FEBS Letters 586 (2012) 459–465

journal homepage: www.FEBSLetters.org

Delivery of *Bordetella pertussis* adenylate cyclase toxin to target cells via outer membrane vesicles

Gina M. Donato [a,*], Cynthia S. Goldsmith [b], Christopher D. Paddock [b], Joshua C. Eby [a], Mary C. Gray [a], Erik L. Hewlett [a]

[a] Department of Medicine, Division of Infectious Diseases, University of Virginia School of Medicine, Charlottesville, VA 22908, USA
[b] Infectious Disease Pathology Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA

ARTICLE INFO

Article history:
Received 19 December 2011
Revised 6 January 2012
Accepted 17 January 2012
Available online 28 January 2012

Edited by Renee Tsolis

Keywords:
Bordetella
Outer membrane vesicles
Adenylate cyclase toxin
cAMP
Intoxication

ABSTRACT

Bordetella pertussis adenylate cyclase toxin (ACT) intoxicates cells by producing intracellular cAMP. _B. pertussis_ outer membrane vesicles (OMV) contain ACT on their surface (OMV–ACT), but the properties of OMV–ACT were previously unknown. We found that _B. pertussis_ in the lung from a fatal pertussis case contains OMV, suggesting an involvement in pathogenesis. OMV–ACT and ACT intoxicate cells with and without the toxin's receptor CD11b/CD18. Intoxication by ACT is blocked by antitoxin and anti-CD11b antibodies, but not by cytochalasin-D; in contrast, OMV–ACT is unaffected by either antibody and blocked by cytochalasin-D. Thus OMV–ACT can deliver ACT by processes distinct from those of ACT alone.

1. Introduction

Bordetella pertussis is the causative agent of whooping cough, which is increasing in incidence, despite high immunization rates [1]. This Gram-negative organism produces a number of virulence factors, including adenylate cyclase toxin (ACT), filamentous hemagglutinin (FHA), pertactin (PRN) and pertussis toxin (PT) [2]. ACT uses the $\alpha_M\beta_2$ integrin CD11b/CD18 as a receptor, but also intoxicates cells not expressing CD11b/CD18 [3–5]. Following binding, regardless of CD11b/CD18, the ACT catalytic domain is translocated into the cell and activated by calmodulin to convert ATP to cyclic AMP (cAMP), a process referred to in this study and elsewhere as "intoxication".

Most Gram-negative bacteria produce outer membrane vesicles (OMV) containing outer-membrane proteins, carbohydrates and lipids. These structures have been extensively studied and recognized to have a role in pathogenesis of some bacterial infectious diseases [6,7]. Hozbor et al. showed that *B. pertussis* produce OMV containing ACT (OMV–ACT) and other virulence factors and proposed use of these OMV as an acellular pertussis vaccine [8,9]. Virtually nothing is known about the effects of OMV–ACT, as illustrated by the fact that neither the Hozbor publications nor two recent reviews on OMV contain information on virulence-associated activities of OMV–ACT. In our studies of intoxication by OMV–ACT, we find that OMV–ACT acts as a delivery system for ACT, but by a process that is different from that used by purified ACT.

2. Materials and methods

2.1. B. pertussis growth

B. pertussis strains (GMT1, GMT1(pTH22) and BP348) were grown on Bordet–Gengou (BG) agar (Difco) containing 10% defibrinated sheep blood (Cocalico) and then modified Stainer–Scholte liquid medium (SSM) [10] at 35.5 °C. GMT1 is a wild-type *B. pertussis* strain [11] and BP348 contains a transposon insertion in *cyaA* [12], rendering it defective in the production of ACT. GMT1(pTH22) was created for this study, as described below.

2.2. Isolation of outer membrane vesicles (OMV)

OMV were isolated from culture supernatants and bacterial cells as described by Hozbor et al. [8]. For OMV from culture supernatant (referred to as "native OMV"), GMT1 was grown in SSM, centrifuged and filtered to remove remaining bacteria. OMV were obtained by centrifugation at 150 000×*g* for 1 h at 4 °C and wash-

* Corresponding author. Address: 1708 MR-6, 345 Crispell Dr, Box 800419, University of Virginia, Charlottesville, VA 22908 USA. Fax: +1 434 982 3830.
E-mail address: gmd2n@virginia.edu (G.M. Donato).

0014-5793/$36.00
doi:10.1016/j.febslet.2012.01.032

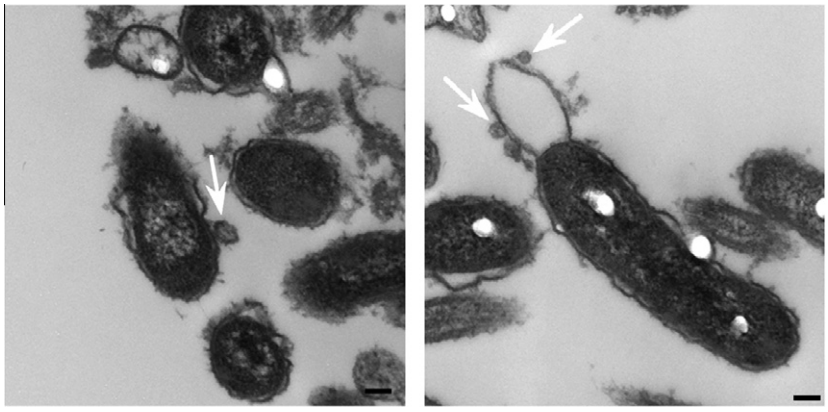

Fig. 1. *B. pertussis* organisms and OMV in human pathology specimens. Electron microscopic images of outer membrane vesicles associated with *B. pertussis* are from lung tissue obtained at autopsy from a child with fatal *B. pertussis* infection. Arrows indicate OMV. Scale bar = 0.1 μm; magnification = 30000×.

ing in TE (20 mM Tris–HCl, 2 mM EDTA pH 8.5). Following the final centrifugation, OMV pellets were stored in TE at −20 °C.

For OMV from treatment of bacteria (designated "enriched OMV"), strains were grown to logarithmic phase, centrifuged and resuspended in TE. OMV were released by sonication on ice in five, 1-min bursts. The bacteria were removed by centrifugation and the OMV-containing supernatants were centrifuged at 100000×*g* for 2 h at 4 °C. The pellets were resuspended in 2% deoxycholate (DOC) and further purified by centrifugation on a 60% sucrose cushion at 100000×*g* for 2 h at 4 °C. The OMV band was collected from the interface, washed and stored in TE at −20 °C. OMV protein concentrations were determined by BCA protein assay (Pierce). The absence of viable bacteria in the OMV was established by lack of growth on BG plates.

2.3. Electron microscopy

For *B. pertussis* cultured in vitro, an aliquot of GMT1 or isolated OMV was added to formvar and carbon-coated, nickel-mesh grids for 1 min, excess liquid removed and samples negatively stained

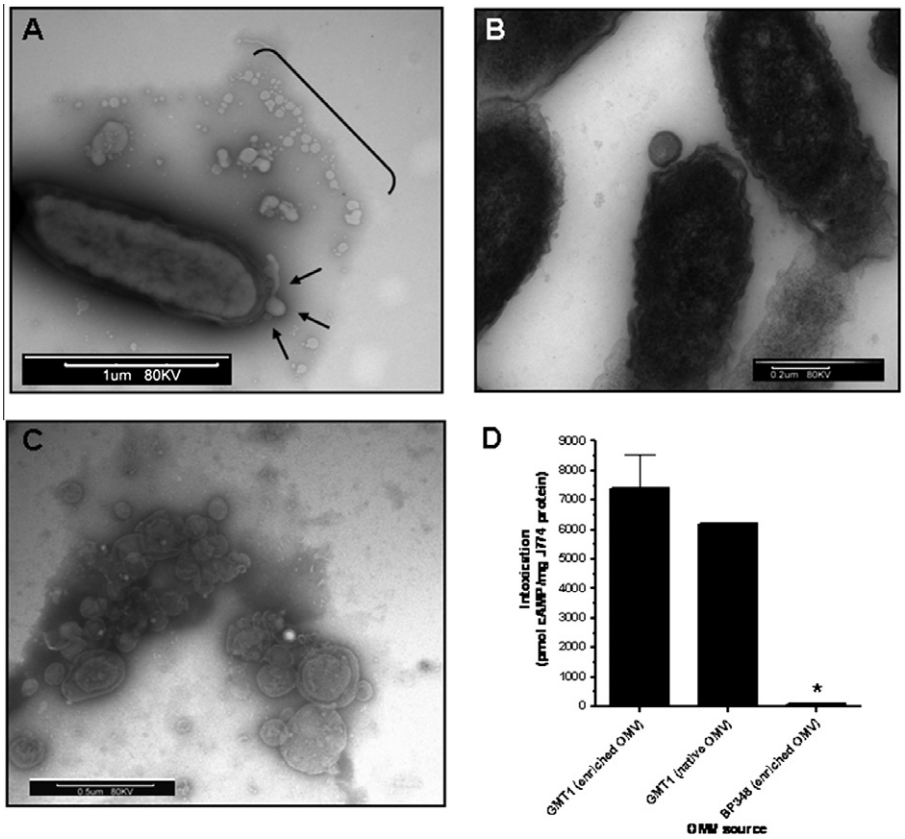

Fig. 2. Transmission EM images of *B. pertussis* and OMV. (A) *B. pertussis* GMT1 and associated OMV were negatively stained with 2% NanoVan. Arrows indicate OMV attached to the bacterial surface; bracket represents OMV released into the culture medium; scale bar = 1 μm; magnification = 15000×. (B) Ultrathin section of embedded bacteria and attached OMV stained with uranyl acetate and lead citrate. Scale bar = 0.2 μm; magnification = 40000×. (C) OMV prepared and purified from GMT1 by the method of Hozbor et al. [8] were negatively stained with 2% PTA pH 7.0. Scale bar = 0.5 μm; magnification = 30000×. (D) J774.A1 cells were intoxicated comparably by enriched and native OMV from GMT1. The comparison of intoxication was based on equalized AC enzymatic activities. Enriched OMV from the ACT-negative strain, BP348, did not cause intoxication. *$P < 0.0001$ compared to GMT1 enriched OMV.