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Release of Outer Membrane Vesicles from *Bordetella pertussis*

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Abstract. The aim of the study reported here was to investigate the production of *Bordetella pertussis* outer membrane vesicles (OMVs). Numerous vesicles released from cells grown in Stainer-Scholte liquid medium were observed. The formation of similar vesicle-like structures could also be artificially induced by sonication of concentrated bacterial suspensions. Immunoblot analysis showed that OMVs contain adenylate cyclase-hemolysin (AC-Hly), among other polypeptides, as well as the lipopolysaccharide (LPS). Experiments carried out employing purified AC-Hly and OMVs isolated from *B. pertussis* AC-Hly$^-$ showed that AC-Hly is an integral component of the vesicles. OMVs reported here contain several protective immunogens and might be considered a possible basic material for the development of acellular pertussis vaccines.

Bordetella pertussis, the causative agent of whooping cough, produces several polypeptides implicated in the pathogenesis of the disease [33]. Among them is the adenylate cyclase-hemolysin (AC-Hly)—a potential vaccine candidate [4, 9, 11], which is released into the culture medium [2]. Although the release of polypeptides facilitates their isolation and purification, vaccine production based on AC-Hly has been rather difficult because of the low yield of the polypeptide secreted into the extracellular medium. Therefore, research directed to gain a better understanding of the mechanisms of AC-Hly secretion into the medium might be useful to devise strategies to improve AC-Hly yields. We have previously reported that the addition of methyl-β-cyclodextrin [7, 15, 16, 32] to the culture medium increases the extracellular level of the AC-Hly [13]. Since the latter compound also enhances the release of lipopolysaccharide (LPS), one of the major outer membrane components [14, 26], it seemed probable that the increased shedding of outer membrane blebs or vesicles (OMVs) might explain the increased levels of both cell-free AC-Hly and LPS in the presence of methyl-β-cyclodextrin. Many Gram-negative bacteria shed the outer membrane as vesicles containing LPS [17–20] into the culture medium. The evidence of relationship of the vesicles with several biological activities suggests that active subtances might be secreted from bacterial cells, either separately from or associated with the OMVs [23]. Nevertheless, the release and composition of OMVs has not been extensively investigated in *Bordetella* species. Morse and Morse reported the presence of naturally secreted OMVs in supernatants of *B. pertussis* grown in Hedley-Wright medium [24]. Vesicles prepared from whole cells of *B. pertussis* by sonication behave as protective antigens in the intracerebral mouse challenge model [27]. Whether these vesicles are antigenically similar to OMVs naturally present in the extracellular medium has not been elucidated as yet.

This study has shown the presence of *B. pertussis* OMVs in the supernatant of the Stainer Scholte synthetic medium used for vaccine production (SS) [30]. Protein composition of the vesicles isolated from culture supernatants was compared with that of OMVs prepared from bacterial cells by sonication. The presence of protective antigens such as adenylate cyclase, pertussis toxin, and filamentous hemagglutinin in the OMVs was investigated as well.

Materials and Methods

Strains and growth conditions. *B. pertussis* Tohama strain (CIP 8132) and *B. pertussis* mutant deficient in AC-Hly expression (Bp 118 ΔB) [3]

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were used throughout this study. *B. pertussis* strains were grown in Stainer-Scholte liquid medium (SS) [36] as indicated previously [13]. In some experiments, 3 mg of methyl-β-cyclodextrin per ml were added to the SS medium (SS + MeβCD) [13].

Isolation of outer membrane vesicles (OMVs). OMVs were isolated either from culture supernatants or from bacterial cells. To examine the presence of vesicles in the supernatant, the Stainer Scholte medium supplemented with cyclodextrin was used. This medium, in which the levels of extracellular proteins [7, 13, 16] and LPS [14] were enhanced, would increase the possibility of finding OMVs. Culture samples from the decelerating growth phase were centrifuged at 10,000 *g* for 20 min at 4°C. Glutaraldehyde was then added to the cell-free supernatant thus obtained (1% v/v, final concentration), and the suspension was incubated for 45 min at room temperature. The resulting suspension was finally centrifuged at 100,000 *g* for 2 h at 4°C to collect the OMVs. The pellet obtained was examined by means of negative staining, i.e., suspending the sample in 0.1 M ammonium acetate (pH 7.0).

To obtain OMVs from bacterial cells, bacteria grown in SS medium were collected by centrifugation as described above and resuspended in 20 mM Tris-HCl, 2 mM EDTA pH 8.5 (TE buffer). Five milliliters of TE buffer were used to resuspend approximately 1 g (wet weight) of bacteria. The suspension was sonicated in cool water for 20 min. After two centrifugations at 10,000 *g* for 20 min at 4°C, the supernatant was pelleted at 100,000 *g* for 2 h at 4°C. This pellet was resuspended in 1.5% (w/v) deoxycholate (DOC) in TE buffer. Six milliliters of this suspension were added on 2 ml of sucrose 60% (w/v). After centrifugation at 100,000 *g* for 2 h at 4°C, the OMV band was observed at TE/sucrose interphase. This procedure was repeated once. Then, the OMVs were stored with glycerol 1% and sodium azide 0.001% at 4°C. The sample obtained from cells was negatively stained and then examined with an electron microscope.

Electron microscope—negative stains. Electron microscopy was performed by suspending OMVs in 0.1 M ammonium acetate (pH 7.0). A droplet of this suspension was placed on a grid coated with a carbon-reinforced fomvar film. After 30 s, the excess fluid was removed by absorbing with filter paper and the grids stained with 2% (w/v) phosphotungstic acid pH 5.2 (with KOH). Examination was done with an JEM 1200 EX Jeol microscope.

Protein assay. Protein content was estimated by the Lowry method, using bovine serum albumin as standard [22].

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The Laemmli discontinuous buffer system was used [21]. The acrylamide concentration of separating gels is indicated in the legends of the figures. OMV preparations were solubilized by heating at 100°C for 5 min in 0.0625 M Tris/HCl buffer, pH 6.8, containing SDS (2%, w/v), glycerol (10%, w/v), bromphenol blue (0.001%, w/v), and mercaptoethanol (5%, w/v).

LPS isolated from cells or from OMVs were solubilized in the sample buffer described above and heated at 100°C for 10 min. Twenty-five micrograms of proteinase K in 10 µl of buffer were added per 50 µl of LPS suspension. The mixtures were incubated in a water bath at 60°C for 1 h with occasional vortexing. Proteinase K–treated samples were applied to gels.

Conditions under which electrophoresis had to be used were performed at room temperature and constant voltage. Polypeptides were stained overnight in a solution of Coomassie Blue R250 (0.2%, w/v) in methanol 40% (v/v) and acetic acid 10% (v/v). Molecular weights were estimated by means of a Pharmacia Calibration Kit. Hitchcock and Brown [12] silver stain technique was used to spot lipopolysaccharide.

Detection of AC-Hly, PTx, and FHA on Western immunoblots. Samples of OMV obtained from *B. pertussis* cells were treated with Laemmli sample buffer [21] and run on 10% SDS gels. Proteins were transferred from polyacrylamide gels to polivynilidene difluoride (Immobilon PVDF, Millipore) sheets and incubated with mouse polyclonal immune sera directed against the adenylate cyclase (AC-Hly), pertussis toxin (PTx), or filamentous hemagglutinin (FHA) of *B. pertussis*. The immunochemical detection was performed using alkaline phosphatase-labeled sheep anti-mouse immunoglobulins.

Results and Discussion

Glutaraldehyde was used to investigate the presence of OMVs in supernatants of *B. pertussis* Tohama strain (CIP 8132) cultures since this fixing agent produced a significant increase of the amounts of vesicles obtained from other Gram-negative bacteria [19, 20]. The SS medium was used supplemented with cyclodextrin (3 mg/ml), a condition that promotes the release of LPS [14] and probably the production of OMVs. Figure 1A shows the results of the electron microscopy. Many vesicles with sizes ranging from 150 to 250 nm were observed. Although the yield of these vesicles was high, further studies with this material were not feasible because these OMVs prepared in the presence of glutaraldehyde could not be properly solubilized in SDS-PAGE sample buffer. Therefore, OMVs were prepared in the absence of glutaraldehyde (unfixed OMVs). However, the unfixed vesicles were scarce and their sizes varied within a wide range. Moreover, these preparations contained significant amounts of amorphous material (Fig. 1B), which could derive from a partial degradation of the vesicles, as suggested by Kondo et al. [20] for *Vibrio cholerae*.

To avoid the low yield of OMVs and the presence of amorphous material, vesicle formation from cell pellets was induced by sonication as is indicated in Materials and methods [6]. Samples obtained were negatively stained and then examined with an electron microscope. Figure 1C shows vesicles which are similar in size to the OMVs obtained from the supernatant with glutaraldehyde. To confirm that those vesicles derived from the outer membrane, the presence of LPS in the OMVs was determined by SDS-PAGE (Fig. 2). Vesicles containing LPS with an electrophoretic pattern similar to that exhibited by LPS preparations extracted from whole cells were observed. This result was in agreement with those reported by Gu and Tsai for LPS purified from both cells and OMVs of *Neisseria meningitidis* [8]. The LPS profiles shown in Fig. 2 consist of two bands (rough-type LPS), an upper dominant (LPSa) and a lower, small band (LPSb), which closely resemble those reported by Peppler et al. [25] for *B. pertussis*. As shown in Fig. 2, the LPS content of the OMVs appears to be reduced when 2.25% (w/v) of DOC instead of 1.5% (w/v) was used during the isolation procedure.

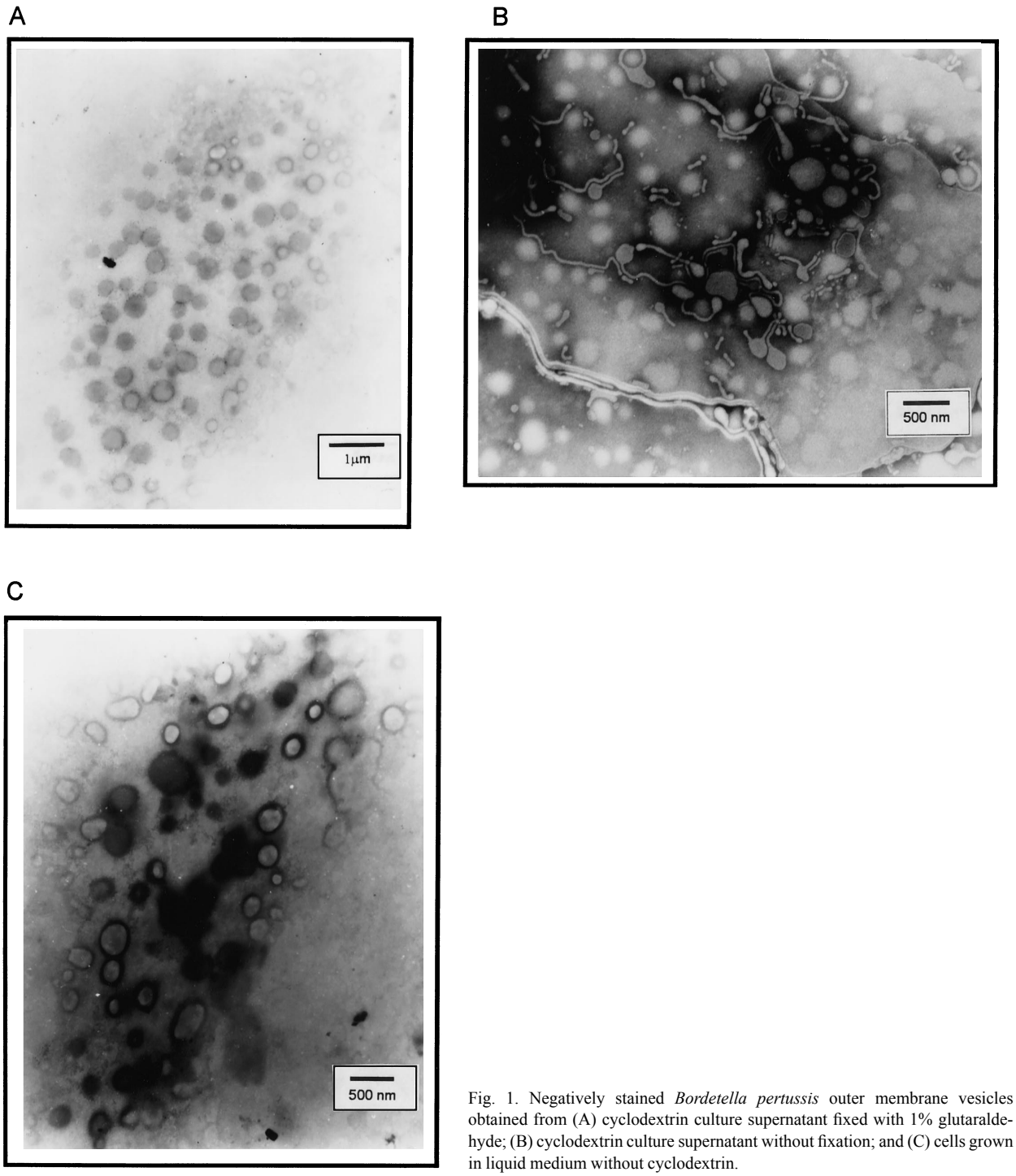

Fig. 1. Negatively stained *Bordetella pertussis* outer membrane vesicles obtained from (A) cyclodextrin culture supernatant fixed with 1% glutaraldehyde; (B) cyclodextrin culture supernatant without fixation; and (C) cells grown in liquid medium without cyclodextrin.

It is worth noticing that the polypeptide profile in SDS-PAGE of OMVs obtained from *B. pertussis* cells was similar to that obtained from unfixed OMVs sedimented from supernatants (Fig. 3). Then, antigenic studies were performed using OMVs prepared by cell sonication, since the yield of these OMVs was higher than that corresponding to the unfixed OMVs. A Western blot analysis of these OMVs is shown in Fig. 4. Antibodies directed against purified AC-Hly recognized antigenic components exhibiting an electrophoretic mobility corresponding to proteins of 200 kDa. Minor components with higher mobility were also recognized. This pattern of

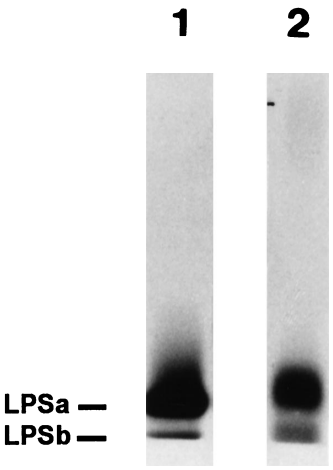

Fig. 2. SDS-PAGE (15% w/v) comparing LPS from *Bordetella pertussis* outer membrane vesicles isolated with different concentration of DOC: 1.5% (line 1) and 2.25% (line 2). LPSa and LPSb are indicated.

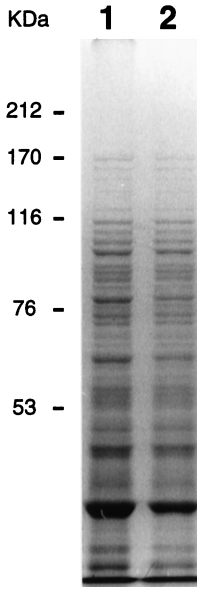

Fig. 3. SDS-PAGE (10% w/v) comparing polypeptides of *Bordetella pertussis* outer membrane vesicles isolated from cells grown in liquid medium without cyclodextrin (line 1) and cyclodextrin culture supernatant without fixation (line 2). The position of molecular size markers are shown on the left side of the figure.

antigenic recognition is in agreement with that reported by Betsou et al. for *B. pertussis* cells [4].

It could not be ruled out that the AC-Hly detected by Western blot might correspond to molecules that were released from the bacterial cells and then associated with the OMVs. To evaluate this possibility, the ability of soluble AC-Hly to associate with the OMVs prepared from a *B. pertussis* AC-Hly-mutant (Bp 118 ΔB) was studied. The addition of purified AC-Hly at the beginning of the OMVs isolating procedure, resulted in no modification of the electrophoretic profiles of the crude OMVs preparations. Furthermore, the anti–AC-Hly antiserum did not recognize polypeptides from either preparation (Fig. 4). These results suggest that AC-Hly is released as an ubiquitous component of the OMVs.

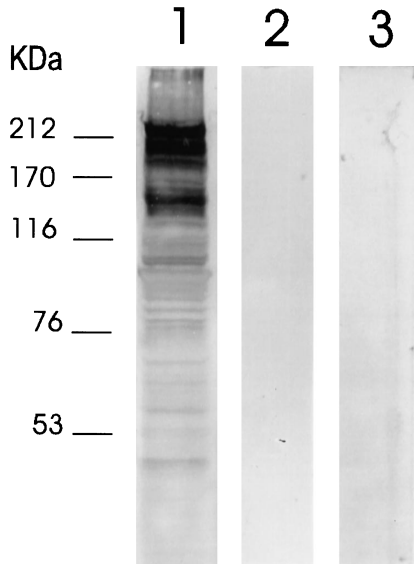

Fig. 4. Western blot analysis of AC-Hly of outer membrane vesicles isolated from *Bordetella pertussis* wild-type cells (line 1), *B. pertussis* AC-Hly mutant (Bp 118 ΔB) (line 2), and from Bp 118 ΔB plus purified AC-Hly added at the beginning of the OMVs isolation procedure (line 3). The position of molecular size markers are shown on the left side of the figure.

The presence of Ptx and FHA in OMVs was also assessed by using specific polyclonal antisera. As shown in Fig. 5, the mouse polyclonal immune sera directed against FHA or PTx recognized single polypeptides with molecular weights corresponding to the respective *B. pertussis* immunogens.

Results described here show the presence of outer membrane vesicles in culture supernatants of *B. pertussis*. The composition of these vesicles seems to be similar to that of the OMVs induced by cell pellet sonication. The OMVs contain several antigens inducing protective immunity, including AC-Hly in addition to the mitogenic LPS. As reported with regard to other Gram-negative bacteria [31], the LPS from *B. pertussis* is a good adjuvant for the stimulation of antibodies against various antigens [1, 10, 28, 29]. It is known that whole-cell pertussis vaccine that contains LPS acts as an adjuvant for other vaccines (diphteria, tetanus toxoids, and *Haemophilus influenzae* type b). It was recently shown by Eskola et al. [5] that immune response against *H. influenzae* type b (Hib) is lower when the acellular pertussis vaccine replaces a whole cell vaccine.

Evidence reported here suggests that OMVs, which

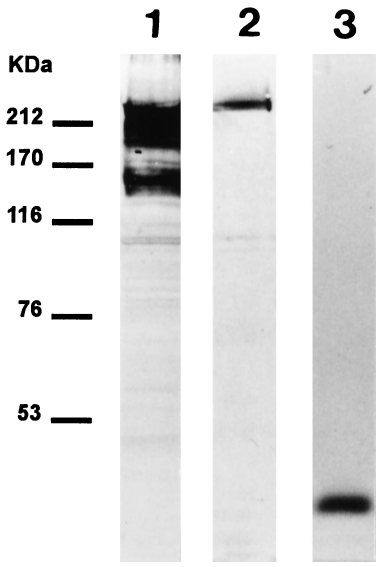

Fig. 5. Western blot analysis of outer membrane vesicles isolated from *Bordetella pertussis* cells using an antiserum against AC-Hly (line 1), FHA (line 2), and PTx (line 3). The position of molecular size markers are shown on the left side of the figure.

contain LPS at a level which could be reduced using desoxycolate, could be successfully used for development of *B. pertussis* acellular immunogens. This strategy has already been used for the development of acellular vaccines of *N. meningitidis* [6].

The OMVs in which the conformation of the antigens could be stabilized close to those present in the living organisms seems to become an interesting subject to be further studied from the point of view of the host immune response and considered as a future vaccine candidate.

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