

Adenylate cyclase influences filamentous haemagglutinin-mediated attachment of *Bordetella pertussis* to epithelial alveolar cells

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Abstract

Attachment to epithelial cells in the respiratory tract is a key event in *Bordetella pertussis* colonization. Filamentous haemagglutinin (FHA) is an important virulence factor mediating adhesion to host cells. In this study, the relevance of the interaction between FHA and adenylate cyclase toxin (ACT) during bacterial attachment was investigated. Mutants lacking either FHA or ACT showed significantly decreased adherence to epithelial respiratory cells. The use of several ACT-specific monoclonal antibodies and antiserum showed that the decrease in attachment of strains lacking ACT expression could not be explained by the adhesin-like activity of ACT, or a change of any of the biological activities of ACT. Immunoblot analysis showed that the lack of ACT expression did not interfere with FHA localization. An heparin-inhibitable carbohydrate-binding site is crucial in the process of FHA-mediated bacterial binding to epithelial cells. In the presence of heparin attachment of wild-type *B. pertussis*, but not of the isogenic ACT defective mutant, to epithelial cells was significantly decreased. These results suggest that ACT enhances the adhesive functions of FHA, and modifies the performance of the FHA heparin-inhibitable carbohydrate binding site. We propose that the presence of ACT in the outer membrane of *B. pertussis* to play a role in the functionality of FHA.

Introduction

Bordetella pertussis, the etiological agent of whooping cough, has several virulence factors under the control of a two-component signal transduction system encoded by the *bvgAS* regulatory locus. The BvgAS system mediates the transition between virulent (Bvg⁺), intermediate (Bvgⁱ) and avirulent (Bvg⁻) phases characterized by distinct patterns of gene expression (Lacey, 1960; Cotter & DiRita, 2000; Deora *et al.*, 2001; Williams *et al.*, 2005). Some of these virulence factors are regarded as adhesins mainly involved in bacterial attachment to the host cells, i.e. filamentous haemagglutinin (FHA), pertactin and fimbriae, while others, such as adenylate cyclase-hemolysin toxin (ACT) contribute to subsequent host colonization (Mattoo & Cherry, 2005)

The Bvg⁺ phase is characterized by the presence of the majority of the virulence factors that are encoded by the so-called vir-activated genes (*vags*), which can be induced experimentally by growing bacteria at 37 °C in standard

Bordetella growth medium. The Bvg⁻ phase can be induced by the exposure to modulators, including MgSO₄, nicotinic acid or low temperatures. This results in the suppression of *vags* and the expression of vir-repressed genes (*vrgs*), which encode proteins whose function is still under investigation. Exposure to 'semi-modulating' concentrations of modulators or temperatures between 30 and 37 °C results in the transition to the Bvgⁱ phase. This phase is characterized by activation of early, including *fhaB*, but not late, such as *cyaA* (which encodes ACT), *vags*, and the lack of transcription of *vrgs* (Cotter & DiRita, 2000; Deora *et al.*, 2001).

Previous studies suggested that changes in the microenvironment regulate BvgAS-mediated expression of specific virulence factors *in vivo*. This might be relevant for *Bordetella* pathogenesis and persistence (Kinnear *et al.*, 2001; Veal-Carr & Stibitz, 2005; Vergara-Irigaray *et al.*, 2005).

It has been demonstrated that a physical association occurs between two of the main virulence factors under the BvgAS control, FHA and ACT, and that FHA is necessary for

ACT retention in the outer membrane. FHA localization was shown unchanged in the absence of ACT, as determined by Western blot analysis (Zaretsky *et al.*, 2002). However, the functional relevance of ACT-FHA coexpression has not been studied in detail.

The purpose of this study was to investigate the influence of the association between FHA and ACT, two virulence factors with different kinetics of expression during phase modulation, on the bacterial interaction with respiratory epithelial cells, a key event during *Bordetella pertussis* colonization of the host.

Materials and methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. All *B. pertussis* strains were grown at 37 °C for 2–3 days on Bordet–Genou agar plates (Difco Laboratories) supplemented with 15% defibrinated sheep blood (ABG). Bacteria were then subcultured on ABG for 24 h. Antibiotics were added to culture media for experiments as indicated, using the following concentrations: kanamycin, 25 µg mL⁻¹; streptomycin, 50 µg mL⁻¹.

For comparative studies of *B. pertussis* Bvg phenotypes, Tohama I was grown on ABG containing either 50 mM of MgSO₄ or no modulator, and further subcultured in Stainer–Scholte (SS) medium with or without 50 mM of modulator, respectively. Next, both bacterial cultures were replicated in SS medium without modulator, harvested at 0, 6, 9, 12 and 24 h, washed and suspended in either Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY), or PBS at a final concentration of 10⁷ bacteria mL⁻¹, and were used in attachment assays or Western blot analysis.

Cells and growth conditions

The human alveolar epithelial cell line A549 (human lung type II pneumocyte) (ATCC CCL185, Rockville, MD) was cultured in DMEM supplemented with 10% of FCS, 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin (Sigma, St Louis, MO) (complete medium) at 37 °C in 5% CO₂. Routine subcultures for A549 pneumocytes were done at 1:3 split ratios by incubation with 0.05% trypsin–0.02%

EDTA for 5 min at 37 °C. For *Bordetella* adherence assay, epithelial cells were cultured to 80% confluence.

Sera and monoclonal antibodies

The following monoclonal antibodies (mAb) against *B. pertussis* proteins were used: 2A12 (IgG2a) against *B. pertussis* ACT, 6E1 (IgG1) against *B. pertussis* ACT (both kindly provided by Dr Hewlett, University of Virginia, USA), and 37F3 (IgG1) against *B. pertussis* FHA kindly provided by The Netherlands Vaccine Institute (Bilthoven, the Netherlands). Polyclonal mouse anti-ACT *B. pertussis* antiserum was generated as described by Hozbor *et al.* (1995). All antibodies were used in nonagglutinating concentrations.

Attachment assays

Attachment assays were performed as described in Rodriguez *et al.* (2006) with minor modifications. Briefly, cells were seeded on glass coverslips in 24-well tissue culture plates (Nunc, Roskilde, Denmark) and incubated for 18 h. Monolayers were washed twice with DMEM supplemented with 10% FCS without antibiotics prior to incubation with selected strain of *B. pertussis* (ratio: 100 bacteria per cell) in the absence or presence of monoclonal antibodies (30 µg mL⁻¹), mouse anti-ACT serum (10% v/v) or murine preimmune serum, or bovine mucosal heparin (Mr; 3000; Sigma; 1 mg mL⁻¹). Bacterial inocula were quantified by plating appropriate dilutions on ABG. To facilitate bacterial interaction with epithelial cells, plates were centrifuged for 5 min at 640 g. Adherence of bacteria to A549 cells was determined after 2 h of incubation at 37 °C with 5% CO₂. No changes in the number of viable A549 cells were detected after incubation with bacteria. Experiments were stopped by washing the monolayers with DMEM medium supplemented with 10% FCS, prior to fixation with methanol. Cells were stained using crystal violet and bacterial adherence was quantified by microscopy. The number of adherent bacteria per cell was estimated by a microscopic examination of 20 randomly selected fields showing a minimum of seven epithelial cells per field. All experiments were carried out at least three times in duplicate.

Immunoblot analysis

Bacterial suspensions were lysed with Laemmli sample buffer and run on 12.5% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE; 12.5% polyacrylamide; Laemmli, 1970). Proteins were transferred to polyvinylidene fluoride (Immobilon PVDF Millipore) sheets (Towbin *et al.*, 1992) and incubated with mouse monoclonal anti-FHA or polyclonal anti-ACT antibodies. The immunochemical detection was performed using alkaline phosphatase-

Table 1. Strains of *Bordetella pertussis* used in this study

Strain	Phenotype	Reference
Tohama I	Virulent phase (wild type)	Sato & Arai (1972)
BpGR4	FHA –, derivative of Tohama I	Locht <i>et al.</i> (1992)
Bp3183	ACT –, derivative of Tohama I	Weiss <i>et al.</i> (1989)
Bp537	Bvg – phase locked, derivative of Tohama I	Relman <i>et al.</i> (1989)

conjugated goat antimouse IgG (Jackson Immuno Research, BaltimorePike).

Statistical analysis

Differences between the results of the experiments were evaluated by means of ANOVA or *t*-test. Significance was accepted at $P < 0.05$. Results are shown as means and SD.

Results

Lack of ACT expression decreases *B. pertussis* attachment to alveolar epithelial cells

In order to evaluate the relevance of ACT expression on FHA-mediated *B. pertussis* interaction with human respiratory epithelial cells we compared the attachment of the *B. pertussis* wild-type strain and the isogenic ACT-deficient mutant (Bp3183) to A549 cells. *Bordetella pertussis* FHA deficient mutant (BpGR4) and *B. pertussis* phase-locked Bvg⁻ mutant (Bp537) were included as controls. The lack of FHA (BpGR4) caused a significant decrease ($P < 0.05$) in the number of attached bacteria (Fig. 1). Attachment ratios of the *B. pertussis* strain lacking the expression of all virulence factors (Bp537) and BpGR4 to epithelial cells were very similar, indicating that FHA is the main Bvg-controlled

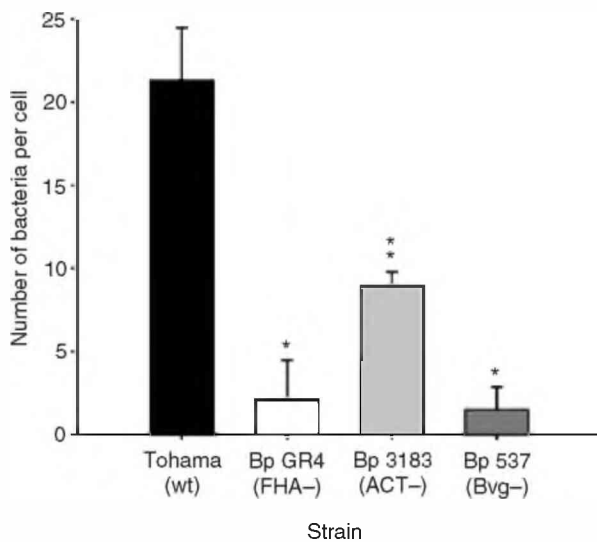


Fig. 1. Role of virulence factors on *Bordetella pertussis* attachment to human epithelial respiratory cells. Wild-type *B. pertussis* and mutant strains defective in the expression of FHA (BpGR4), ACT (Bp3183), or all Bvg-regulated virulence factors (Bp537) were allowed to adhere to A549 cells at a multiplicity of infection (MOI) of 100. Data represents mean \pm SD of at least four independent experiments. The attachment levels of Bp537 and BpGR4 were significantly different from the attachment of both Bp3183 and the wild-type strain ($*P < 0.05$). Furthermore, attachment of Bp3183 was different from that exhibited by the wild-type strain ($**P < 0.05$).

adhesin in this system. However, the absence of ACT significantly reduced bacterial adhesion compared the wild-type strain ($P < 0.05$), but to a lesser extent than bacteria lacking FHA.

Antibodies against ACT fail to block *B. pertussis* attachment to A549

Next, we evaluated the attachment of wild-type *B. pertussis* in the presence of several ACT-specific antibodies. Mouse anti-ACT serum and anti-ACT monoclonal antibodies with different specificities were tested. MAb 6E1, was shown to reduce the hemolytic activity (Hly) but did not change adenylate cyclase enzymatic activity (AC) of ACT. MAB 2A12, blocks the binding of ACT to the host cell membrane, and both enzymatic activities of ACT, Hly and AC (Lee *et al.*, 1999). These ACT-specific mAbs, as well as the ACT-specific polyclonal antiserum, did not reduce the level of attachment of wild-type *B. pertussis* to epithelial cells ($P < 0.05$; Fig. 2). In contrast, the attachment level of wild-type *B. pertussis* was significantly decreased in the presence of anti-FHA ($P < 0.05$). To further evaluate a possible role of ACT as a minor adhesin we tested the effect of ACT-specific antibodies on the epithelial attachment of the FHA deficient

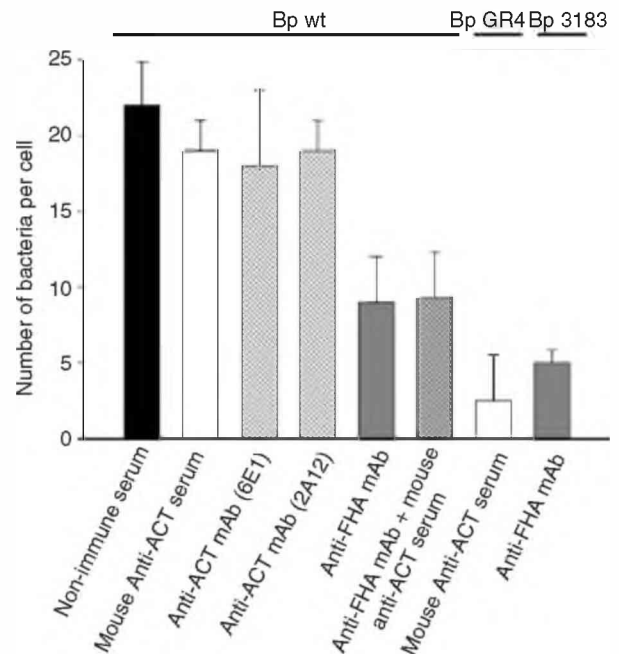


Fig. 2. Effect of anti-ACT and anti-FHA antibodies on the attachment of *Bordetella pertussis* to human respiratory epithelial A549 cells. *Bordetella pertussis* wild type or a mutant strain defective in the expression of ACT (Bp3183) were added to A549 cell monolayers (MOI of 100) in the presence of murine preimmune serum (10% v/v), murine ACT-specific antiserum (10% v/v), two different monoclonal antibodies against ACT (30 $\mu\text{g mL}^{-1}$), or monoclonal antibodies against FHA (30 $\mu\text{g mL}^{-1}$). Values are the mean \pm SD of at least four separate experiments.

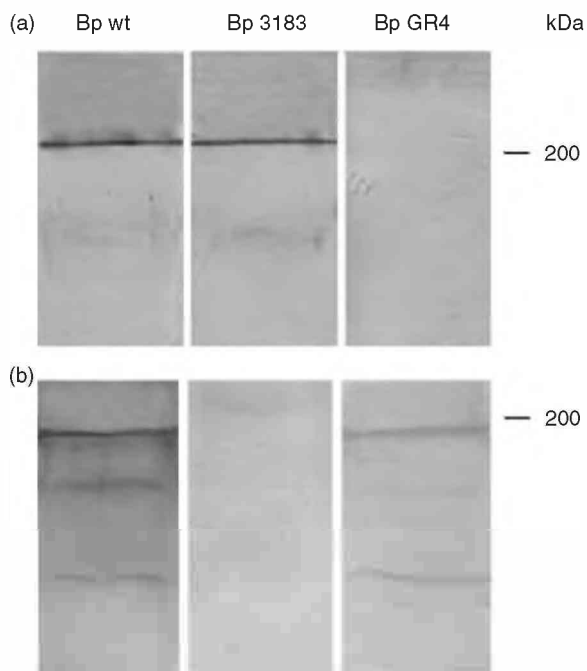


Fig. 3. The absence of ACT does not affect cellular FHA expression. Western blot analysis of whole-cell lysates of *Bordetella pertussis* wild type; Bp3183 (ACT⁻) and BpGR4 (FHA⁻). Bacterial cell suspensions were run in SDS-PAGE 12.5%, and transferred to PVDF membranes. Immunoblot was performed with mouse anti-FHA (a) or anti-ACT (b) antibodies. Migration of molecular mass markers (kDa) is indicated on the right.

mutant, and the effect of anti-FHA in combination with anti-ACT antibodies on the wild-type strain attachment level. The addition of anti-ACT antibodies did not decrease adherence of the FHA deficient mutant to epithelial cells. Similarly, addition of ACT-specific antibodies did not further decrease the attachment level of wild-type bacteria in the presence of anti-FHA antibodies (Fig. 2). These results show that ACT does not act as an adhesin in the interaction of *B. pertussis* with A549 cells. The attachment level of *B. pertussis* lacking the expression of ACT was found to be lower in the presence of anti-FHA antibodies, confirming the involvement of FHA in the attachment of ACT-deficient mutant *B. pertussis* (Fig. 2).

The lack of ACT does not affect FHA cellular expression

FHA expression is essential for ACT association with the bacterial surface (Zaretsky *et al.*, 2002). Therefore, we investigated whether the lack of ACT affects FHA expression, which could explain the decreased bacterial attachment of the ACT-deficient mutant. As found by others (Zaretsky *et al.*, 2002), western blot analysis of wild-type and the FHA-deficient BpGR4 strain showed a reduction of bacterial ACT

in the absence of FHA. Conversely, the lack of ACT did not affect the expression of FHA (Fig. 3).

Differential expression of ACT in response to modulating factors affects bacterial attachment to epithelial cells *in vitro*

The differential regulation of the *bvg*-activated promoters shows that *B. pertussis* transiently expresses FHA but not ACT during phase modulation. We here investigated the effect of a *B. pertussis* phase shift on the level of attachment to A549 cells in order to further evaluate the effect of the lack of ACT expression. Bacteria grown in the presence of modulating concentrations of MgSO₄ for 21 h were further subcultured under nonmodulating conditions for different periods of time (Bp out-mod) and tested for their ability to attach to respiratory epithelial cells. Virulent *B. pertussis* wild-type and ACT-deficient mutant strains were grown in parallel under nonmodulating conditions to be used as controls. Additionally, the ACT-deficient mutant was also grown in the presence of modulating concentrations of MgSO₄ for 21 h, further subcultured under nonmodulating conditions for different periods of time (Bp3183 out-mod) and tested for its ability to attach to respiratory epithelial cells. Figure 4 shows that immediately after shifting to nonmodulating conditions, bacteria (Bp out-mod/0) attached significantly less ($P < 0.05$) than both the virulent *B. pertussis* wild-type strain and Bp3183. Bacteria collected after 6 h of culture under nonmodulating conditions (Bp out-mod/6) showed significantly higher attachment ratios ($P < 0.05$) than those exhibited by Bp out-mod/0, but similar to those of the *B. pertussis* ACT-deficient mutant. Bacteria cultured for 9 h under nonmodulating conditions attached to epithelial cells significantly better ($P < 0.05$) than bacteria harvested after 6 h, although the attachment ratio of these bacteria was still lower ($P < 0.05$) than that of the wild-type strain. After 12 h of incubation under nonmodulating conditions, bacterial attachment reached the level exhibited by wild-type bacteria. After 6 h of culture under nonmodulating conditions, no further increase in the expression of cellular FHA was detected (Fig. 4). Notably, the evolution of bacterial attachment ratios seems to correlate with the time-dependent level of ACT expression. Accordingly, the increase of adherence of the ACT mutant strain after shifting to nonmodulating conditions (Bp3183 out-mod) proved similar to that exhibited by the wild-type strain during the first 6 h. After 9 h of incubation under nonmodulating conditions, the attachment level of the wild-type strain, already expressing ACT (Fig. 4a), proved significantly higher ($P < 0.05$) than that exhibited by the ACT-deficient mutant under the same culture conditions.

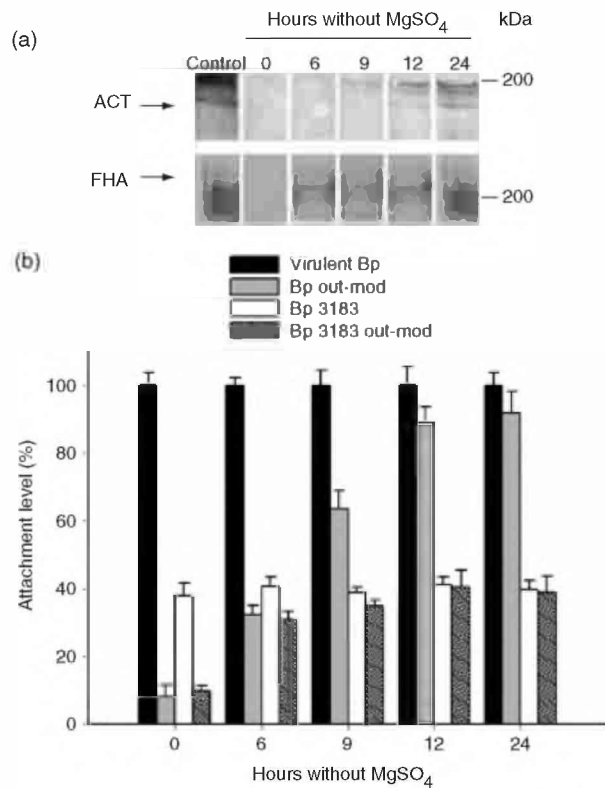


Fig. 4. Phenotypic modulation and its effect on the attachment of *Bordetella pertussis* to epithelial respiratory cells. *Bordetella pertussis* wild-type and ACT-deficient mutant (Bp3183) strains were grown in presence of 50 mM of MgSO₄ for 21 h and then cultured in the absence of MgSO₄ (Bp out-mod and Bp3183 out-mod, respectively) for 0, 6, 9, 12 or 24 h. Additionally, wild-type (virulent Bp) and ACT-deficient mutant (Bp3183) strains were grown in parallel in the absence of modulator agent for 0, 6, 9, 12 or 24 h, to be used as controls. ACT and FHA expression of Bp out-mod grown under nonmodulating for different periods of times was analysed by Western blot analysis, as described in the legend of Fig. 3. ACT and FHA expression of virulent Bp was tested as a control (a). Bp out-mod, Bp3183 out-mod, virulent Bp, and Bp3183 grown under nonmodulating conditions for different periods of times were allowed to attach to the A549 cells (MOI 100) (b). Virulent Bp attachment level (control) was set as 100%. Data are the mean of at least three independent experiments.

ACT expression affects heparin-inhibitable binding activity of FHA

The heparin-inhibitable binding activity of FHA is largely responsible for the attachment of *B. pertussis* to epithelial cells (Menozi *et al.*, 1991; Hannah *et al.*, 1994; Menozzi *et al.*, 1994). We therefore evaluated whether the interaction between ACT and FHA is relevant for the functionality of this binding domain. To this end, the attachment of *B. pertussis* wild-type and ACT-deficient mutant strains was compared in the presence of heparin. The presence of heparin led to a significant decrease ($P < 0.05$) in adherence of the *B. pertussis* wild-type strain but did not alter the

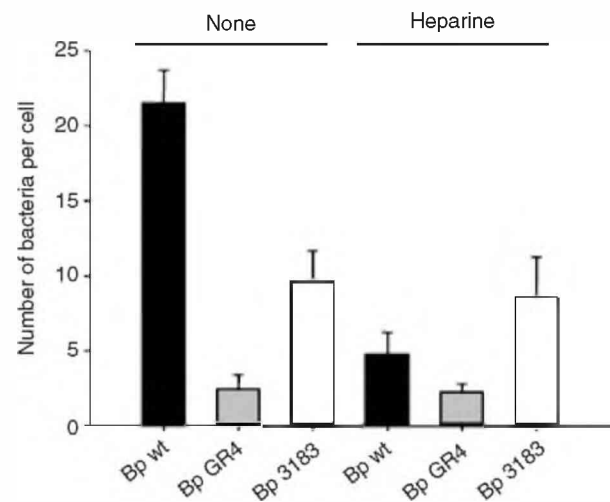


Fig. 5. Effect of heparin on *B. pertussis* attachment to human respiratory epithelial A549 cells. Wild-type *B. pertussis*, FHA-deficient mutant (BpGR4), or ACT-deficient mutant (Bp3183) strains were added to A549 cell monolayer (MOI of 100) in the presence, or absence of heparin (1 mg mL⁻¹). Results are expressed as the number of bacteria adhered per cell. Values are the mean \pm SD of at least four separate experiments.

attachment level of the isogenic FHA-deficient mutant (Fig. 5). These results confirmed the involvement of the heparin-binding domain in the FHA-mediated bacterial attachment to respiratory cells. The attachment level of the *B. pertussis* ACT-deficient mutant to A549 cells was not affected ($P < 0.05$) by the presence of heparin, suggesting that FHA-ACT interaction on bacterial surfaces is relevant for the activity of this FHA binding domain (Fig. 5).

Discussion

Bordetella pertussis is an obligate human pathogen that resides in the respiratory tract during infection. Bacterial adhesins promote attachment to host cells, which is a key event in pathogenesis. *Bordetella pertussis* is able to adhere to various cell types through different adhesins. Fimbrial-type protein (Fimbriae) was found implicated in *B. pertussis* binding to HEp-2 (van den Berg *et al.*, 1999) cells and monocytes (Hazenbos *et al.*, 1995a,b), while pertactin, an outer membrane protein, contributes to the binding to HEp-2 (van den Berg *et al.*, 1999) and CHO (Leininger *et al.*, 1991) cell lines. FHA, the main adhesin of *B. pertussis*, was shown to be involved in bacterial attachment to both (human) ciliated (Tuomanen & Weiss, 1985; Tuomanen *et al.*, 1988) and nonciliated cells (Prasad *et al.*, 1993; Menozzi *et al.*, 1994; van den Berg *et al.*, 1999; Rodriguez *et al.*, 2006).

Apart from adhesins, *B. pertussis* produces toxins that are involved in host immune evasion and cause pertussis symptoms. ACT is a bacterial enzyme that contains two

functionally separated domains (Hanski *et al.*, 1989; Ehrmann *et al.*, 1991). One of them is activated by host cell calmodulin and catalyses the production of intracellular cAMP from ATP, thus intoxicating the host cell. The second domain induces hemolysis of erythrocytes. ACT has been recently shown to be physically associated with FHA on the *B. pertussis* surface (Zaretsky *et al.*, 2002). Although *B. pertussis* mutants lacking FHA were found to release significantly more ACT due to a defective retention on the bacterial surface (Weiss *et al.*, 1983), our results confirm that the lack of ACT does not affect cellular FHA expression (Zaretsky *et al.*, 2002), and additionally show that ACT-deficiency causes a significant decrease in bacterial attachment. Interestingly, similar results were obtained using ciliated epithelium to test the attachment of *Bordetella* mutant strains lacking ACT expression in the outer membrane of the bacteria (Tuomanen & Weiss, 1985; Edwards *et al.*, 2005), whereas a *B. pertussis* ACT mutant expressing nontoxic ACT on the bacterial surface was found to attach to respiratory cells in a similar way to the wild-type strain (Ishibashi *et al.*, 2001). Combined, these results seem to indicate that expression of ACT in the bacterial surface is relevant for the efficiency of bacterial attachment.

Bacteria transiently expressing FHA, but not ACT, during phase modulation showed similar attachment ratios as ACT-deficient strains. In agreement with previous studies, the expression of FHA was detected earlier than ACT expression after changing to nonmodulating conditions. Attachment ratios increased in parallel with ACT expression. Interestingly, Edwards *et al.* (2005) observed that *B. bronchiseptica* strains lacking adenylate cyclase-hemolysin toxin displayed an 'intermediate' level of attachment to a rabbit tracheal epithelial cell, i.e. in between that of Bvg⁺ and Bvg⁻ strains.

A direct role of ACT as an adhesin could not be demonstrated. *Bordetella pertussis* lacking the expression of all Bvg-regulated virulence factors attached to epithelial cells in a way similar to the isogenic FHA mutant strain suggesting that FHA, but not ACT or any other Bvg-regulated virulence factor, acts as an adhesin under the experimental conditions used in this study. Consequently, the presence of polyclonal anti-ACT did not modify the attachment of wild-type or FHA deficient mutant strains to respiratory epithelial cells. These data suggest that ACT does not display adhesive activity.

Increases in intracellular cAMP could induce changes in the target cell which might eventually lead to an increase of bacterial attachment. To investigate this possibility we used blocking monoclonal antibodies against either AC or Hly activities of ACT. None of the blocking monoclonal antibodies decreased adherence of wild-type bacteria indicating that ACT enzymatic activities are not involved in the attachment of *B. pertussis* to epithelial cells. Gray *et al.*

(2004) reported that blocking AC toxin activity by the use of a monoclonal antibody abolished the induction of high levels of cAMP in mouse monocytes but did not influence *B. pertussis* attachment. Similarly, Ishibashi *et al.* (2001) showed that the level of cell-associated *B. pertussis* mutant expressing inactive ACT on the bacterial membrane was comparable to that exhibited by wild-type strains. Taken together, these results seem to exclude the idea that enzymatic activities of ACT influence bacterial attachment.

The reported association of ACT and FHA on the bacterial surface raises the possibility that ACT expression facilitates FHA-mediated bacterial binding. FHA is a multifunctional adhesin with several docking molecules, including lactosyl ceramides of ciliated respiratory cells, sulphated sugars at the surface of epithelial cells and extracellular matrix, and integrins on leukocytes. Menozzi *et al.* (1994) have shown that the heparin-inhibitable binding activity of FHA is primarily responsible for the attachment of *B. pertussis* to epithelial cells. Attachment levels of the wild-type strain decreased to the level of the FHA-deficient mutant, whereas adherence of the FHA-deficient strain was not further modified by the presence of heparin. Interestingly, heparin did not significantly modify the attachment rates of the ACT-defective strain. These results suggest that the ACT modifies and amplifies the FHA function, presumably by inducing a conformational change that involves, at least, one of the binding domains. The lack of ACT seems to modify FHA-cells interaction in a way that is less efficient in mediating bacterial attachment but no longer inhibitable by heparin. Heparin and heparan sulphates are abundant in extracellular matrices and interact with many eukaryotic proteins which regulate important physiological processes. Heparan sulphate is present on the surface of almost all animal cells.

Published data seem to indicate that the differential regulation of Bvg-activated virulence factors plays a role in *B. pertussis* pathogenesis (Kinneer *et al.*, 2001; Vergara-Irigaray *et al.*, 2005). A recently reported study (Veal-Carr & Stibitz, 2005) further supports this hypothesis and suggests that the Bvg¹ phase is important during infection. This phenotype is characterized by the expression of FHA but not ACT. The resulting change in functionality of surface associated FHA in the absence of ACT expression might be relevant during infection, when sulphated polysaccharides are present in large amounts, e.g. in mucus of the respiratory tract.

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