

Vesicle-Mediated Export and Assembly of Pore-Forming Oligomers of the Enterobacterial ClyA Cytotoxin

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Summary

The ClyA protein is a pore-forming cytotoxin expressed by *Escherichia coli* and some other enterobacteria. It confers cytotoxic activity toward mammalian cells, but it has remained unknown how ClyA is surface exposed and exported from bacterial cells. Outer-membrane vesicles (OMVs) released from the bacteria were shown to contain ClyA protein. ClyA formed oligomeric pore assemblies in the OMVs, and the cytotoxic activity toward mammalian cells was considerably higher than that of ClyA protein purified from the bacterial periplasm. The redox status of ClyA correlated with its ability to form the oligomeric pore assemblies. In bacterial cells with a defective periplasmic disulphide oxidoreductase system, the ClyA protein was phenotypically expressed in a constitutive manner. The results define a vesicle-mediated transport mechanism in bacteria, and our findings show that the localization of proteins to OMVs directly may contribute to the activation and delivery of pathogenic effector proteins.

Introduction

Several distinct types of secretion pathways for proteins that are important for the bacterial interaction with host environments have been identified in gram-negative bacteria. The type I, and type III pathways are one-step mechanisms by which the secreted proteins cross directly from the cytoplasm to the bacterial surface. For example, the cytolytic HlyA and Ehx hemolysins are

utilizing the type I pathway (Koronakis et al., 1991, 2000). In the type II pathway, also known as the general secretion pathway, proteins are first translocated through the inner membrane to be accumulated in the periplasm and are then transported across the outer membrane by either of several distinct “terminal branches” of the process (Pugsley, 1993; Sandkvist, 2001). The type III system functions both as a pathway for secretion across the bacterial membranes and for translocation of the secreted bacterial proteins across the plasma membrane of eukaryotic host/target cells (Galan and Collmer, 1999; Hueck, 1998). The type IV system also usually translocates proteins in a single step from the cytoplasm to the intracellular compartment of a target cell (Christie and Vogel, 2000). The type V pathway is utilized by so-called autotransporter proteins (Henderson et al., 2000). Such proteins possess an N-terminal signal sequence and utilize the Sec system for translocation across the inner membrane, whereas they mediate their own translocation across the outer membrane by forming a pore structure. The Tat system allows (in a Sec-independent way) translocation of folded proteins carrying redox cofactors across the inner membrane (Sargent et al., 1998). All of the mentioned pathways include some accessory proteins that may be commonly utilized by separate pathways (e.g. signal sequence peptidase; the Sec apparatus) or specifically assembled for the particular pathway.

Recently, it became evident that even derivatives of the common laboratory strain *E. coli* K-12 can express a hemolytic/cytolytic phenotype. Activation of a gene denoted *clyA* (*hlyE*, *sheA*) results in expression of the ClyA (cytolysin A) protein (Uhlin and Mizunoe, 1994; Oscarsson et al., 1996, 1999; del Castillo et al., 1997; Green and Baldwin, 1997; Ludwig et al., 1999). Intact *E. coli* cells expressing ClyA show cytotoxic effects upon direct contact with mammalian cells and ClyA appeared to be a contact-dependent activity (Oscarsson et al., 1999; Lai et al., 2000). Structural studies revealed that ClyA represents a new type of bacterial cytotoxins with a protein folding different from previously studied toxins (Wallace et al., 2000). In association with lipid membranes, in vitro ClyA forms an oligomeric pore-like assembly that was suggested to be the active pore-forming cytotoxin complex. Earlier studies with overproducing strains suggested that ClyA could accumulate in the periplasmic space (Ludwig et al., 1999; Oscarsson et al., 1999). Results from recent studies with ClyA protein fusions indicate that the protein has an intrinsic ability to get translocated across the cytoplasmic membrane, and this does not require the cytolytic activity per se (del Castillo et al., 2001). However, the ClyA protein is translocated without cleavage of any N-terminal signal sequence, and it has remained unknown how it is surface exposed and released from the bacterial cells.

Gram-negative cell walls have a dynamic feature that is not seen in gram-positive counterparts. Outer-membrane vesicles (OMVs) are constantly being discharged from the surface of the cell during bacterial growth (reviewed by Beveridge, 1999). OMVs include outer-mem-

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brane proteins, lipopolysaccharide (LPS), and phospholipids and, as the vesicles are being released from the surface, they entrap some of the underlying periplasm. Previous studies showed that bacterial OMVs had fusion activity to mammalian target cells. For example, Kadurugamuwa and Beveridge (1998) reported that the vesicles from *Shigella flexneri* loaded with antibiotic are capable to deliver the antibiotic into the cytoplasm of Henle cells after the internalization of the vesicles. The release of such vesicles has been suggested to be associated with the surface exposure of a protein toxin in *E. coli* (Wai et al., 1995; Horstman and Kuehn, 2002). Fiocca et al. (1999) observed that the vesicles released from *Helicobacter pylori* bind to epithelial cells in vitro, as well as to the gastric mucosal surface in vivo. Thus, they suggested that *H. pylori* vesicles may play a role in the delivery of bacterial components to host gastric tissue. Recently, Kato et al. (2002) observed that the OMVs secreted by *Actinobacillus actinomycetemcomitans* are enriched in leukotoxin, and they suggested that the formation of vesicles occurs by a process that results in the enrichment of leukotoxin.

Here, we present evidence that the ClyA cytotoxin is exported from the bacterial cell in OMVs and that it is adopting a cytolytically active, oligomeric conformation in the vesicles. We suggest that the results define a vesicle-mediated transport mechanism in bacteria that is responsible for the activation and delivery of pathogenic effector proteins.

Results

Surface Exposure of ClyA

The surface exposure of ClyA on different *E. coli* K-12 strains was observed by immunofluorescence microscopy as shown in Figure 1. ClyA was detected on the surface of most cells of MC1061/pYMZ80, which is the derivative carrying a multicopy plasmid clone of the *clyA* structural gene (Figures 1A and 1B). In order to assess the surface expression and export of ClyA protein without the overproduction obtained from the cloned *clyA* gene, we made use of strain MWK11, a derivative in which the chromosomal *clyA* locus contains a better recognition site for the CRP-cAMP regulatory complex and thereby expresses phenotypically detectable levels of ClyA (Westermarck et al., 2000). ClyA was detected on the surface of the majority of MWK11 cells (Figures 1C and 1D). There was very little immunofluorescence detected in the case of the vector control strain (MC1061/pUC18), but a small subpopulation of cells (1%) apparently expressed high enough levels to be seen in this analysis (Figures 1E and 1F). The chromosomal *clyA:kan* knockout mutant was completely negative (Figures 1G and 1H). Taken together, the immunofluorescence studies showed that the ClyA protein evidently was exposed on the surface of the bacterial cells.

To get more detailed information about the surface characteristics of cells expressing ClyA, we examined the bacteria by electron microscopy (EM) and by atomic force microscopy (AFM). We then found that there were small outer-membrane vesicles surrounding the bacterial cells. The AFM images of MC1061/pYMZ80, MWK11, and YMZ19 cells in Figures 2A–2C, and the EM micro-

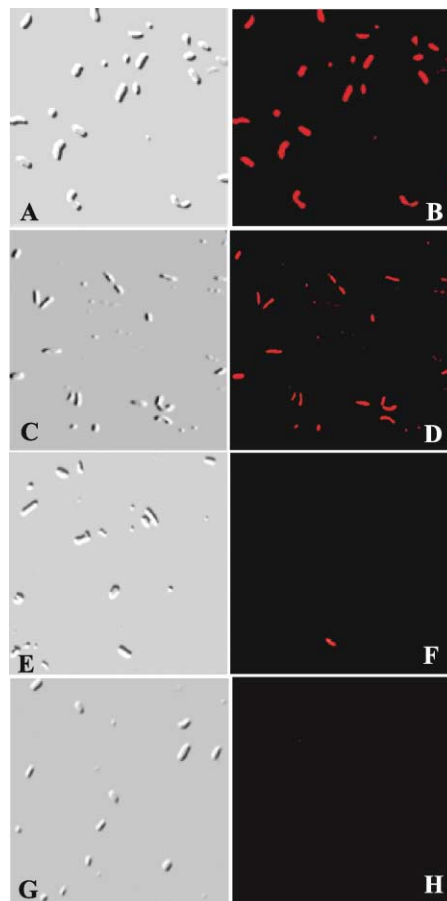


Figure 1. Immunofluorescence Microscopic Analysis of ClyA Expression

Bacterial cells grown overnight at 37°C in LB were treated with mouse monoclonal anti-ClyA antibodies and subsequently with rhodamine-conjugated anti-mouse IgG. Bacterial strains used: (A) and (B), MC1061/pYMZ80; (C) and (D), MWK11; (E) and (F), MC1061/pUC18; (G) and (H), YMZ19. (A), (C), (E), and (G) show phase contrast microscopy and (B), (D), (F), and (H) show fluorescence microscopy of the preparations in a pairwise manner.

graphs of MWK11 and YMZ19 in Figures 2D and 2E show representative examples of vesicles near the bacterial cells. At higher magnification using EM, we observed small ring-like structures with a central electron-dense core in vesicles from the ClyA-expressing strains (Figures 2F and 2G). These structures were suspected to represent ClyA pore assemblies since they resembled what had earlier been found after addition of purified ClyA protein to membrane vesicles or planar membrane bilayers in vitro. Eventually such preparations result in formation of pore structures that can be observed by EM (Wallace et al., 2000; Wai et al., 2003).

ClyA Accumulation in OMVs

To further analyze the relationship between ClyA and vesicles, we purified vesicles from cell-free culture supernatants of ClyA-positive and -negative strains (see Experimental Procedures). These preparations were enriched in vesicles with a diameter within the range 50–200 nm. In the case of the ClyA-producing bacteria (e.g.,

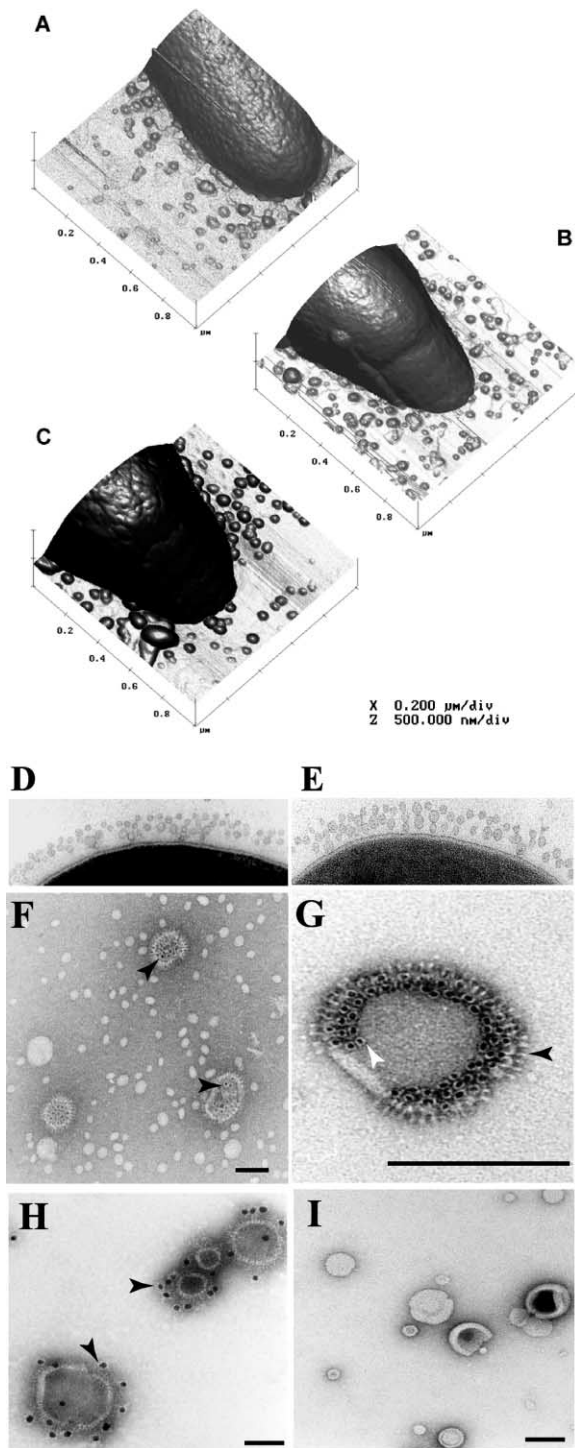


Figure 2. ClyA in OMVs of *E. coli* Cells
(A–C) AFM images of vesicles surrounding cells of the *E. coli* strains: MC1061/pYMZ80 (A), MWK11 (B), and YMZ19 (C).
(D–E) Electron microscopy (EM) of bacterial cells and associated vesicles of strains: MWK11 (D) and YMZ19 (E).
(F) EM analysis of OMVs from strain MWK11. Arrowheads point at two of the pore structures.
(G) Higher-resolution micrograph of a vesicle from strain MWK11 showing typical ClyA pore assemblies appearing as ring-like structures with a central electron-dense core (white arrow) and as spikes protruding from the margin of OMVs (black arrow).

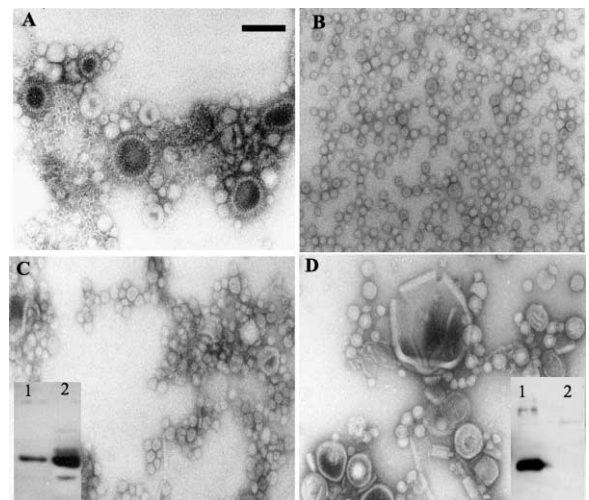


Figure 3. Electron Microscopy of Isolated OMVs from *E. coli* K-12 and from Pathogenic *E. coli* and *Salmonella*
(A) OMVs from strain MC1061/pYMZ80.
(B) OMVs from strain YMZ19.
(C) OMVs from the *E. coli* O111:H⁻ isolate CCUG29196; the insert shows immunoblot analysis using anti-ClyA antibodies of a whole-cell extract (lane 1) and of the OMV preparation (lane 2).
(D) OMVs from the *S. typhi* strain JON42 carrying the ClyA⁺ plasmid pMWK21; the insert shows immunoblot analysis using anti-ClyA antibodies of the OMVs from (D) in lane 1 and of OMVs from vector control in lane 2. The bar in (A) represents 200 nm in length.

strain MWK11; Figures 2F and 2G), there appeared some larger vesicles in which the structures resembling ClyA pore assemblies were observed. That the observed structures really represented ClyA protein was shown by immunolocalization using anti-ClyA antibodies in the immunogold labeling method. An intense deposition of gold particles was detected on the vesicles obtained from ClyA- producing bacteria, whereas there was no labeling in case of the ClyA-negative strain (Figures 2H and 2I). We concluded that the pore-like structures were ClyA protein assemblies.

Vesicles from the strains producing ClyA were somewhat heterogenous in size, and the larger vesicle structures were more common in the case of strains overproducing the cytotoxin (e.g., MC1061/pYMZ80; Figure 3A). In addition to the pore-like structures, side views of the presumed pore assemblies could be seen as spikes protruding from the margin (Figures 2G and 3A). There were no such structures observed with the vesicles obtained from the ClyA-negative strain, but they retained their more uniform size and appearance (Figure 3B).

Analyses of the protein content of the vesicle preparations are shown in Figure 4. Vesicles from the strains MC1061/pYMZ80 and MWK11 contained relatively large amounts of ClyA protein. In fact, ClyA appeared to be the most abundant protein present as judged by the intensity of silver staining (Figure 4A). Immunoblot analy-

(H–I) EM analysis by immunogold-labeling of ClyA in OMVs from strains MWK11 (H) and YMZ19 (I). Arrowheads point at two of the 10 nm gold particles. The bars shown at the lower right corners of Figures 2F–2I represent a length of 200 nm.

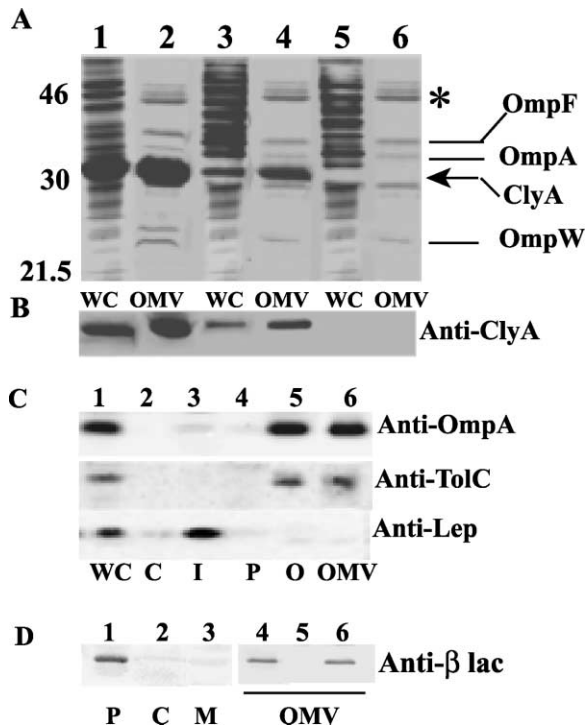


Figure 4. Accumulation of ClyA Protein in OMVs

(A) Silver staining after SDS-PAGE analysis of total cell extracts (lanes 1, 3, and 5) and of OMV preparations (lanes 2, 4, and 6). Strains: MC1061/pYMZ80 (lanes 1 and 2), MWK11 (lanes 3 and 4), and YMZ19 (lanes 5 and 6). The positions of molecular size markers (kD) are shown along the left side. Bands representing proteins (ClyA, OmpA, OmpF, and OmpW) identified by N-terminal amino acid sequencing are indicated along the right side. The band marked by an asterisk represents flagellin.

(B) Immunoblot analysis of the ClyA content in whole cells (WC) and in preparations of outer-membrane vesicles (OMVs), as in (A).

(C) Immunoblot analysis of two outer-membrane proteins (OmpA and TolC) and one inner-membrane protein (Lep) in subcellular fractions and OMVs from strain MWK11. Samples: whole-cell proteins (WC), cytosolic proteins (C), inner membrane proteins (I), periplasmic proteins (P), outer-membrane proteins (O), and outer-membrane vesicles (OMV).

(D) Immunoblot analysis of β -lactamase in subcellular fractions (Abbreviations: P, periplasmic proteins; C, cytosolic proteins; M, membrane proteins) of strain MC1061/pYMZ80 (lanes 1–3) and in OMVs from MC1061/pYMZ80 (lane 4), YMZ19 (lane 5), and YMZ19/pYMZ80 (lane 6).

sis using anti-ClyA antibodies confirmed the presence of the ClyA protein in both the whole-cell preparations and in vesicles (Figure 4B). Determination of the N-terminal amino acid sequence of each of the major protein bands showed that in addition to ClyA protein present in case of the ClyA-producing bacteria, bands detected in all of the preparations represented three outer-membrane proteins: sequences obtained were unambiguously showing that the proteins were OmpA, OmpF, and OmpW. The preparations also contained some flagella, as evidenced by presence of the flagellin protein. Additional evidence that they were OMVs was obtained by immunoblot analysis using anti-OmpA and anti-TolC antibodies. We also compared the OMVs with different subcellular fractions of the bacteria. As a specific inner-

membrane protein marker we used the leader peptidase protein detected with anti-Lep antibodies. The results clearly confirmed that the OMVs were derived from the outer membrane (Figure 4C). That OMVs also contained other periplasmic proteins was evident from immunoblot analysis of the plasmid-encoded β -lactamase that was detected in the case of strains MC1061/pYMZ80 and YMZ19/pYMZ80 (Figure 4D).

The EM and AFM analyses suggested that there were similar numbers of OMVs associated with cells of the different bacterial strains (Figures 2A–2E and data not shown). The outer-membrane porin proteins appeared equally abundant in the different OMVs according to the silver staining (Figure 4A). We concluded that there was no obvious difference in the amount of OMVs obtained from the different ClyA-positive and -negative derivatives. On the basis of the relative amount of porin proteins present in the preparations, we estimated that OMVs represented about 1% of the total outer-membrane material. We suggest that the surface expression of ClyA involves export from the periplasm via such vesicles.

ClyA Cytotoxin in OMVs from Pathogenic *E. coli* and *Salmonella*

Recently, the ClyA cytotoxin was found in pathogenic *E. coli* and in *Salmonella enterica* serovars Typhi and Paratyphi A (Oscarsson et al., 2002a; 2002b). We also studied the localization of ClyA cytotoxin in such isolates. Immunoblot analysis of OMVs from the enterohemorrhagic strain CCUG29196 (serotype O111:H⁻) confirmed that they contained appreciable amounts of ClyA protein (Figure 3C insert, lane 2). Similarly, we could isolate OMVs from the *S. typhi* strain JON42, which is a derivative of strain Ty21a where the *clyA* gene was abolished by an in-frame deletion. Vesicles from JON42 carrying a vector plasmid were quite uniform in size and shape, whereas the OMVs from JON42 carrying the trans-complementing *clyA*⁺ plasmid pMWK21 were more similar to the OMVs from *E. coli* cells expressing ClyA (Figure 3D and data not shown). Immunoblot analysis confirmed that there was ClyA protein present in OMVs from the ClyA positive *S. typhi* (Figure 3D insert, lane 1). We conclude from these experiments that the ClyA cytotoxin is exported via OMVs also in the case of natural isolates of these different enterobacteria.

ClyA Cytolytic Activity Is Associated with OMVs

To determine hemolytic activity, we used a contact hemolytic assay (see Experimental Procedures). We compared the activity with that of purified ClyA protein obtained from the periplasm of the bacteria. Both the OMVs and the purified ClyA elicited a dose-dependent response on the blood cells (Figure 5A). While the OMVs contained a similar amount of ClyA protein, we noted that there was a clear difference in lytic activity. The results suggested that ClyA in the OMVs had more than 8-fold higher activity than the purified ClyA.

To assay the cytotoxic effect on growing target cells, we studied how cultured HeLa cells were affected by preparations of OMVs or by the purified ClyA protein. A considerable level of toxicity toward the HeLa cells was observed by microscopy as soon as 15 min after the start of the treatment with ClyA contained in OMVs (Figure 5B,

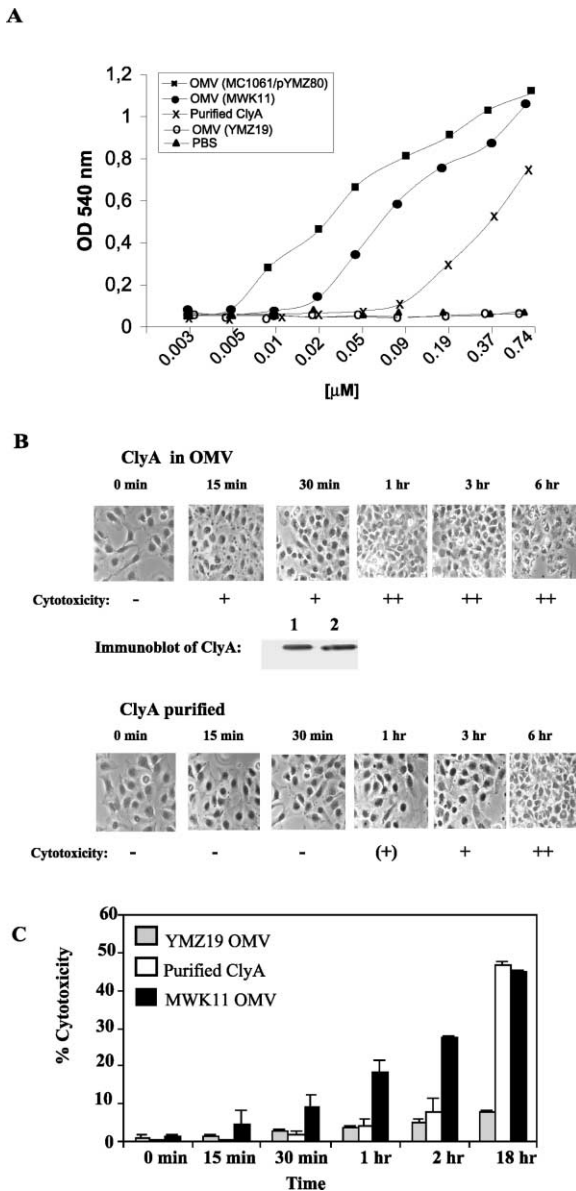


Figure 5. Cytolytic and Cytotoxic Activity of ClyA in OMVs
(A) Quantification of hemolytic activity of ClyA in OMVs and of ClyA purified from the bacterial periplasm as monitored by a contact hemolytic assay (see Experimental Procedures).
(B) HeLa cell cytotoxicity of ClyA in OMVs (top) and in purified form (bottom). Nonconfluent layers of HeLa cells were treated with 150 nM ClyA and examined at different time points in the microscope for signs of cytotoxicity as indicated below each frame (“-,” no cytotoxicity; “+,” signs of cytotoxicity observed; “++,” most cells affected). An immunoblot analysis of the ClyA preparations is shown as an insert; lane 1, ClyA in OMVs; lane 2, purified ClyA.
(C) Cytotoxicity as manifested by LDH release from HeLa cells after treatment with OMVs from strains MWK11 and YMZ19 or with purified ClyA protein as in (B). The plotted values are the mean from three separate experiments and bars show the standard error.

top). Cells with altered, more round morphology started to appear, and there was detachment of cells from the surface. However, in the case of pure protein, the toxic effect on HeLa cells only appeared after longer treatment (Figure 5B, bottom). The first clear signs of cytotoxicity

were observed after about 60 min and it took more than 3 hr before the majority of cells were affected. In control experiments with OMVs from the ClyA defective strain (YMZ19), there was no obvious cytotoxicity observed during the 6 hr duration of the studies (data not shown).

To quantify the ClyA cytotoxicity, we used an assay determining the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) from the HeLa cells upon treatment with either the purified ClyA or with the OMVs from strains MWK11 and YMZ19. The results were consistent with the observations described above, and only the ClyA-containing OMVs caused clear cytotoxicity during the initial 60–120 min (Figure 5C). A cytotoxic response also to the purified ClyA appeared after prolonged treatment (e.g., 18 hr), as seen earlier with macrophages (Lai et al., 2000).

A more-sensitive method to monitor cellular responses such as cell lysis is to study whether toxin exposure alters the ion homeostasis of target cells. Thus, we used a ratiometric imaging system to monitor the intracellular calcium concentration in primary epithelial cells from rat. This approach allows a rapid detection of cell lysis, within the minutes range, on a single-cell level. Cells preloaded with the calcium-sensitive dye Fura-2/AM were exposed to ClyA at 30 nM in an OMV preparation from strain MC1061/pYMZ80. Approximately 10 min after addition, a sustained elevation of intracellular calcium was observed (Figure 6A). Within 30 min, all cells in the cell cluster showed a sustained elevation of the intracellular calcium concentration, and several of the cells had lysed. Cell lysis was shown as an abrupt drop in immunofluorescence (ratio = 0) due to leakage of the calcium-sensitive dye out of the cell, and this occurred normally within 1 hr (data not shown). The cytotoxic effect was indeed due to the effect of ClyA, since OMVs from the *clyA* mutant strain YMZ19 had no effect on the intracellular calcium concentration (Figure 6B), not even at amounts twice as high as when ClyA-containing OMVs were used. As shown in Figure 6C, the purified ClyA (60 nM) was unable to introduce any alterations of the cells calcium homeostasis during the duration of the experiment. These results are in keeping with the above-described hemolysis and cytotoxicity assays. Taken together, the results demonstrate that the ClyA protein exposed by the vesicle preparations was presented in a considerably more cytolytically and cytotoxicity active form than when the protein was purified from the periplasmic compartment of the bacteria.

Monomeric ClyA in the Periplasm and Oligomer Formation in the OMVs

We conducted crosslinking experiments in order to assess the occurrence of monomeric and oligomeric ClyA protein in the periplasm and in the OMVs (Figure 7A). The ClyA protein isolated from the periplasmic fraction was mainly in the monomeric form (Figure 7A, lanes 1–3). However, ClyA present in OMVs appeared in the form of larger (more than 220 kDa) oligomeric complexes (Figure 7A, lanes 4–9) presumably representing the octameric ClyA complexes suggested to be the active form of pore assemblies (Wallace et al., 2000).

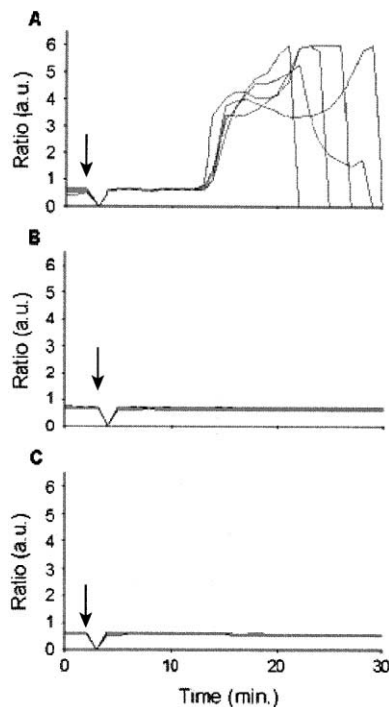


Figure 6. ClyA Cytotoxicity Monitored by Ratiometric Calcium Imaging

Intracellular calcium measurements in renal proximal tubule cells after treatment with (A) a sample with 30 nM ClyA in vesicles prepared from MC1061/pYMZ80, (B) a corresponding amount of vesicles from the ClyA-negative strain YMZ19, or (C) purified ClyA protein (60 nM). Arrows indicate addition of stimuli. Arbitrary units (au) represent ratio changes corresponding to changes in intracellular $[Ca^{2+}]$. One representative experiment is shown for each stimulus, and measurements from five cells in each case were plotted.

The monomeric ClyA in the periplasm was shown to have an intramolecular disulphide bond formed between the cysteine residues at positions 87 and 285 in the polypeptide (Atkins et al., 2000). The presence of the disulphide bond was suggested to prevent oligomerization of ClyA. To directly assess the status of the cysteine residues and their involvement in disulphide bond formation, we carried out alkylation of free thiols using the high molecular mass molecule AMS (4-acetamido-4' maleimidystilbene-2-2'-disulfonic acid) as described in the Experimental Procedures. Samples of the ClyA protein purified from periplasm and of the OMVs containing ClyA were subjected to the AMS alkylation and were subsequently analyzed by immunoblot analysis with anti-ClyA antibodies (Figure 7B). The purified ClyA protein was not susceptible to become AMS derivatized, as judged by the fact that it was not altered in its gel-electrophoretic migration even after treatment with a high concentration of AMS (Figure 7B lanes 1–3). We take this as evidence that the cysteine residues in ClyA were engaged in disulphide bonding. In tests after prior treatment of the protein with the reducing agent dithiothreitol (DTT), we found that thiols became available for AMS derivatization (data not shown), and the results were consistent with the notion that the majority of ClyA in the periplasm is in oxidized form. In the case of ClyA in

the OMVs, there were clear indications that AMS groups were added to free thiols in the protein. It appeared that the majority of ClyA was present in reduced form and migration was altered even when lower AMS concentrations and shorter incubations were tested (Figure 7B, lanes 4–9).

It was conceivable that the observed difference in oligomer formation could be due to a local concentration effect since, presumably, the effective concentration of the protein may be much higher in the OMVs relative to the ClyA in solution. We estimated from the relative yields of ClyA protein in the different preparations that the concentration can be 10-fold higher in the OMVs as compared with that of the periplasm. After concentrating the ClyA obtained from the periplasmic compartment by vacuum evaporation and thereby obtaining a 10-fold more concentrated solution, we repeated the cross-linking test. The test, which was performed with two different, independently isolated preparations, did not provide evidence for oligomer formation due to the higher protein concentration, but the ClyA protein remained as monomers (data not shown). The result does not rule out that a local concentration effect might contribute to higher bioactivity of ClyA in OMVs, but it did not seem to be the main mechanism.

To test if the lack of formation of oligomers in the case of ClyA from the periplasm depended on the redox status per se, we performed crosslinking analysis with the reduced protein after DTT treatment. As shown in Figure 7C, the reducing treatment of the ClyA protein resulted in formation of oligomers that efficiently were detected both after short (10 min) and more prolonged crosslinking, and it was evident that formation of such complexes did not require higher concentration of ClyA. The oligomers appeared similar (more than 220 kDa) to those detected with ClyA present in OMVs (compare Figure 7A). Furthermore, we found that the oligomerization of the DTT treated protein resulted in higher cytolytic activity. It showed more than 16-fold higher hemolytic activity than the untreated ClyA protein (data not shown).

We conclude that the ClyA protein had a different redox status when present in the OMVs in comparison with the ClyA protein from the periplasm, and evidently this difference correlated with the difference in formation of oligomers and active pore assemblies.

Dsb Pathway-Dependent Redox Regulation of ClyA Activity

In the periplasm of *E. coli* there are two complementary pathways that catalyze the formation of disulphide bonds (reviewed by Ritz and Beckwith, 2001). DsbA and DsbB in the disulphide bond formation pathway add new disulphide bonds to folding proteins. The DsbC and DsbG proteins in the disulphide isomerization pathway rearrange existing bonds between incorrectly paired cysteines. Since our analysis of ClyA indicated that the pore-forming activity of the protein might be dependent on the redox status, we tested if expression of ClyA activity was affected in *E. coli* strains with deficiency in the disulphide bond formation pathway using the strains HK205 (wild-type), HK229 (*dsbA::Kan* mutant), and HK227 (*dsbB::Kan* mutant). We then made the remarkable observation that the *dsb* mutant derivatives ex-

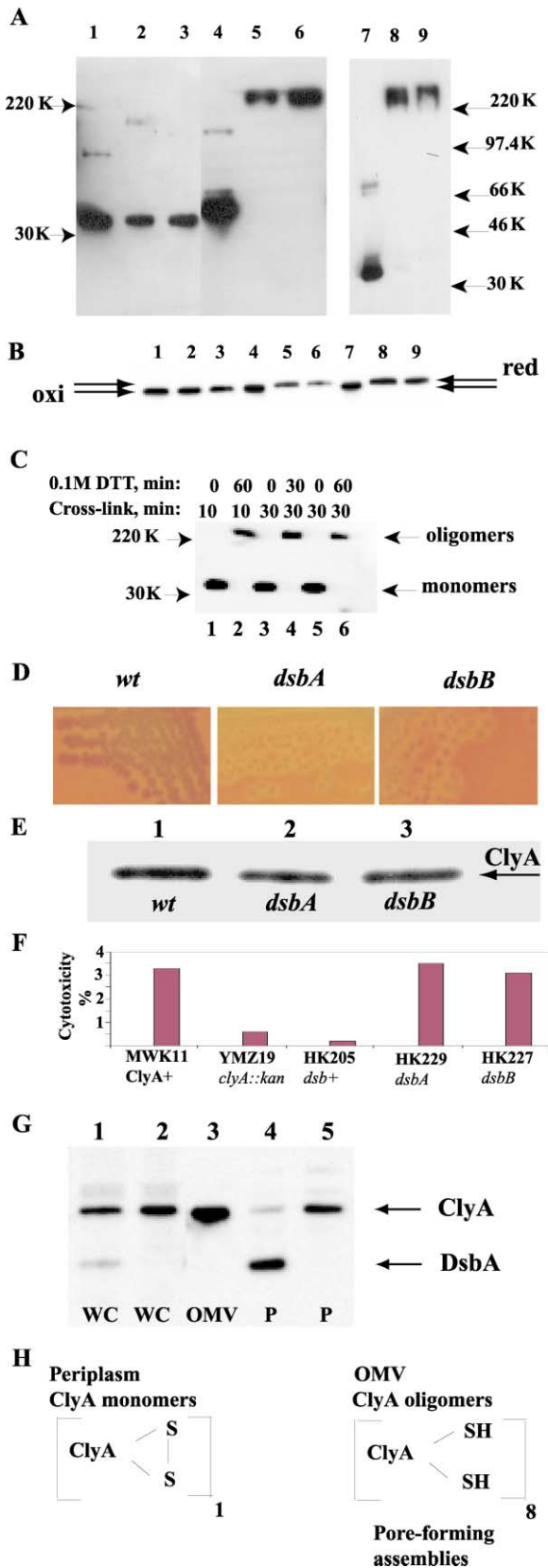


Figure 7. Control of ClyA Redox Status and Oligomer Formation by the Dsb System

(A) Glutaraldehyde crosslinking and immunoblot analysis of mono-

pressed hemolysis on blood agar plates (Figure 7D). There were clear zones of lysis around the bacterial colonies of the mutant strains, whereas the wild-type strain was negative. The level of expression of ClyA protein in *E. coli* K-12 strains is normally not high enough to cause such a recognizable phenotype on blood agar (Oscarsson et al., 2002a). To check if the ClyA protein level was altered, we carried out immunoblot analysis of total bacterial cell extracts using anti-ClyA antibodies. The ClyA protein levels in the *dsb* mutant bacteria were not increased in comparison with the wild-type strain (Figure 7E). The quantitative LDH release assay was used to test cytotoxicity of the different bacterial strains to HeLa cells. For comparison, we included the ClyA-expressing strain MWK11 and the ClyA-deficient strain YMZ19. The results from assays after treatment of HeLa cells with the bacteria are summarized in Figure 7F. In keeping with the hemolysis phenotypes, we found that the *dsb* mutant strains displayed cytotoxicity, whereas the wild-type strain did not.

meric and oligomeric ClyA forms in preparations from the periplasm and from OMVs. ClyA purified from the periplasmic fraction of strain MWK11: lane 1, no treatment; lane 2, 2.5 mM glutaraldehyde treatment during 10 min; lane 3, 2.5 mM glutaraldehyde treatment during 30 min. ClyA in OMVs from strain MWK11: lanes 4 and 7, no treatment; lanes 5 and 8, 2.5 mM glutaraldehyde treatment during 10 min; lanes 6 and 9, 2.5 mM glutaraldehyde during 30 min. Lanes 1–6 are showing samples in a 13% gel and lanes 7–9 are showing samples in a 8% gel. Positions of molecular size standards are shown along the sides.

(B) Analysis of redox status of the purified ClyA protein from the periplasm and ClyA in OMVs. Alkylation of free thiols using the high-molecular mass molecule AMS was carried out as described in the Experimental Procedures. The samples were subsequently subjected to SDS-PAGE and immunoblot analysis with anti-ClyA antibodies, and the positions of oxidized (oxi, lower arrow) and reduced (red, upper arrow) forms are indicated. Lanes 1–3: Purified ClyA from periplasm treated with 45 mM AMS during 0, 30, and 60 min, respectively. Lanes 4–6: ClyA in OMVs treated with 45 mM AMS during 0, 30, and 60 min, respectively. Lanes 7–9: ClyA in OMVs treated with 15 mM AMS during 0, 30, and 60 min, respectively.

(C) Oligomer formation by the ClyA preparation from the periplasm after treatment with the reducing agent DTT. ClyA untreated (lanes 1, 3, and 5) or DTT treated (lanes 2, 4, and 6) for the indicated amount of time was subjected to the cross-linking analysis with glutaraldehyde (compare Figure 7A).

(D) Effect of *dsb* mutations on the phenotypic expression of ClyA. The strains HK205, HK229, and HK227 which have wild-type, *dsbA::Kan*, or *dsbB::Kan* loci, respectively, were grown on blood agar plates at 37°C.

(E) Immunoblot analysis of the ClyA protein levels in total cell extracts from the *dsb*⁺ (lane 1), *dsbA::Kan* (lane 2), and *dsbB::Kan* (lane 3) strains grown as shown in (C).

(F) Cytotoxicity expressed by *dsb* mutant *E. coli* as monitored using the LDH release assay with HeLa cell cultures. Bacterial cells were incubated together with HeLa cells (at a multiplicity of infection of ten) during 150 min before determining the release of LDH (see Experimental Procedures for details).

(G) Differential localization of the DsbA and ClyA proteins in the periplasm and OMVs. Cell extracts of the *dsb*⁺ strain MWK11 and a *dsbA::Kan* transductant (SNW200) were analyzed by immunoblotting using both anti-DsbA antibodies and anti-ClyA antibodies. Lanes 1 and 2 show whole-cell (WC) extracts of MWK11 and SNW200, respectively. Lane 3 shows the OMV fraction of strain MWK11. Lanes 4 and 5 show periplasmic (P) fractions of strain MWK11 and SNW200, respectively.

(H) Summary of the DsbA-dependent redox status and the monomer versus oligomer status of ClyA in the periplasm and in OMVs.

Genetic analyses that included derivatives with an in-frame deletion of the *clyA* locus verified that the hemolytic/cytolytic phenotype was due to the activity of ClyA. The *dsbA::Kan* mutation was introduced by transduction into strains M182 and MWK7, which are wild-type and Δ *clyA* derivatives, respectively. All *dsbA::Kan* transductants of M182 were showing the positive hemolytic phenotype, whereas the MWK7 transductants did not. The results confirmed that the activity of ClyA was dependent on the disulphide bond formation pathway in a manner that was consistent with the redox status of the protein.

To assess if ClyA and DsbA were colocalized in both periplasm and OMVs, we assayed whole-cell extracts, OMVs, and periplasmic extracts of the ClyA-producing strain MWK11 in immunoblot analysis (Figure 7G, lanes 1, 3, and 4). A *dsbA::Kan* mutant transductant (SNW200) of MWK11 was included as control (Figure 7G, lanes 2 and 5). The ClyA protein was detected together with the DsbA protein in whole-cell extracts and periplasmic extracts. However, in the OMVs, there was no detectable DsbA protein (compare lanes 3 and 4 in Figure 7G). We obtained the same result with two separate preparations of OMVs from strain MWK11 (Figure 7G and data not shown). While we do not exclude that a small amount of DsbA might be localized to the OMVs, the results clearly demonstrated that the ratio between DsbA and ClyA was very different in the periplasmic and OMV compartments, and this difference was consistent with the redox status of ClyA.

Discussion

This work defines a vesicle-mediated transport mechanism in bacteria that is responsible for the activation and delivery of pathogenic effector proteins, seemingly independent of the previously described type I–V secretions systems. Our studies demonstrate that the ClyA cytotoxic protein is found in the form of active oligomeric assemblies in outer-membrane-derived vesicles released from the bacterial cells during normal growth. We conclude that the ClyA protein, which is present in monomeric form in the periplasm, is undergoing an oligomerization and forms the active pore assemblies in the OMVs and that this change into the active form is dependent upon the altered redox status (Figure 7H). We may consider this assembly of ClyA oligomers in OMVs as a process, which exports ClyA in an active form to the external environment.

It is feasible that the OMVs containing ClyA were fusing with the target cells and that the cytotoxin thereby also was more efficiently delivered to the target cell membranes. However, more importantly, the present findings establish that both export and the formation of cytotoxically active pore assemblies are facilitated by the release of OMVs. We conclude that our studies of the ClyA cytotoxin have revealed a bacterial export-dependent activation pathway. Since the Dsb system is important for many periplasmic bacterial proteins we must consider the possibility that localization to OMVs may be of relevance for the action and function also in other cases where the redox status of the protein can be affected.

A possible reason for a change with respect to the redox status of proteins in OMVs is suggested when we look at the proposed role and location of the different Dsb proteins involved in disulphide bond formation in the periplasm (reviewed by Ritz and Beckwith, 2001). The periplasmic space in *E. coli* is favorable for disulphide bond formation as it contains the thiol oxidant DsbA protein and the disulphide bond isomerase DsbC. The DsbB protein is a cytoplasmic membrane protein responsible for maintaining DsbA in an oxidized state. Using immunoblot analysis, we found that little or no DsbA protein was localized in the OMVs together with the ClyA cytotoxin while it was readily detected in the periplasmic compartment (Figure 7G). The β -lactamase, which is another periplasmic protein, was clearly detectable in both compartments, although the ratio of β -lactamase between OMV and periplasm was lower than that of ClyA (Figures 4D and 7G). Assuming that some undetected amount of DsbA was localized in the OMVs, the protein would no longer be in contact with the DsbB protein and, consequently, the disulphide oxidase activity of DsbA could be expected to be less efficient. We should emphasize that it is not ruled out that there could be some costimulatory component(s) of ClyA action in the membrane vesicles. However, the observed difference in redox status and the findings with the *dsb* mutant bacteria suggested that the Dsb components were important here. We do not have any evidence for direct involvement of some disulphide reductase in the OMVs, but it may be considered. In any case, by virtue of the separation from some of the Dsb components, the oxidation of ClyA and other proteins would be expected to be less efficient in the OMV compartment. Our data are consistent with such a model, and it was evident that the ClyA protein might thereby become more active in the formation of the oligomeric pore-forming assemblies. While it remains to be elucidated, our findings raise intriguing questions about the possibility that there could be some mechanism of crude sorting, in which some periplasmic proteins are more or less excluded from OMVs.

A different role for OMVs released by *E. coli* in connection with toxin secretion has been suggested in previous studies. Secretion of the heat labile toxin (LT) from enterotoxigenic *E. coli* (ETEC) to the external environment was associated with release of OMVs (Wai et al. 1995; Horstman and Kuehn, 2000). Secretion of LT from *E. coli* occurs via the GSP, and recent studies suggested that the majority of LT subsequently associates with LPS on OMVs (Tauschek et al., 2002; Horstman and Kuehn, 2002). The ClyA protein is not dependent on the GSP secretion system as evidenced by tests with a *gspF* knockout mutant, and none of the other known secretion systems (types I–V) appear to be involved directly (our unpublished data). It was proposed that LPS tethered LT to OMVs and that the interaction facilitated intoxication (Horstman and Kuehn, 2002). These studies also showed that the LT interaction with LPS neither attenuates nor enhances toxicity per se. In the case of the ClyA protein, we suggest that it is incorporated in the OMVs as part of the periplasm that is released from the cells by the vesicle formation. It should be emphasized that our findings with the ClyA cytotoxin thereby provided evidence

for physiological relevance of prokaryotic vesicle formation.

Experimental Procedures

Bacterial Strains and Growth Conditions

Bacterial strains used were (1) *E. coli* K-12: strain MC1061 (Casadaban and Cohen, 1980), strain M182 (Casadaban, 1976), the M182 Δ clyA derivative MWK7 (Westermarck et al., unpublished construction), the clyA::Kan strain YMZ19 (Oscarsson et al., 1996), the ClyA⁺ strain MWK11 (Westermarck et al., 2000), the dsb⁺ strain HK205 and the dsbA::Kan and dsbB::Kan mutants HK229 and HK227, respectively (Kadokura et al., 2000), and SNW200 (a dsbA::Kan mutant transductant of MWK11, this work); (2) the *E. coli* O111:H⁻ isolate CCUG29196 (Whittam et al., 1988); and (3) the *Salmonella enterica* serovar Typhi derivative JON42 (Oscarsson et al., 2002b). The pYMZ80 clone was described earlier (Oscarsson et al., 1999). The bacterial strains were grown aerobically, with shaking, at 37°C in Luria-Bertani (LB) broth or on agar. Blood agar plates contained 5% (v/v) horse erythrocytes. Antibiotics were added to LB broth or agar at the following final concentrations: 50 µg ml⁻¹ carbenicillin or 30 µg ml⁻¹ kanamycin.

Immunofluorescence Microscopy

Using monoclonal anti-ClyA antibody and rhodamine-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc.), we performed immunofluorescence labeling of bacterial cells as described earlier (Xia et al., 2000).

Electron Microscopy

Procedures for immunogold labeling and electron microscopy were essentially as described earlier (Wai et al., 1998).

Atomic Force Microscopy

Imaging was done on a Nanoscope IIIa Atomic Force Microscope (Digital Instruments) using Tapping Mode. The final images were plane fitted in both axes and presented in a surface plot of the height mode.

Isolation of OMVs

OMVs were isolated from culture supernatants as previously described with some modifications (Wai et al., 1995). After centrifugation at 5000 × g for 15 min at 4°C, the supernatants were filtered through a 0.45 µm vacuum filter. OMVs were then collected by centrifugation at 150,000 × g for 3 hr at 4°C in a 45 Ti rotor (Beckman Instruments, Inc.). The pellets were suspended in 20 mM Tris-HCl (pH 8.0) and used as the OMVs' preparation.

Cell Fractionation and Purification of ClyA Protein

Different subcellular fractions were obtained using procedures described recently (Oscarsson et al., 2002b; Wai et al., 2003). ClyA protein from the periplasmic fraction was purified by fast-protein liquid chromatography (FPLC) with a MonoQ ion exchange column (Pharmacia) as described (Wai et al., 2003).

SDS-PAGE Electrophoresis

The standard SDS-PAGE procedure was used (Laemmli, 1970). Gels were stained with Coomassie blue and/or silver stain. Protein standards were from Bio-Rad.

Western Blot Analysis

For immunoblotting, we used the methods of Towbin et al. (1979) and different antibodies: polyclonal anti-ClyA (Oscarsson et al., 1999), monoclonal anti-ClyA (S.N.W. and B.E.U. unpublished data), polyclonal anti-β-lactamase (Oscarsson et al., 2002b), polyclonal anti-DsbA (Akiyama et al., 1992), polyclonal anti-OmpA (Henning et al., 1979), polyclonal anti-TolC (Thanabalu et al., 1998), and polyclonal anti-Lep (de Gier et al., 1996). For detection we used the ECL⁺ chemiluminescence system (Amersham Pharmacia Biotech).

N-Terminal Protein Sequencing

Determination of the N-terminal amino acid sequence of proteins was achieved by automated Edman degradations of samples blot-

ted onto PVDF membranes. The sequencing was performed on a Procise 494 Sequencer (Applied Biosystems) with an on-line PTH-analyzer. A sufficient number of residues were determined to allow unambiguous identification (100% identity; using the NCBI BLAST database).

Assay of Hemolytic Activity

Phenotypic test of hemolytic activity on blood agar plates and the quantitative contact hemolytic assay were performed as previously described (Oscarsson et al., 1999).

Cytotoxicity Assays with HeLa Cells

HeLa cells were cultured in Ham's F-10 medium supplemented with 10% fetal calf serum using standard procedures. Cells treated with pure ClyA or OMVs were fixed in PBS containing 3% paraformaldehyde for 15 min at room temperature before the samples were observed using a Zeiss Standard WL phase-contrast microscope. The quantitative lactate dehydrogenase (LDH) release assay was performed using the CytoTox 96 nonradioactive cytotoxicity assay kit (Promega). In tests with bacterial cells, the multiplicity of infection was ten, and bacterial growth was inhibited by addition of 15 µg/ml chloramphenicol. At the onset of the incubation, the bacteria and HeLa cells were centrifuged at 2800 rpm for 15 min.

Calcium Imaging

The animal studies were approved by the Swedish Ethical Committee, Karolinska Institutet. Primary proximal tubule cells were prepared from kidneys from 20-day-old female Sprague-Dawley rats and used in ratiometric calcium imaging experiments as previously described (Larsson et al., 1986; Uhlén et al., 2000). Cells loaded with 2 µM Fura-2 acetylmethyl ester (Fura-2/AM) (Molecular Probes) were exposed to OMVs or purified ClyA.

Protein Crosslinking

ClyA preparations from the periplasm or as OMVs were subjected to the treatment with 2.5 mM glutaraldehyde during 10 or 30 min as described (Chen et al., 1996).

Determination of ClyA Redox Status

Treatment of ClyA protein with the alkylating high molecular mass molecule AMS (4-acetamido-4' maleimidystilbene-2-2'-disulfonic acid; Molecular Probes) was carried out essentially as described (Joly and Swartz, 1997). 15 mM or 45 mM AMS was added to protein samples in 50 mM Tris-HCl (pH 8.0), 1% SDS-PAGE, and 1 mM EDTA and incubation was done at 37°C during 30 or 60 min. The ClyA protein was examined by immunoblot analysis. The AMS-derivatized ClyA migrated slightly slower in SDS-PAGE as expected since each AMS conjugated moiety increases the molecular mass by 490 Da (Joly and Swartz, 1997).

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