matrix Science). Mascot was run on a public database (National Center for Biotechnology Information non-redundant (NCBInr), Gram-negative, release June 19, 2007; 5,043,617 sequences) or a database containing protein sequences (18,478 sequences) deduced from four sequenced *E. coli* genomes downloaded from NCBInr. We used the genome of the two commensal *E. coli* K-12 strains MG1655 and W3110 (accession numbers NC_000913 and AC_000091, respectively (43, 44)) and the two extraintestinal pathogenic strains *E. coli* 536 (accession number NC_008253) and CFT073 (accession number NC_004431 (45)). Search parameters were as follows: fixed modifications, propionamide (Cys); variable modifications, oxidation (Met); cleavage by trypsin (cuts C-terminal side of Lys and Arg unless next residue is Pro); mass tolerance, 300 ppm; missed cleavage, 0; mass values, MH^+ monoisotopic. Known contaminant ions (trypsin, m/z = 842.509400, 1045.563700, 1165.585300, 1179.601000, 1300.530200, 1713.808400, 1716.851700, 1774.897500, 1993.976700, 2083.009600, 2211.104000, 2283.180200, and 2825.405600) were excluded. Identifications were validated when the Mowse score was significant according to Mascot (46). If peptides matched to multiple members of a protein family we reported the accession number of the protein identified as the first hit (top rank) by Mascot. Strain of reference, accession number, annotation, Mowse score, percentage of protein coverage, number of unique peptides matched, number of masses not matched, score for the highest ranked hit to a non-homologous protein, and score for search in a decoy database (Mascot) are reported in Supplemental Table 1.

Bioinformatics—Prediction of protein localization was carried out using the PSORTb algorithm (47) and Lipo program (48).

Western Blot Analysis—Western blot was carried out on 0.22-μm filtered total culture supernatant, ultracentrifuged supernatant (secreted proteins), and pellet from ultracentrifuged supernatant (OMVs). In the case of the Δ*tolR* mutant, 10 ml of Δ*tolR* IHE3034 culture supernatant were filtered to remove residual bacterial cells. 15 μl were collected for subsequent electrophoretic analysis. The remaining material was ultracentrifuged at 200,000 × *g* for 90 min. After ultracentrifugation, the pellet was resuspended in 10 ml of PBS. 15 μl of the ultracentrifuged supernatant and of the resuspended pellet were loaded onto an SDS gel (see below). As far as the wild type IHE3034 strain is concerned, because the amount of released OMVs was much lower, protein concentration was required. In particular 200 ml of wild type culture supernatant was filtered through a 0.22-μm filter, and 100 ml were collected for subsequent TCA precipitation. The remaining 100 ml were ultracentrifuged at 200,000 × *g* for 90 min. The pellet was resuspended in 30 μl of 200 mM Tris-HCl, pH 8.8, whereas the proteins from the ultracentrifuged supernatant and the filtered culture supernatant were precipitated by adding TCA and deoxycholate at a final concentration of 10 and 0.04%, respectively. The precipitation was allowed to proceed for 30 min at 4 °C. Precipitate was recovered by 10-min centrifugation at 20,000 × *g* at 4 °C. The pellet was washed once with 10% TCA (w/v) and twice with absolute ethanol, dried with a SpeedVac (Labconco, Kansas City, KS), and resuspended in 30 μl of 200 mM Tris-HCl, pH 8.8. The 15-μl fractions from both wild type and mutant samples were boiled in loading buffer and loaded on a 4–12% (w/v) polyacrylamide-SDS gel (Bio-Rad). The gel was run in MOPS buffer (Bio-Rad) and transferred to nitrocellulose membrane (Trans-blot transfer medium, Bio-Rad). Samples were then incubated, after membrane saturation in PBS containing 3% (w/v) powdered milk, with mouse polyclonal antisera diluted (1:1000) in PBS containing 3% (w/v) powdered milk for 90 min at 37 °C. Membranes were washed three times with PBS containing 0.1% (v/v) Tween 20 and then incubated with sheep anti-mouse horseradish peroxidase-conjugated IgG (GE Healthcare) diluted (1: 7500) in PBS containing 3% (w/v) powdered milk. Colorimetric staining was performed, after washing the membranes, with SuperSignal West Pico Chemiluminescent Substrate kit (Pierce.) as described by the manufacturer.

RESULTS

OMVs Can Be Purified from the Supernatant of the ΔtolR IHE3034 E. coli Mutant—The inactivation of the *tolR* gene in *E. coli* has been shown previously to promote the release of large amounts of OMVs (32). With the aim of utilizing OMVs to characterize the membrane protein composition of the pathogenic *E. coli* IHE3034 strain, the entire *tolR* coding sequence

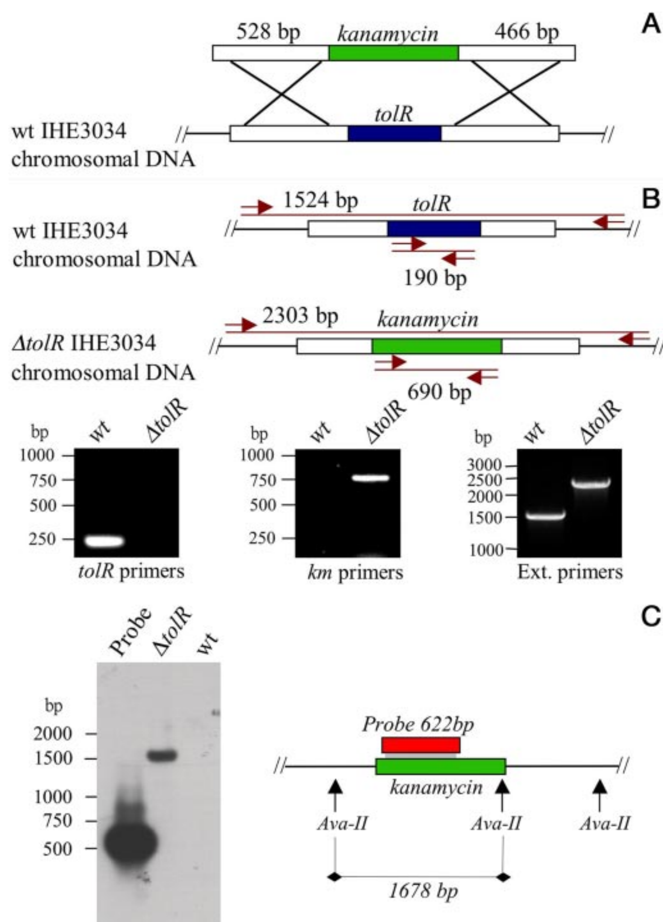

FIG. 1. *A*, schematic representation of *tolR* gene replacement. A linear double strand DNA fragment was amplified to contain the km^r cassette flanked by the 528 bp upstream and the 466 bp downstream of the *tolR* gene at its 5′- and 3′-ends, respectively. The linear fragment was electroporated into the IHE3034 strain, and km^r clones were selected. *B*, PCR analysis of wild type (*wt*) and Δ*tolR* mutant. Chromosomal DNA from wild type IHE3034 and Δ*tolR* IHE3034 was used as template of PCRs with primers specifically annealing to *tolR*, to kanamycin resistance gene, and in regions external (*Ext.*) to the sequences used for homologous recombination. *C*, Southern blot analysis of wild type and Δ*tolR* mutant strains. Chromosomal DNA from wild type IHE3034 and Δ*tolR* IHE3034 was digested with AvaII restriction enzyme. Fragments were separated on an agarose gel and transferred to nitrocellulose membrane for Southern blot analysis using as probe a 622-bp PCR product partially overlapping the kanamycin resistance gene. The restriction fragment of 1678 bp, containing the kanamycin resistance gene, is visible in the lane corresponding to the Δ*tolR* genomic DNA. As control of the Southern blot efficacy, a PCR fragment of 622 bp from the km^r gene was loaded onto the agarose gel.

was replaced with a km^r cassette using a modified version of the previously published three-step PCR method (37, 49, 50). We first amplified a linear double strand DNA fragment containing the km^r cassette flanked by the 528-bp *tolR* upstream region at its 5′-end and by the 466-bp *tolR* downstream region at its 3′-end (Fig. 1A). The linear DNA fragment was electroporated into the IHE3034 strain expressing the highly proficient homologous recombination system (*red* operon) encoded by plasmid pAJD434 (39), and IHE3034 mutants, in which the *tolR* gene was replaced with the km^r cassette, were selected on kanamycin-containing plates. Gene deletion in one Km-resistant clone was confirmed by PCR genomic amplification using primers specifically annealing to *tolR* and kanamycin resistance gene and to regions further upstream and downstream from the sequences used for homologous recombination (Fig. 1). As expected, the 690-bp fragment corresponding to the km^r gene, but not the 190-bp fragment of the *tolR* gene, was amplified from the chromosomal DNA of the mutant strain. The opposite was true when the amplification was carried out using the DNA from IHE3034 wild type strain (Fig. 1*B*). Finally PCR products obtained with the primers annealing to the external regions were in agreement with the predicted size of 1524 and 2303 bp for the wild type and mutant strains, respectively (Fig. 1*B*). To exclude a second recombination event occurring in other chromosomal regions, a Southern blot analysis of chromosomal DNA from both wild type and mutant strains was performed using the kanamycin resistance gene as probe. As shown in Fig. 1*C*, the probe hybridized with a unique AvaII restriction fragment of 1678 bp of genomic DNA from the mutant strain, whereas no hybridization was detected with the wild type DNA.

To establish whether the mutant strain had acquired the capacity to release OMVs, the presence of proteins in the culture supernatant was analyzed by SDS-PAGE. Indeed a number of protein bands, not visible in the supernatant of the wild type strain, were detected in the culture supernatant of the mutant strain. Subsequent mass spectrometry analysis of a few of the visible bands confirmed that they corresponded to the major *E. coli* outer membrane proteins (results not shown). The supernatant of a 200-ml culture of Δ*tolR* IHE3034 was then passed through a 0.22-μm filter and subjected to ultracentrifugation at 200,000 $\times$ *g* for 3 h. The pellet, typically containing about 2 mg of proteins, was analyzed by electron microscopy. As shown in Fig. 2*A*, the particulate material was constituted by vesicles of a size ranging from 50 to 100 nm in diameter. This is consistent with what was originally reported for *E. coli* mutants impaired in the Tol/Pal system (32).

Proteomics Analysis Reveals 100 OMV-associated Proteins—To characterize the protein content of the released OMVs, 20 and 200 μg of OMV proteins were separated by SDS-PAGE and 2D electrophoresis, respectively (Fig. 2, *B* and *C*). Bands and spots, visible after Coomassie staining, were excised and digested with trypsin. Proteolytic peptides were then analyzed by MALDI-TOF MS. Because the genome sequence of IHE3034 is not available yet, protein identification was carried out by comparing the experimental peptide mass fingerprints with the theoretical fingerprints deduced from the predicted proteins of four available *E. coli* genomes: the genome sequences of the two commensal *E. coli* K-12 strains MG1655 and W3110 (accession numbers NC_000913 and AC_000091, respectively (43, 44)) and the genomes of the two

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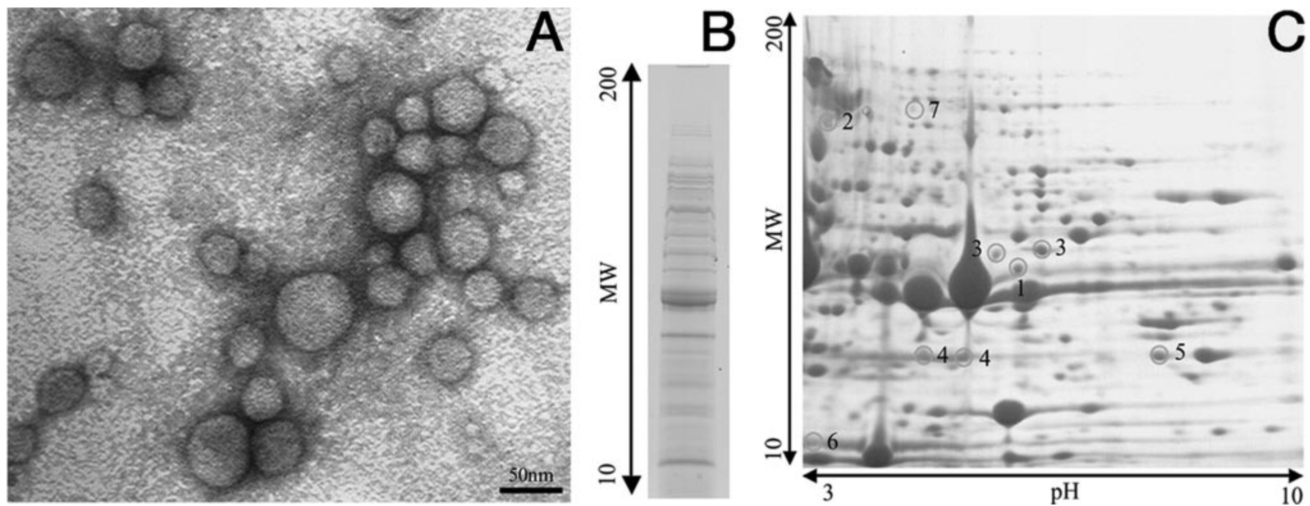

FIG. 2. *A*, electron microscopy of Δ*tolR* IHE3034 OMVs. Δ*tolR* IHE3034 OMVs were isolated from the growth culture medium by ultracentrifugation, fixed overnight in 2.5% glutaraldehyde in PBS, prepared for negative staining, and viewed by electron microscopy. *Bar length*, 50 nm. *B*, SDS-PAGE of OMVs. 20 μg of OMV proteins were separated in a 4–12% polyacrylamide gradient gel and stained with Coomassie Blue R-250. *C*, 2D gel electrophoresis of OMVs. 200 μg of OMV proteins were first focused on a non-linear pH 3–10 gradient and then separated on a 9–16% polyacrylamide gradient. The gels were stained with Coomassie Blue G-250. The seven proteins specific for pathogenic strains are *circled* (see text and Table II).

extraintestinal pathogenic strains *E. coli* 536 (accession number NC_008253) and CFT073 (accession number NC_004431 (45)). When a protein could not be identified using the four annotated genomes, fingerprint comparative analysis was performed against all Gram-negative bacterial proteins available in the NCBInr database. In total, 288 spots and 29 bands were analyzed, and the analysis allowed the unambiguous identification of 100 unique proteins (Table I). Of the 100 proteins identified, 79 were found in all four genomes, six were found in common with three of the four strains, six were found in common with the two pathogenic strains, and two were specific for strain 536. Finally the seven remaining proteins were identified after analysis of all Gram-negative bacterial proteins deposited in NCBInr (Fig. 3*A*).

Interestingly, it should be noted that the highest Mowse scores were obtained using protein sequences deduced from the pathogenic 536 strain genome, whereas the lowest Mowse scores were obtained using protein sequences deduced from the two commensal bacteria, *E. coli* K-12 strains MG1655 and W3110 (Supplemental Table 1). This would suggest that IHE3034 is phylogenetically more related to the pathogenic strains and particularly to strain 536.

OMVs Are Constituted by Outer Membrane and Periplasmic Proteins—Recently we demonstrated that *N. meningitidis* Δ*gna33* mutant spontaneously released high amounts of OMVs in the growth medium (28). These vesicles were mainly, if not exclusively, constituted by outer membrane and periplasmic proteins (28). To assess whether a similar protein composition characterized the OMVs of the Δ*tolR* IHE3034, the 100 identified proteins were subjected to computer analysis using PSORTb and Lipo protein localization predictor programs (47, 48). As shown in Fig. 3*B* and in Table I, 72% of the proteins were classified as either outer membrane proteins (41 proteins) or periplasmic proteins (31 proteins). Of the remaining 28%, two proteins were predicted to be secreted, one protein was assigned to the inner membrane compartment, 18 proteins had an uncertain cellular localization, and seven proteins were predicted to be cytoplasmic. The latter group includes the elongation factor Tu (gi|110643580) and the 60-kDa chaperone (gi|110644502) found to be associated to the OMVs of *N. meningitidis* Group B (28), and the chaperone protein HtpG (gi|110640734) found to be membrane-associated and important for virulence in *Porphyromonas gingivalis* (51).

In conclusion, the protein composition of the Δ*tolR* IHE3034 OMVs is in line with a mechanism of OMV formation according to which the vesicles are generated by a "budding out" process of the outer membrane most likely favored by the alteration, caused by the *tolR* mutation, of the inner-outer membrane interactions (32). In this budding process, periplasmic proteins are entrapped into the vesicles, whereas inner membrane and cytoplasmic proteins remain excluded.

The Cytolethal Distending Toxin Is Associated to OMVs—The 100 OMV-associated proteins include the three subunits CdtA, CdtB, and CdtC constituting the cytolethal distending toxin. According to PSORTb (47) and Lipo (48), CdtA and CdtC belong to the lipoprotein family, whereas no localization was predicted for CdtB. CDT was first identified in the culture supernatants of some *E. coli* (21) and *Campylobacter* spp. (52) clinical isolates, which were found to induce distension and death of mammalian cells. However, secretion of the toxin as a soluble three-subunit CDT complex has never been demonstrated. Failures in toxin purification from the bacterial culture supernatants were attributed either to the propensity

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TABLE I

List of proteins identified by proteomics analysis of ΔtolR IHE3034 OMVs

Protein identification was carried out by comparing experimentally generated monoisotopic peaks of peptides with computer-generated fingerprints using the Mascot program. Mascot was run on protein sequences deduced from four sequenced *E. coli* genomes downloaded from NCBInr: *E. coli* K-12 strains MG1655 and W3110 (accession numbers NC_000913 and AC_000091, respectively (43, 44), *E. coli* 536 (accession number NC_008253), and *E. coli* CFT073 (accession number NC_004431 (45)). When possible, *E. coli* 536 was used as the reference strain. When a protein was not identified using the four *E. coli* genomes, Mascot was run on the Gram-negative bacteria NCBInr public database, and as reference strain we used the one representing the first identification hit of Mascot. Protein localizations were as predicted by PSORTb (47) and the Lipo program (48). FKBP, FK506-binding protein.

	Reference strain	Protein annotation	Mowse score	Sequence coverage	No. of matched peptides	No. of unmatched peptides	Protein accession no.
				%			
Extracellular							
1	UTI89	Type 1 fimbriae major subunit FimA (*E. coli* UTI89)	105	74	6	57	gi|91213959
2	536	Putative lipoprotein AcfD precursor	58	5	5	4	gi|110643204
Outer membrane							
3	536	Protease VII precursor	134	54	13	87	gi|110640796
4	536	Outer membrane protein TolC precursor	176	49	16	31	gi|110643281
5	536	Ferrichrome-iron receptor precursor	254	48	30	27	gi|110640371
6	536	Putative outer membrane antigen	237	48	29	41	gi|110640396
7	536	Nucleoside-specific channel-forming protein tsx precursor	98	48	10	24	gi|110640672
8	536	Ferrienterobactin receptor precursor	109	29	12	29	gi|110640814
9	536	Peptidoglycan-associated lipoprotein precursor	66	38	6	26	gi|110640949
10	536	Probable tonB-dependent receptor ybiL precursor	123	28	15	31	gi|110641009
11	536	Outer membrane protein X precursor	61	34	4	14	gi|110641018
12	536	Outer membrane protein A (*E. coli* 536)	164	55	14	28	gi|110641146
13	536	Outer membrane protein W precursor	64	39	5	40	gi|110641486
14	536	MltA-interacting protein precursor	104	58	12	38	gi|110641904
15	536	Colicin I receptor precursor	111	34	19	80	gi|110642365
16	536	Outer membrane protein C precursor	81	31	10	39	gi|110642425
17	536	Putative outer membrane lipoprotein	60	38	5	44	gi|110643800
18	536	Phospholipase A_1 precursor	134	49	12	26	gi|110644146
19	536	Vitamin B_{12} receptor precursor	169	43	18	38	gi|110644310
20	536	Maltoporin precursor	97	43	16	80	gi|110644375
21	536	Probable tonB-dependent receptor YncD precursor	151	42	18	43	gi|110641630
22	536	Long-chain fatty acid transport protein precursor	63	21	7	28	gi|110642547
23	536	Outer membrane porin protein LC precursor	91	47	10	86	gi|110641339
24	536	TonB-dependent outer membrane siderophore receptor	119	28	14	32	gi|110640508
25	536	Putative outer membrane receptor for iron compound or colicin	71	19	11	50	gi|110642164
26	536	Outer membrane hemin receptor	211	46	24	49	gi|110643744
27	536	Hypothetical type II secretion protein GspD	66	22	12	55	gi|110643200
28	B171	Outer membrane receptor proteins, mostly iron transport	71	26	7	57	gi|75207813
29	536	Putative autotransporter	73	11	13	22	gi|110643163
30	536	Hypothetical protein YddB	210	33	21	23	gi|110641669
31	536	Hypothetical protein YraM (putative LppC, putative lipoprotein)	172	26	13	8	gi|110643388
32	536	Organic solvent tolerance protein	303	52	37	34	gi|110640268
33	536	Tail-specific protease precursor	111	30	15	65	gi|110641948
34	536	Hypothetical protein YhjL	80	16	14	42	gi|110643777
35	536	Hypothetical lipoprotein yjbH precursor	109	29	16	56	gi|110644368
36	536	putative lipoprotein	113	33	12	43	gi|110640975
37	536	Outer membrane lipoprotein, Slp family	89	58	8	63	gi|110641923
38	536	Hypothetical protein YfdY precursor	88	52	6	45	gi|110642613
39	536	Lipoprotein-34 precursor	66	37	9	88	gi|110642651
40	536	Hypothetical lipoprotein YfiO precursor	110	45	12	25	gi|110642758
41	536	Outer membrane lipoprotein	69	47	5	47	gi|110641763

Table I—*continued*

	Reference strain	Protein annotation	Mowse score	Sequence coverage	No. of matched peptides	No. of unmatched peptides	Protein accession no.
				%			
42	E6468/62	CdtA (*E. coli*)	101	42	8	34	gi|416214
43	E6468/62	Cytolethal distending toxin C (*E. coli*)	112	66	7	57	gi|121582252
Periplasmic							
44	536	Protease	113	43	14	57	gi|110640382
45	536	TolB protein precursor	175	41	12	18	gi|110640948
46	536	Glutamine-binding periplasmic protein precursor	96	35	7	24	gi|110641015
47	536	Outer membrane lipoprotein carrier protein precursor	90	38	7	11	gi|110641091
48	536	Periplasmic glucan biosynthesis protein MdoG precursor	83	33	13	75	gi|110641225
49	536	Periplasmic oligopeptide-binding protein precursor	134	43	18	75	gi|110641472
50	536	Periplasmic β-glucosidase precursor	143	33	26	60	gi|110642341
51	536	D-Galactose-binding periplasmic protein precursor	121	52	13	42	gi|110642359
52	536	Ecotin precursor	95	50	7	37	gi|110642418
53	536	Histidine-binding periplasmic protein precursor	76	42	9	69	gi|110642513
54	536	Glycine betaine-binding periplasmic protein precursor	94	33	6	23	gi|110642805
55	536	Thiol:disulfide interchange protein DsbC precursor	64	35	8	45	gi|110643041
56	536	Suppressor of FtsI	151	53	20	65	gi|110643260
57	536	Protease DegQ precursor	137	41	13	44	gi|110643468
58	536	FKBP-type peptidyl-prolyl cis-trans isomerase FkpA precursor	92	37	7	34	gi|110643588
59	536	Peptidyl-prolyl cis-trans isomerase A precursor	73	51	5	34	gi|110643603
60	536	Nickel-binding periplasmic protein precursor	103	29	12	57	gi|110643718
61	536	Periplasmic dipeptide transport protein precursor	79	21	10	47	gi|110643793
62	536	D-Ribose-binding periplasmic protein precursor	160	59	13	22	gi|110644091
63	536	Thiol:disulfide interchange protein DsbA precursor	108	50	11	33	gi|110644199
64	536	Maltose-binding periplasmic protein precursor	104	41	12	41	gi|110644373
65	536	Osmotically inducible protein Y precursor	66	31	4	46	gi|110644812
66	536	Soluble lytic murein transglycosylase precursor	201	40	27	34	gi|110644831
67	536	Membrane-bound lytic murein transglycosylase B	78	49	12	43	gi|110642821
68	CFT073	Survival protein surA precursor	135	47	15	37	gi|26245979
69	536	Polysialic acid transport protein KpsD precursor	112	27	13	29	gi|110643176
70	536	Putative sulfatase YdeN precursor	127	38	12	20	gi|110641672
71	536	Hypothetical protein YggE	68	29	6	22	gi|110643066
72	536	Glutamate/aspartate periplasmic binding protein precursor	71	33	9	64	gi|110640877
73	536	Protein YdcG precursor (putative periplasmic glucan biosynthesis)	82	17	8	18	gi|110641607
74	536	Hypothetical protein YraP precursor	66	52	8	62	gi|110643391
Inner membrane							
75	536	Hypothetical protein YfgC precursor	85	31	11	41	gi|110642656
Cytoplasm							
76	536	Chaperone protein HtpG (*E. coli* 536)	91	25	12	65	gi|110640734
77	536	Alkyl hydroperoxide reductase C22 protein	62	42	6	41	gi|110640837
78	536	Elongation factor Tu	178	55	21	30	gi|110643580
79	536	Succinate dehydrogenase flavoprotein subunit	213	40	22	13	gi|110640932
80	536	Dihydrolipoamide dehydrogenase	116	36	12	32	gi|110640334
81	536	Dihydrolipoamide succinyltransferase component	106	32	9	16	gi|110640935
82	536	60-kDa chaperonin	60	31	9	61	gi|110644502
Unknown							
83	536	Hypothetical protein YaiO	69	36	8	62	gi|110640627
84	536	Putative lipoprotein	62	38	7	45	gi|110640694
85	536	Succinate dehydrogenase iron-sulfur protein	59	30	6	56	gi|110640933
86	536	Hypothetical protein ECP_0753	76	44	8	47	gi|110640950
87	536	Putative periplasmic protein	91	42	8	29	gi|110641023
88	536	Protein YceI precursor	105	57	7	26	gi|110641233
89	536	Hypothetical protein YcfS precursor	156	61	15	62	gi|110641289

TABLE I—*continued*

	Reference strain	Protein annotation	Mowse score	Sequence coverage	No. of matched peptides	No. of unmatched peptides	Protein accession no.
				%			
90	536	Protein YdgH precursor	59	26	7	37	gi|110641726
91	536	Fructose-bisphosphate aldolase class I	102	41	13	53	gi|110642305
92	536	Membrane-bound lytic murein transglycosylase A precursor	81	36	11	44	gi|110642954
93	536	Hypothetical protein YrbC (putative toluene tolerance protein)	69	38	6	64	gi|110643432
94	536	ATP synthase α chain	190	45	17	40	gi|110644075
95	CFT073	Hypothetical protein yfdQ	155	60	13	33	gi|26249043
96	536	Major coat protein	56	30	11	70	gi|110641354
97	536	Putative metalloprotease YggG	60	25	9	73	gi|110643085
98	APEC01	Hypothetical protein APECO1_4044 (*E. coli* APECO1)	89	32	10	85	gi|117624709
99	EDL933	Hypothetical protein (*E. coli*)	96	25	9	39	gi|50983080
100	E6468/62	CdtB (*E. coli*)	123	54	11	58	gi|416215

of CDT to form aggregates or to associate with bacterial membranes (53, 54). The fact that our proteomics analysis revealed the presence of the toxin in the vesicles might suggest that, rather than being released outside the cell through one of the classical secretory mechanisms, CDT could leave the pathogen in association with OMVs. To further support this hypothesis, we collected the supernatant from an exponentially growing Δ*tolR* IHE3034 culture. The supernatant was filtered through a 0.22-μm-pore size filter and then subjected to high speed centrifugation. The OMV pellet and supernatant were then analyzed by Western blot using a mouse polyclonal antibody against the CdtC subunit of the toxin. As shown in Fig. 4*A*, a band corresponding to the CdtC subunit was visible only in the OMV fraction. A similar result was obtained using, as control, an antibody against the outer membrane porin protein Lc, a protein known to be associated to the *E. coli* outer membrane (55).

The release of CDT from IHE3034 cells via OMVs appeared to occur also in the wild type strain as demonstrated by Western blot analysis of wild type OMVs obtained from a large culture volume (see "Experimental Procedures") (Fig. 4*B*). Anti-CdtC antibodies recognized a protein of the right molecular weight in the pellet of the ultracentrifuged supernatant, whereas no reactive material was evidenced in the TCA-precipitated supernatant after ultracentrifugation even if the protein precipitation was carried out using a large culture volume.

DISCUSSION

Bacterial surface proteins play a fundamental role in the interaction between the bacterial cell and its environment (56–60). They are involved in adhesion and invasion into host cells, in sensing the chemical and physical conditions of the external milieu and sending appropriate signals to the cytoplasmic compartment, and in mounting defenses against host responses and in toxicity. Hence surface proteins are potential targets of drugs aimed at preventing bacterial infections (61) and are ideal candidates to become components of effective vaccines.

Despite the biological relevance of bacterial surface proteins, their characterization is still incomplete because of the intrinsic difficulties in defining the protein composition and topology of the bacterial surface. In Gram-negative bacteria, several protocols based on detergents or carbonate extraction have been developed for the analysis of outer membrane proteins, but none of them are fully satisfactory, and in general, poor enrichment of membrane proteins and frequent contamination by cytosolic proteins occur (62, 63).

We recently described a new and effective approach to identify outer membrane proteins in the Gram-negative bacterium *N. meningitidis* based on the analysis of OMVs. We showed that the vesicles abundantly produced by a specific *N. meningitidis* mutant were mostly constituted by outer membrane and periplasmic proteins (28), and we predicted that the proteomics characterization of OMVs could become a general strategy to define membrane protein composition in Gram-negative bacteria.

It was known that *E. coli* mutants altered in the structure of the Braun's (murein) lipoprotein (33, 34) or in proteins constituting the Tol-Pal system (32, 35) spontaneously released vesicles in the growth medium. However, no detailed analysis of OMV protein composition had been reported so far. In this work, we deleted the *tolR* gene of the meningitis-associated ExPEC IHE3034 strain, and we fully characterized the protein content of the released vesicles. In total, 100 proteins were identified, the majority of which are predicted to be either associated to the outer membrane or localized to the periplasmic region. Interestingly, 18 of the identified proteins could not be assigned to any cellular compartment by PSORTb and Lipo software, and therefore our analysis offers the opportunity to investigate the function of these mostly hypothetical proteins in the context of their membrane association.

Despite the fact that *E. coli* is one of the most thoroughly

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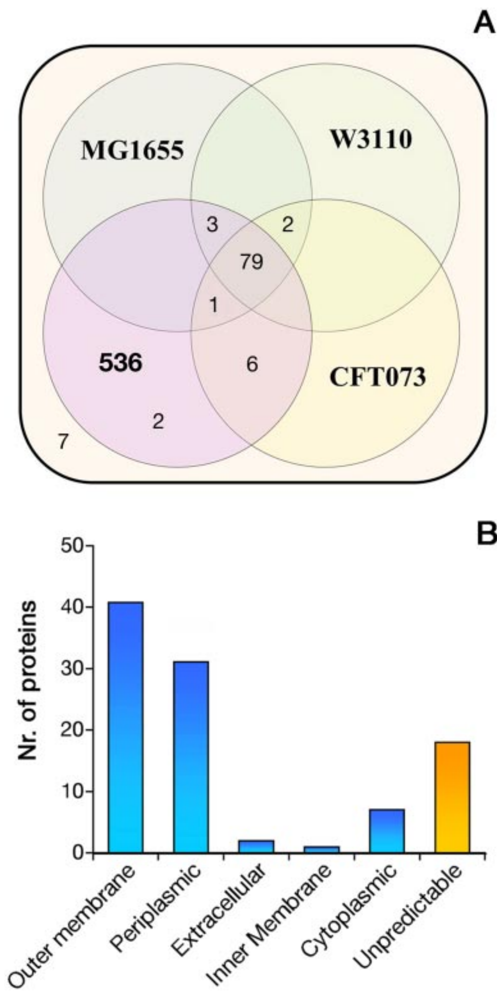

FIG. 3. *A*, distribution of OMV proteins among available ExPEC genomes. Experimental peptide mass fingerprints were compared with the genomes of the two commensal *E. coli* strains MG1655 and W3110 and the two uropathogenic *E. coli* strains 536 and CFT073. Profiles not matching any protein of the four strains were compared with the whole Gram-negative NCBI database. Of the 100 proteins identified, 79 were found in all four genomes, six were found in common with three of the four strains, six were found in common with the two pathogenic strains, and two were specific for strain 536. Seven proteins were identified among all Gram-negative bacteria proteins deposited in NCBInr. *B*, prediction of cellular localization of OMV proteins. Protein localization was established using either PSORTb software or the Lipo program. Seventy-two proteins (72%) were classified as either outer membrane proteins (41 proteins, 41%) or periplasmic proteins (31 proteins, 31%). Of the remaining proteins, two were predicted to be extracellular (2%), one was predicted to be inner membrane (1%), and seven were predicted to be cytoplasmic (7%). For 18 proteins (18%) the cellular localization was unpredictable. *Nr.*, number.

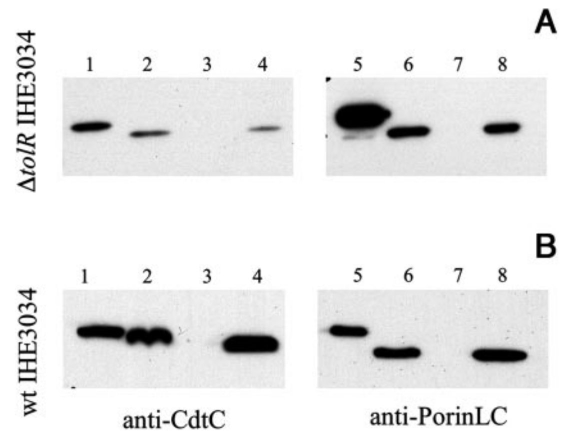

FIG. 4. **Association of CdtC subunit to OMVs.** *A*, OMVs from the supernatant of a Δ*tolR* IHE3034 culture were filtered through a 0.22-μm-pore size filter and were either loaded onto an SDS-polyacrylamide gel (15 μl, *lanes 2* and *6*) or centrifuged at 200,000 × *g* for 90 min. After centrifugation, the pellet was resuspended in the original volume, and both the resuspended pellet (*lanes 4* and *8*) and the supernatant (*lanes 3* and *7*) were loaded on the same gel (15 μl each). *B*, the same fractions were analyzed from wild type culture supernatant with the exception that proteins loaded in *lanes 2* and *6* and *lanes 3* and *7* were derived from 50 ml of TCA-precipitated supernatant (see "Experimental Procedures"). As controls, recombinant His-tagged CdtC (*lane 1*, 1 μg in *A* and 50 ng in *B*) and outer membrane porin protein LC (*lane 5*, 1 μg in *A* and 10 ng in *B*) were loaded on the same gel. In both panels, proteins were blotted on a nitrocellulose membrane and incubated with polyclonal antibody against either CdtC or outer membrane porin protein LC.

studied organisms, only a few systematic analyses of its outer membrane and periplasmic compartments have been reported. To the best of our knowledge, the most extensive studies on *E. coli* membrane proteins have been carried out by Molloy *et al.* (64) and Xu *et al.* (65), who described only 30 outer membrane-associated proteins. Very recently, Alteri and Mobley (66) have presented 30 proteins expressed on the bacterial surface during growth in human urine. Our analysis confirms the presence of most of the proteins identified by these and other authors and expands the number of proteins experimentally demonstrated to be associated to the outer membrane fraction. Furthermore we provide the first systematic study on the protein composition of the *E. coli* periplasm.

Although the OMV production appears to be the result of membrane budding, and therefore OMVs should genuinely represent the real protein composition of the bacterial membrane and periplasmic compartments (32, 34), alteration of the outer membrane organization due to the Δ*tolR* mutation cannot be ruled out. To exclude this, a direct comparison with OMV protein composition from wild type strain would be necessary. Unfortunately this analysis is complicated by the minute amount of OMVs released from the wild type strain during *in vitro* growth. However, three pieces of evidence support the hypothesis that Δ*tolR* released OMVs truly represent the outer membrane and periplasmic compartments. First, in preliminary experiments, we have introduced the same mutation in CFT073, a strain with a different genetic background, purified the OMVs, and analyzed their protein content. The 2D maps of the OMVs from the two Δ*tolR* mutants appeared relatively conserved (Supplemental Fig. 1), and the proteomics analysis indicated that 77% of Δ*tolR* CFT073-derived proteins were also identified in Δ*tolR*

TABLE II

ΔtolR IHE3034 OMV proteins exclusively expressed in pathogenic bacteria

The 100 OMV proteins were blasted against all sequences from Gram-negative bacteria present in NCBInr to search for those proteins that have homologs only in pathogenic strains (>80% identity to pathogenic bacterial proteins and <40% identity to proteins from commensal strains).

	Protein accession no.	Protein annotation	ExPEC	IPEC	APEC	*Shigella*	*Enterobacter*
1	gi|110641354	Major coat protein	●	●		●	
2	gi|110643744	Outer membrane hemin receptor	●	●	●	●	●
3	gi|117624709	Hypothetical protein APECO1_4044		●	●		
4	gi|416214	Cytolethal distending toxin A	●	●	●		
5	gi|416215	Cytolethal distending toxin B	●	●	●		
6	gi|121582252	Cytolethal distending toxin C	●	●	●		
7	gi|110642164	Putative outer membrane receptor for iron compound or colicin	●	●			

IHE3034 OMVs (Supplemental Table 2). Second and particularly interestingly, CDT, which was unexpectedly found in Δ*tolR* IHE3034 OMVs, was also found in the wild type-derived vesicles, suggesting a common mechanism of OMV production for both the wild type and mutant strains. Finally very recently Lee *et al.* (67) published a proteomics analysis of OMVs released from the culture supernatant of wild type *E. coli* strain. Although their OMVs were prepared from late stationary phase culture and cell lysis occurred (with 43% of the identified proteins belonging to the cytoplasmic and inner membrane compartment), it is remarkable that 73% of the outer membrane proteins identified in our study were also found by these authors.

As previously noted, well expressed surface exposed proteins are good candidates for vaccine development (68, 69). Indeed vaccines based on surface-exposed and secreted proteins are already commercially available, and others are in development. Therefore, the availability of a detailed map of the *E. coli* outer membrane proteins is expected to become useful information for the selection of vaccine candidates against pathogenic *E. coli*. In this context, it is worth pointing out that using a reverse vaccinology approach we recently succeeded in identifying protective antigens against Group B *N. meningitidis* (69), and five of these antigens are currently in the clinics to test their efficacy as human vaccine (70). Interestingly the most protective MenB antigens now in clinical trials were all found to be part of the *N. meningitidis* OMV proteome (28), highlighting the usefulness of OMV protein characterization as an effective and rapid approach to vaccine candidate discovery.

In the case of ExPEC, because it is a pathogen that shares high homology with commensal *E. coli* strains, it is generally accepted that ExPEC vaccine candidates should be selected among the pathogen-specific membrane-associated proteins to avoid mounting an immune response against the commensal population. A BLAST analysis of the 100 OMV proteins against the currently available bacterial genomes indicates that seven proteins (Table II) share more then 80% identity to pathogenic bacterial proteins and less than 40% identity to proteins from commensal strains. These seven proteins, shown in the 2D map of Fig. 2*C*, include the three components of the cytolethal distending toxin, CdtA, CdtB, and CdtC, identified in ExPEC and in several intestinal pathogenic *E. coli* (IPEC) strains; the outer membrane hemin receptor found in ExPEC, IPEC, and in the enteric pathogens *Shigella* and *Enterobacter*; a putative iron outer membrane receptor found in ExPEC and IPEC; the major coat protein found in ExPEC, IPEC, and *Shigella*; and the hypothetical protein APECO1_4044 found in IPEC. Interestingly five of the seven pathogen-specific proteins have homologs in avian pathogenic *E. coli* (APEC) strains (see Table II). This is in agreement with other studies showing clonal relationships between human pathogenic *E. coli* strains and virulent strains isolated from animals, including chickens (71, 72). Furthermore Johnson *et al.* (73) recently reported that *E. coli* strains from retail chicken products have virulence profiles similar to those of ExPEC isolates. Therefore, as in the case of influenza virus where birds are the most important reservoir of human influenza infection, the data seem to support the hypothesis that APEC potentially serves as a source of virulence-associated genes for human pathogenic *E. coli*.

The identification of the CDT found in association with the OMVs from Δ*tolR* mutant and, most importantly, from the wild type strain deserves a final comment. CDT is a bacterial toxin produced by a variety of Gram-negative pathogenic bacteria. The mechanism of cytotoxicity of CDT is unique in that one of its subunits, CdtB, which has a nuclease activity, enters into the eukaryotic cell nucleus and breaks double-stranded DNA. This results in the arrest of the cell cycle at the G_2/M boundary and the consequent apoptosis of the target cell. CDTs are encoded by three linked genes (*cdtA*, *cdtB*, and *cdtC*), which are required to constitute the fully active holotoxin (for recent reviews, see Heywood *et al.* (74) and Thelestam and Frisan (75)). CdtA and CdtC subunits have a lipo domain for bacteria membrane binding and a ricin B lectin domain, which may help the binding to a not yet identified galactose-containing receptor (76). CdtB has no prokaryotic localization domain, whereas it possesses a eukaryotic nuclear localization signal domain (77). The fact that we found the toxin associated to the OMVs and not in the culture supernatant as soluble pro-

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tein in appreciable quantities suggests that OMVs represent a natural vehicle for toxin release into eukaryotic cells. According to this model, the two membrane-anchored subunits, CdtA and CdtC, might serve as ligands of the vesicles to cellular receptors. Once bound, the vesicles are internalized by the cell, and the CdtB subunit reaches the nucleus exploiting its unique nuclear localization signal domain in a not yet defined manner (78). Very recently, the cytotoxic necrotizing factor-1 and the cholera toxin homolog LT have been demonstrated to be associated to the vesicles and delivered to the host cells through OMV internalization into eukaryotic cells (22, 36). In particular, Kesty *et al.* (22) demonstrated that LT is released by *E. coli* associated with OMVs, which bind to the host cells using the toxin as ligand and get internalized via lipid rafts. Once inside the cell, the toxin is trafficked via retrograde transport through the Golgi and the endoplasmic reticulum (22). Altogether our data and previous studies strongly suggest that OMV release represents a common mechanism through which bacteria deliver toxins into the host cells.

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