Role of the *Bordetella pertussis* P.69/pertactin protein and the P.69/pertactin RGD motif in the adherence to and invasion of mammalian cells

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The role of the *Bordetella pertussis* P.69/pertactin protein in mammalian cell adhesion and invasion was investigated. *Salmonella* strains expressing surface-associated P.69/pertactin from a chromosomally located *prn* gene were significantly more invasive than isogenic parental strains. This effect was most pronounced in the poorly invasive, semi-rough *S. typhimurium* strain LB5010. *Escherichia coli* K-12 strain HB101 harbouring the plasmid p41869D, which encodes the full-length *prn* gene under the control of the *tac* promoter on the broad-host-range plasmid pMMB66EH, was significantly more adhesive to HEp-2 and Chinese Hamster Ovary (CHO) cells growing in culture than *E. coli* HB101(pMMB66EH). However, the ability of *E. coli* to invade mammalian cells was not affected by P.69/pertactin expression. P.69/pertactin-mediated adhesiveness of HB101 to HEp-2 and CHO cells was not influenced by the viability of the bacterial cells. However, adherence was markedly reduced when assays were performed for less than 3 h, at 4 °C or in the presence of cycloheximide, suggesting the active participation of the eukaryotic cell in bacterial adhesion. Site-directed mutagenesis was used to mutate Asp to Glu in an Arg-Gly-Asp (RGD → RGE) sequence present in mature P.69/pertactin and the mutated gene was cloned in the same broad-host-range vector (plasmid p41869E). This mutation had no detectable influence on the ability of P.69/pertactin to mediate adhesion of HB101 to HEp-2 or CHO cells. Plasmids p41869D and p41869E were introduced into the *bvg*-negative *B. pertussis* strain BP347. Expression of P.69RGD or P.69RGE did not enhance the adhesiveness of BP347 for epithelial (HEp-2 and CHO) cells.

Keywords: *Bordetella pertussis*, pertactin, RGD motif, adhesion, invasion

INTRODUCTION

Bordetella pertussis, the causative agent of whooping cough, colonizes the respiratory tract mucosa of the host by adhering to host tissues, in particular ciliated epithelial cells and possibly alveolar macrophages (Tuomanen & Weiss, 1985; Rappuoli, 1994; Friedman *et al.*, 1992). A number of bacterial surface-associated proteins, including fimbriae (Mooi *et al.*, 1992), filamentous haemagglutinin

(FHA; Tuomanen & Weiss, 1985; Relman *et al.*, 1989, 1990; Arico *et al.*, 1993; Loch *et al.*, 1993; Sandros & Tuomanen, 1993), pertussis toxin (Tuomanen & Weiss, 1985) and pertactin (Leininger *et al.*, 1991, 1992; Roberts *et al.*, 1991; Arico *et al.*, 1993) have been implicated in the adhesion process. Indeed, several proteins may cooperate to increase the efficiency of adhesion and hence colonization (Rappuoli, 1994). FHA is a large filamentous protein that has both a secreted and cell-associated form and is involved in adherence to ciliated and non-ciliated cells (Tuomanen & Weiss, 1985; Relman *et al.*, 1989, 1990; Arico *et al.*, 1993; Loch *et al.*, 1993; Sandros & Tuomanen, 1993). FHA appears to contain multiple adhesive domains, including an Arg-Gly-Asp (RGD) sequence that may target receptors at the surface of

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Abbreviations: CHO, Chinese Hamster Ovary; FACS, fluorescence-activated cell sorting; FHA, filamentous haemagglutinin; FITC, fluorescein isothiocyanate; SDM, site-directed mutagenesis.

macrophages (Relman *et al.*, 1990; Locht *et al.*, 1993; Sandros & Tuomanen, 1993). The sequence RGD has been implicated in the interactions of a number of extracellular matrix proteins such as fibronectin and vitronectin with integrin receptors on the surface of eukaryotic cells (Hynes, 1987).

B. pertussis pertactin, also known as P.69/pertactin after the apparent molecular mass of the mature polypeptide in SDS-polyacrylamide gels (Makoff *et al.*, 1990), has also been implicated in attachment to and invasion of eukaryotic cells (Leininger *et al.*, 1991, 1992; Roberts *et al.*, 1991; Arico *et al.*, 1993). The *B. pertussis* pertactin gene, *prn*, encodes a polypeptide of 93000 Da that is processed to yield the polypeptides P.69, derived from the amino-terminus, and P.30, derived from the carboxy-terminus (Charles *et al.*, 1989, 1994; Makoff *et al.*, 1990). Both P.69 and P.30 can be detected in the outer membrane of cells of *B. pertussis* and enteric bacteria expressing the *prn* gene (Charles *et al.*, 1994). P.69/pertactin has been used as a subunit vaccine and as a component in a live *Salmonella*-based vaccine to protect mice against respiratory tract infection by virulent *B. pertussis* (Shahin *et al.*, 1990; Romanos *et al.*, 1991; Roberts *et al.*, 1992, 1993; Strugnell *et al.*, 1992; Novotny *et al.*, 1991; Anderson *et al.*, 1996). P.69/pertactin has recently been shown to be a critical component of effective acellular whooping cough antigens in humans (Marwick, 1996; Poland, 1996).

The mechanism by which P.69/pertactin mediates adhesion to human cells is poorly understood. No receptor has been identified to date. However, sequencing of the *prn* gene revealed a number of interesting features in the primary amino acid sequence (Charles *et al.*, 1989). P.69/pertactin contains two regions of repeated amino acid sequences that are immunodominant in the sense that several anti-pertactin mAbs and polyvalent antibodies recognize the repeat regions (Charles *et al.*, 1991). P.93/pertactin possesses two RGD sequences, although only one is present in the mature P.69 polypeptide, at residues 225–227 from the amino-terminus (Charles *et al.*, 1989; Makoff *et al.*, 1990).

The role of P.69/pertactin in adherence of *B. pertussis* to cultured mammalian cells has been investigated by constructing defined mutants of *B. pertussis* lacking expression of P.69/pertactin, FHA or both these proteins (Arico *et al.*, 1993; Leininger *et al.*, 1991; Roberts *et al.*, 1991). A role for P.69/pertactin in adherence to HEp-2 cells could only be demonstrated in the absence of FHA expression (Arico *et al.*, 1993; Roberts *et al.*, 1993). Experiments using Chinese Hamster Ovary (CHO) and HeLa cells showed a 30–40% reduction in adherence of P.69/pertactin-deficient mutants (Leininger *et al.*, 1991). Studies using purified P.69/pertactin have implicated a role for the RGD sequence in attachment as synthetic peptides corresponding to the RGD sequence can partially inhibit bacterial adhesion to eukaryotic target cells (Leininger *et al.*, 1991, 1992). Interestingly, synthetic peptide inhibition studies suggested that the P.69/pertactin RGD sequence may play a role in the entry of *B. pertussis* into HeLa cells (Leininger *et al.*, 1992). In this report, we describe further studies on the interaction of

P.69/pertactin with mammalian cells using different bacterial species expressing surface-associated P.69/pertactin.

METHODS

Bacterial strains, plasmids and growth conditions. *Escherichia coli* K-12 HB101 (Davis *et al.*, 1980) was used for propagation of plasmids directing P.69/pertactin expression. *Salmonella typhimurium* strain BRD641 is a derivative of LB5010 harbouring a single copy of the full-length *prn* gene integrated into the *Salmonella* chromosome at the *aroC* locus (Strugnell *et al.*, 1992). P.69/pertactin is expressed at the bacterial cell surface from the *trc* promoter in this strain. *S. typhimurium* strain BRD640 is a derivative of BRD509 (*aroA aroD*) constructed by transducing the *aroC::prn* locus of BRD641 into BRD509 using bacteriophage-P22-mediated transduction (Strugnell *et al.*, 1992). The construction of BRD641 and BRD640 has been described previously by Strugnell *et al.* (1992). *E. coli* HB101(pR1203) expresses the *Yersinia pseudotuberculosis* invasin protein at its surface (Isberg & Falkow, 1996).

E. coli and *Salmonella* strains were grown in L-broth or on L-agar (Davis *et al.*, 1980) supplemented with antibiotics as appropriate. Antibiotics were used at a concentration of 100 µg ml⁻¹. Minimal medium was prepared as described by Cohen & Wheeler (1946). *B. pertussis* strains were cultured on Cohen–Wheeler agar (Cohen & Wheeler, 1946) supplemented with 10% (v/v) defibrinated horse blood and antibiotics as appropriate. BP536 is a streptomycin-resistant derivative of *B. pertussis* Toham 1 (Relman *et al.*, 1989) and BP347 is a Tn5-induced *bug*-negative mutant of Toham 1 (Weiss *et al.*, 1983).

Plasmid pPERtac36 is a vector that encodes P.69/pertactin (Makoff *et al.*, 1990); pAYL1 encodes P.93/pertactin (Charles *et al.*, 1994). Plasmid pMMB66EH is a broad-host-range *Ptac* expression vector (Furste *et al.*, 1986).

Construction of RGD to RGE (Arg-Gly-Glu) site-directed mutants of *prn* and broad-host-range vectors that express wild-type and mutant pertactin. A 753 bp *AccI* fragment flanking the RGD site of P.69/pertactin was isolated and subcloned in M13mp19 for site-directed mutagenesis (SDM) (Fig. 1). SDM was carried out using a mutagenesis kit supplied by Amersham and the oligonucleotide 5'-ATA GCG GCG GGG GAG GCG CCT GCC-3'. Following mutagenesis, the RGD to RGE site-directed change was confirmed by DNA sequencing, and the mutated *AccI* fragment was isolated and subcloned into *AccI*-digested pPERtac36 (Makoff *et al.*, 1990) to generate pPERtac36(E). In order to generate a vector capable of directing the expression of full-length P.93/pertactin to the bacterial cell surface, a *SmaI*–*SacI* fragment of pPERtac36(E) was gel-purified and ligated with *SmaI*–*SacI* pAYL1 to generate pAYL1(E). The whole P.93/pertactin gene can be conveniently excised as a *Bam*HI fragment from either the native (pAYL1) or mutant [pAYL1(E)] plasmids and ligated into the broad-host-range expression vector pMMB66EH to generate either p41869D (expressing RGD-containing P.93) or p41869E (expressing RGE-containing P.93). Plasmids p41869D and p41869E were introduced into *E. coli* HB101 and *B. pertussis* BP347 by electroporation. Expression of P.69/pertactin was analysed with a P.69/pertactin-specific mAb (BB05) by Western blotting to determine the level of expression and by agglutination to determine if P.69/pertactin was surface-exposed as previously described by Strugnell *et al.* (1992). All strains containing plasmids expressing pertactin produced similar levels of P.69/pertactin and were readily agglutinated by the pertactin-specific mAb (data not shown).

Fig. 1. SDM of *prn* (a) and cloning of the mutant *prn* into a broad-host-range vector (b). A fragment of *prn* encoding the RGD site was subcloned into M13mp19 and SDM was performed so that the fragment would now encode RGE. The mutagenized fragment was swapped with the equivalent fragment in the wild-type gene. The mutant *prn* gene was cloned into the broad-host-range vector pMMB66EH. A full description of the mutagenesis and cloning are given in Methods. The letters in parentheses following the plasmid name denote whether the *prn* gene is wild-type (D, RGD) or mutant (E, RGE).

Cell adhesion and invasion assays. For adhesion assays, HEP-2 and CHO cells were used. These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% (v/v) foetal calf serum and 2 mM glutamate (Sigma) at 37 °C in an atmosphere containing 5% CO₂. For adhesion and invasion assays, cells were seeded into 24-well tissue culture plates (Costar). When the monolayers were confluent ($\sim 2 \times 10^5$ cells per well), bacterial strains were added to the cells at a concentration of 1×10^7 and *E. coli* and *B. pertussis*, but not *Salmonella*, were centrifuged at 300 *g* onto the cell monolayer. The monolayers were incubated for at least 3 h at 37 °C in an atmosphere of 5% CO₂ and were then washed at least five times with PBS (Dulbecco's, with calcium and magnesium; Sigma; pH 7.2). The monolayers were then lysed using 1% (v/v) Triton X-100 in PBS for *Salmonella* and *E. coli* strains and with H₂O and vigorous pipetting for *B. pertussis* (Roberts *et al.*, 1991). Bacterial viable counts were then determined. Invasion assays were performed in a similar manner except that gentamicin (200 µg ml⁻¹) was added for 1 h (*Salmonella* and *E. coli*) or 3 h (*B. pertussis*) after the initial incubation step, followed by washing to remove antibiotic, prior to cell monolayer lysis. In each assay, every strain was tested in at least triplicate.

Determination of bacterial adhesion using fluorescence-activated cell sorting (FACS). Bacterial cells were labelled using fluorescein isothiocyanate (FITC; Sigma). FITC was coupled to washed bacteria grown overnight in broth or on plates by dissolving FITC (1 mg ml⁻¹) in dimethyl sulphoxide and adding 1×10^7 bacteria and incubating for 4 h at 37 °C. The FITC-labelled bacteria were washed extensively in PBS and added to tissue-culture-grown eukaryotic cells in a 24-well plate (Costar). The cells were centrifuged at 300 *g* for 30 min and incubated at 37 °C for 3 h. The cells were then washed at least five times in PBS and incubated in cell dissociation solution (Sigma). FITC-labelled bacteria adhering to tissue culture cells were analysed using a FACSsort (Becton Dickinson).

Bacterial adhesion ELISA. HEP-2 or CHO cells were grown to confluence in Costar flat-bottomed 96-well plates. The wells were then blocked using 1% (w/v) bovine serum albumin in DMEM and different numbers of test bacteria were added to the wells. The plates were centrifuged at 300 *g* for 30 min and incubated at 37 °C/5% CO₂ for 3 h. The wells were then washed thoroughly with PBS and fixed in 0.2% glutaraldehyde. Appropriately diluted rabbit anti-*E. coli* or rabbit anti-whole-cell *B. pertussis* sera was added and incubated overnight at 4 °C. The following morning, the wells were washed with PBS

containing 0.05% Tween 20 and anti-rabbit immunoglobulin-horseradish peroxidase conjugate (Sigma) was added (1:1000). Following incubation and washing, bound antibodies were visualized by addition of *o*-phenylenediamine (OPD) substrate (0.04% OPD in citrate/phosphate buffer, pH 5, containing 0.01% H_2O_2). After colour development, the reaction was stopped with 3 M H_2SO_4 and the A_{490} was read (Ceres 900 HDi ELISA plate reader; Bio_tek Instruments).

RESULTS

Invasion of HEp-2 cells by *S. typhimurium* expressing surface-associated P.69/pertactin

S. typhimurium strains BRD640 and BRD641 express surface-associated P.69/pertactin that can be detected using immunoelectron microscopy or agglutination (Strugnell *et al.*, 1992). *S. typhimurium* LB5010 and BRD509 are the parental strains of BRD641 and BRD640, respectively. HEp-2 invasion assays were performed in order to determine the influence, if any, of P.69/pertactin expression on the mammalian cell invasiveness of the *Salmonella* strains. Typical results are shown in Fig. 2. Expression of the pertactin gene significantly ($P < 0.05$) enhanced the invasiveness of both *S. typhimurium* strains. LB5010 is a semi-rough strain and is poorly invasive for HEp-2 cells. In different experiments, BRD641 was between 40 and 70 times more invasive than LB5010. BRD509 is naturally much more invasive for HEp-2 cells than LB5010. Nevertheless, expression of P.69/pertactin could still significantly improve the invasiveness of BRD509. Thus, in two different *S. typhimurium* background strains, the expression of surface-associated P.69/pertactin enhanced entry into HEp-2 cells.

Adherence to and invasion of HEp-2 cells by *E. coli* HB101 expressing surface-associated P.69/pertactin

Since *Salmonella* strains are both adhesive and invasive for HEp-2 cells, it is difficult to distinguish whether P.69/

Fig. 2. Invasion of HEp-2 cells by *S. typhimurium* strains expressing P.69/pertactin. Bacteria were added to cells and incubated for 3 h. Cells were washed to remove unbound bacteria and then incubated in the presence of gentamicin to kill extracellular micro-organisms. Intracellular bacteria were enumerated by performing viable counts on cell lysates. The percentage of organisms recovered compared to the initial inoculum was determined. The columns represent the mean percentage invasion of organisms from three separate wells and the error bars represent the SEM.

Fig. 3. Adhesion of *E. coli* HB101 expressing P.69/pertactin RGD, P.69/pertactin RGE or invasin to HEp-2 cells. Bacteria were centrifuged onto cells for 30 min and then incubated at 37 °C for 3 h in 10% CO_2 . Cells were lysed and the number of cell-associated bacteria was determined by viable count. The percentage of organisms recovered compared to the initial inoculum was determined. The columns represent the mean percentage adhesion of bacteria from four separate wells and the error bars represent the SEM. A, HB101(pMMB66EH); B, HB101(p41869D) (P.69RGD); C, HB101(p41869E) (P.69RGE); D, HB101(pRI203) (invasin protein).

pertactin is promoting adhesion, invasion or both. To address this question, P.69/pertactin was expressed in the poorly adhesive *E. coli* K-12 strain HB101. High-level expression of P.93 is toxic to *E. coli* (Charles *et al.*, 1994). To overcome this, the *prn* gene was cloned into the low-copy-number broad-host-range expression vector pMMB66EH (Fig. 1). *E. coli* HB101 harbouring the resulting plasmid (p41869D) was readily agglutinated by an anti-pertactin mAb (data not shown), indicating that P.69/pertactin was located on the cell surface.

HB101(p41869D) was much more adhesive to HEp-2 cells than HB101 harbouring the control vector plasmid pMMB66EH ($P < 0.05$). This difference could be consistently measured by using normal tissue culture cell adhesion assays based on viable counts (Fig. 3) or by using FACS analysis with FITC-labelled *E. coli* (Fig. 4a). Identical results were seen using CHO cells (Fig. 4b and data not shown).

Role of the RGD site of P.69/pertactin in bacterial adherence

A mutant form of the *prn* gene which expressed P.69/pertactin containing RGE instead of RGD was constructed as described in Methods using SDM of a fragment of the *prn* gene that included the region encoding the RGD motif. A restriction fragment carrying the mutated *prn* region was swapped with the equivalent fragment on pAYL1 to create the plasmid p41869E (Fig. 1). HB101(p41869E) synthesized equivalent levels of P.69/pertactin to HB101(p41869D), as determined by Western blotting and agglutination. HB101(p41869E) and HB101(p41869D) were found to possess indistinguishable levels of adhesiveness to HEp-2 cells when assayed using viable counts or using FACS analysis.

Fig. 4. Comparison of the attachment of fluorescently labelled *E. coli* HB101 expressing P.69/pertactin RGD, P.69/pertactin RGE or invasin to HEP-2 (a) and CHO (b) cells. Bacteria were coupled with FITC as described in Methods. Labelled bacteria were centrifuged onto HEP-2 or CHO cells. Cells were incubated for 3 h at 37 °C in 10% CO₂. After this time, cells were removed from the tissue culture plate and the number of attached bacteria was analysed by FACS. The columns represent the mean count from four separate wells and the error bars represent the SEM. A, HB101(pMMB66EH); B, HB101(p41869D) (P.69RGD); C, HB101(p41869E) (P.69RGE); D, HB101(pRI203) (invasin protein).

In some experiments, slightly higher numbers of HB101(p41869D) were adherent to cells than HB101(p41869E), whereas in other experiments the reverse was true, but in no instance was there a significant difference in the adhesiveness of the two strains.

Effect of P.69/pertactin on the invasiveness of *E. coli*

In contrast to the results obtained using *Salmonella*, P.69/pertactin expression was not found to enhance the invasiveness of HB101 (Fig. 5). The lack of invasiveness was not due to the nature of the assay system as HB101(pRI203), which expresses the *Y. pseudotuberculosis* invasin protein (which mediates both adhesion and invasion; Isberg & Falkow, 1996), was highly invasive for HEP-2 cells under the conditions employed (Fig. 5).

Fig. 5. Effect of P.69/pertactin expression on the invasiveness of *E. coli* for HEP-2 cells. The assay was performed as described in Fig. 2 except that the bacteria were centrifuged onto the cells for 30 min at the start of the assay. The columns represent the mean percentage invasion of bacteria from four separate wells and the error bars represent the SEM. Percentage invasion is defined in Fig. 2. A, HB101(pMMB66EH); B, HB101(p41869D) (P.69RGD); C, HB101(p41869E) (P.69RGE); D, HB101(pRI203) (invasin protein).

Factors affecting adhesion mediated by P.69/pertactin

Since HB101 expressing P.69/pertactin is rendered non-viable by the FITC treatment without destroying enhanced adhesiveness, bacterial viability is not required for adhesion. The influence of a number of other parameters that might affect adherence was analysed; the results are shown in Fig. 6. Frequently centrifugation is employed in adhesion assays to enhance the contact between bacterial and host cells. Usually this augments the adhesiveness of bacteria but is not essential. We found that centrifugation was essential for adhesion of both HB101(p41869E) and HB101(p41869D). Also, an incubation period of at least 3 h was required to achieve detectable binding of *E. coli* expressing P.69/pertactin to HEP-2 cells. Interestingly, adhesion was impaired if assays were performed at 4 °C. These results are in contrast to those found with HB101(pRI203), which does not require centrifugation or incubation for longer than 1 h to detect adhesion to HEP-2 cells. Also HB101(pRI203) is adherent at 4 °C (Fig. 6). These data suggest that P.69/pertactin-mediated adhesion may require metabolic activity associated with the target eukaryotic cell. Further adhesion assays were performed in the presence of the eukaryotic protein synthesis inhibitor cycloheximide (Fig. 6). Cycloheximide greatly reduced the ability of HB101(p41869D) and HB101(p41869E) but not HB101(pRI203) to adhere to HEP-2 cells.

Adhesion of *B. pertussis* expressing P.69/pertactin to HEP-2 cells

Because *B. pertussis* produces a number of potential adhesins, the effect of mutation in one adhesin gene may be unapparent or underestimated because of compen-

Fig. 6. Parameters affecting the adhesiveness of P.69/pertactin-expressing *E. coli* for HEp-2 cells. The effect of incubation time and temperature, centrifugation and eukaryotic protein synthesis on P.69/pertactin-mediated adhesion was examined. Assay conditions: (a) no spin, 1 h, 37 °C; (b) no spin, 3 h, 37 °C; (c) spin, 3 h, 37 °C; (d) no spin, 3 h, 4 °C; (e) spin, 3 h, 37 °C, cycloheximide added at 100 µg ml⁻¹. The columns represent the mean adhesion of bacteria from three separate wells. A, HB101(pMMB66EH); B, HB101(p41869D) (P.69RGD); C, HB101(p41869E) (P.69RGE); D, HB101(pRI203) (invasin protein).

Fig. 7. Effect of P.69/pertactin expression on the adhesiveness of *B. pertussis* bvg. Adhesion of *B. pertussis* strains to HEp-2 cells was measured by adhesion ELISA as described in Methods. Strains: ◆, BP536; □, BP347(pMMB66EH); ○, BP347(p41869D); ▲, BP347(p41869E). Each point represents the mean absorbance of duplicate tests and the error bars represent the SEM.

sation by other adhesins (Roberts *et al.*, 1991). An alternative approach which may overcome this problem is to take a *bvg* mutant of *B. pertussis*, which will not express the majority of the known virulence factors including adhesins, and express the gene for the adhesin under study under the control of a non-*bvg*-controlled promoter. This would allow the particular adhesin to be studied in isolation in a *B. pertussis* background.

The broad-host-range plasmids p41869D and p41869E were transferred into the *bvg*-negative *B. pertussis* strain

BP347. BP347(p41869D) and BP347(p41869E) expressed levels of surface-associated P.69/pertactin equivalent to the wild-type *B. pertussis* strain BP536 (results not shown). However, *B. pertussis* BP347(p41869D) and BP347(p41869E) did not show enhanced adhesiveness or invasiveness to HEp-2 cells compared to BP347(pMMB66EH) in standard adhesion and invasion assays (data not shown). These assays are problematic with *B. pertussis* as detergents cannot be used to lyse the eukaryotic cells because of the exquisite toxicity of detergents for *B. pertussis*. It is difficult to lyse both HEp-2 and CHO cells with water alone. To overcome this problem, we devised a *B. pertussis* cell adhesion ELISA. This not only eliminated the problem of cell lysis, but the 96-well format allowed multiple assays to be performed at one time. This enabled us to determine easily the effect of bacterial numbers on adhesion. As was found in the other assays, expression of neither P.69RGD nor P.69RGE enhanced the adhesiveness of BP347 (Fig. 7). BP347(pMMB66EH), BP347(p41869D) and BP347(p41869E) were much less adhesive to target eukaryotic cells than the parental strain BP536 (Fig. 7). Identical findings were obtained with CHO cells (data not shown). The number of BP536 cells adherent to HEp-2 cells increased in a dose-dependent fashion. BP536 adhered without centrifugation onto HEp-2 cells but the number of adherent bacteria increased with centrifugation (data not shown).

DISCUSSION

The role of P.69/pertactin in mediating the attachment to and invasion of eukaryotic cells was assessed by expressing the full-length pertactin gene in a number of different host bacteria species. The impact of P.69/pertactin expression on adhesiveness or invasiveness was found to differ, depending on the bacterial host cell employed. In *Salmonella*, P.69/pertactin was found to increase the invasiveness of these naturally invasive bacteria. The impact on invasiveness was greatest with the semi-rough, and normally weakly invasive, *S. typhimurium* strain

LB5010. The influence of P.69/pertactin on *S. typhimurium* BRD509, which is naturally highly invasive, was significant but not as great as for LB5010. One probable reason for this is that the rough nature of LB5010 allows P.69/pertactin to be fully exposed on its surface (Strugnell *et al.*, 1992) as it is in *B. pertussis*, which has short-chain lipopolysaccharide (lipooligosaccharide; Chaby & Caroff, 1988). In contrast, surface-associated expression of P.69/pertactin in the normally non-adhesive, non-invasive *E. coli* K-12 strain HB101 increased the adhesiveness of this strain for HEp-2 and CHO cells but did not influence invasiveness. Thus in *E. coli*, P.69/pertactin was found to act as an adhesin but not an invasin. This observation suggests that in *Salmonella*, P.69/pertactin expression is enhancing the invasiveness of strains simply by increasing the efficiency of bacterial/host cell interactions. In fact, we found that pertactin also enhanced the adhesiveness of *Salmonella* strains (data not shown).

Surface-associated expression of P.69/pertactin did not detectably influence the adhesiveness of the *bvg*-negative mutant *B. pertussis* strain BP347. This was initially somewhat surprising since P.69/pertactin has been implicated in *B. pertussis* adhesion. BP347 has lost *bvg* activity and thus does not express a number of surface proteins associated with virulence (Weiss *et al.*, 1983). In addition, the genes for some proteins (*bvg*-repressed genes) are known to be activated in the absence of *bvg* expression (Knapp & Mekalanos, 1988). Thus it is possible that the adhesive activity of P.69/pertactin expressed from a non-*bvg*-regulated promoter in BP347 may be influenced or suppressed by normally *bvg*-repressed proteins.

The receptor targeted by P.69/pertactin has not been identified. Evidence using synthetic peptide inhibition of the binding of epithelial cell lines to isolated P.69/pertactin coated on microtitre plates has implicated a role for the RGD sequence present in mature P.69/pertactin (Leininger *et al.*, 1991). This would suggest that integrins may be one target receptor for P.69/pertactin on eukaryotic cells. By constructing site-directed mutants of P.69/pertactin we were able to assess the influence on eukaryotic cell adherence of this sequence. Interestingly, *E. coli* cells expressing a mutant P.69/pertactin where the RGD site in P.69/pertactin had been mutated to RGE were equally as adherent as *E. coli* expressing wild-type P.69/pertactin. Thus P.69/pertactin can clearly mediate bacterial adhesion to eukaryotic cells, even in the absence of a functional RGD motif, indicating that P.69/pertactin may mediate adhesion to target cells by more than one mechanism.

The reported effect of synthetic peptides based on P.69/pertactin on the invasiveness of *B. pertussis* (Leininger *et al.*, 1992) contradicts our finding that P.69/pertactin is not an invasin. If synthetic peptides are affecting P.69/pertactin, it might be that they are inhibiting adhesion mediated by P.69/pertactin and not invasion *per se*, thereby indirectly affecting the function of another *B. pertussis* factor that promotes invasion.

Analysis of the factors that influence adhesion of bacteria mediated by P.69/pertactin revealed characteristics both

typical and atypical of other bacterial adhesins. A common finding with bacterial adhesion is that as long as the adhesin is already present at the start of the assay then bacterial viability is not important, as we found to be the case with P.69/pertactin-expressing *E. coli*. Centrifugation of bacteria onto host cells was found to be essential for efficient binding by P.69/pertactin-expressing *E. coli* in our assays. This is unusual; centrifugation will often enhance bacterial adherence but is not usually essential, as we found with the HB101 strain expressing invasin. Centrifugation increases the chance of contact between the bacteria and host cell, thereby reducing the time for maximum binding to occur. In the case of bacteria expressing *prn*, it is possible that centrifugation overcomes an electrostatic barrier that causes eukaryotic and bacterial cells to repel, allowing P.69/pertactin to act. In wild-type *B. pertussis* this may be accomplished naturally by other, physically longer, adhesins such as FHA or fimbriae that can bring *B. pertussis* into close enough contact with the host cell to allow P.69/pertactin to act.

Metabolic activity by the target eukaryotic cell is not usually required for bacterial binding as long as the receptor is already present; therefore, attachment can occur at 4 °C and to killed or fixed cells. Also, bacteria or their purified adhesins can attach to their purified cognate receptor. Our finding that attachment of *E. coli* expressing P.69/pertactin required metabolically active host cells, and in particular *de novo* protein synthesis by the eukaryotic cells, was somewhat surprising. This finding is consistent with the prolonged incubation period necessary to observe binding by the P.69/pertactin-expressing strains. One explanation for these findings is that initial contact of P.69/pertactin-expressing *E. coli* with eukaryotic cells, possibly via pertactin-receptor interactions, results in upregulation of the pertactin receptor. Binding of enteric pathogens, such as enteropathogenic *E. coli* and *Salmonella* spp., to eukaryotic cells can activate intracellular signalling pathways in these cells (Biska *et al.*, 1993). Attachment of pertactin to its receptor may induce similar signalling events; this is currently under investigation.

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