

Iron stress increases *Bordetella pertussis* mucin-binding capacity and attachment to respiratory epithelial cells

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Abstract

Whooping cough is a reemerging infectious disease of the respiratory tract caused by *Bordetella pertussis*. The incomplete understanding of the molecular mechanisms of host colonization hampers the efforts to control this disease. Among the environmental factors that commonly determine the bacterial phenotype, the concentration of essential nutrients is of particular importance. Iron, a crucial and scarce nutrient in the natural environment of *B. pertussis*, has been found to induce substantial phenotypic changes in this pathogen. However, the relevance of this phenotype for the interaction with host cells was never investigated. Using an *in vitro* model for bacterial attachment, it was shown that the attachment capacity of *B. pertussis* to epithelial respiratory cells is enhanced under iron stress conditions. Attachment is mediated by iron-induced surface-exposed proteins with sialic acid-binding capacity. The results further suggest that some of these iron-induced surface-associated proteins are immunogenic and may represent attractive vaccine candidates.

Introduction

Iron is an essential nutrient for virtually all bacteria. The concentration of free iron in the human body is too low to support bacterial growth, due to the presence of iron-sequestering proteins. Bacterial pathogens respond to iron starvation stress by up-regulating transcription of genes encoding proteins with a high-affinity iron-binding capacity and other virulence factors (Litwin & Calderwood, 1993; Guerinot, 1994).

Bordetella pertussis, the etiologic agent of whooping cough, is a strictly human pathogen that colonizes the respiratory tract. *Bordetella pertussis* expresses several high-affinity iron-binding proteins, including possible receptors for human transferrin and lactoferrin, and a receptor for hemoglobin, to scavenge this metal in response to the limited free iron concentrations in the human airways (Redhead *et al.*, 1987; Redhead & Hill, 1991; Nicholson & Beall, 1999; Brickman *et al.*, 2006). Iron stress induces the expression of several outer membrane (OM) proteins and siderophore receptors (Gorringe *et al.*, 1990; Agiati & Dyer,

1992; Brickman & Armstrong, 1999). Interestingly, the Vir90 protein, which has significant homology with ferrisiderophore receptors, is regulated by extracellular iron concentrations but also by the *bvgAS* locus, which regulates the expression of virulence factors (Passerini de Rossi *et al.*, 2003). The BvgAS system mediates the transition between virulent (Bvg+) and avirulent (Bvg-) phases of *B. pertussis* characterized by distinct patterns of gene expression (Lacey, 1960; Deora *et al.*, 2001). Virulence factors encoded by the so-called vir-activated genes (*vags*), such as the main adhesin Filamentous hemagglutinin (FHA), or Pertussis toxin (PT), are expressed in the Bvg+ phase but not in Bvg- phase. While most described *B. pertussis* iron uptake systems are not Bvg-dependent, Vir90 is only expressed in the virulent phase, and is up-regulated when iron availability is low (Passerini de Rossi *et al.*, 2003). Interestingly, recent studies provided evidence that iron starvation also modulates the expression of one of the main *B. pertussis* virulence factors, PT (Thalen *et al.*, 2006).

Iron limitation was found to induce phenotypes of several human pathogens with increased virulence, characterized by

enhanced host cell-binding capacity (Scharfman *et al.*, 1996; Moreira *et al.*, 2003). In this study, it was investigated whether iron limitation influences *B. pertussis* interaction with epithelial respiratory cells. Furthermore, it was evaluated whether iron stress induces immunogenic surface-exposed proteins that may represent attractive vaccine candidates.

Materials and methods

Bacterial strains and growth conditions

Bordetella pertussis strains Tohama I and BpGR4, a Tohama derivative mutant lacking the expression of FHA (Locht *et al.*, 1992), were used in this study. For selected experiments, *B. pertussis* strain Tohama I was transformed with plasmid pCW505 (Weingart *et al.*, 1999) (kindly supplied by Dr Weiss, Cincinnati, OH), which induces cytoplasmic expression of green fluorescent protein (GFP) without affecting growth or antigen expression (Weingart *et al.*, 1999).

Bacteria were stored at -70°C and recovered by growth at 37°C for 3 days on Bordet–Gengou agar (ABG) (Difco laboratories, Detroit, MI) supplemented with 15% defibrinated sheep blood. Bacteria were then subcultured in Stainer–Scholte liquid medium (SS) at an initial cell density corresponding to an $A_{650\text{nm}}$ of 0.2. SS cultures were grown at 37°C under shaking conditions for 24 h. Bacterial cells were harvested by centrifugation (10 000 g for 15 min at room temperature), washed with sterile, iron-free saline solution, and diluted to an estimated concentration of 2×10^8 CFU mL⁻¹. Equal volumes of bacterial cell suspensions were used to inoculate 100 mL of iron-replete SS (36 μM iron) and iron-depleted SS (without addition of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$). Bacterial cultures were performed at 37°C under shaking conditions (150 r.p.m.) for 20 h, and subcultured twice in the respective culture media. Bacteria were then harvested by centrifugation and used in attachment or lectin-binding assays.

For comparative studies of *B. pertussis* Bvg phenotypes, Tohama I was grown on ABG containing either 50 mM of MgSO_4 (modulator) or no modulator, and further subcultured in either iron-replete SS (SS) or iron-depleted SS (SS-Fe) medium with or without 50 mM of modulator.

Iron-depleted SS media were prepared using sterile polypropylene tubes (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) or Pyrex glass containers sequentially washed with 3 M nitric acid and deionized water to remove surface-bound iron. All water used for preparing iron-free media was deionized. Iron-depleted SS was further treated with the cation exchange resin Chelex 100 (Bio-Rad, Hercules, CA) as described by West and Sparling (1987).

Siderophore assays

The presence of siderophores in culture supernatants of *B. pertussis* grown in iron-depleted SS medium was used to

confirm the iron-starved status of the bacteria. Briefly, samples of *B. pertussis* grown in iron-depleted SS or iron-replete SS (to be used as a negative control) were centrifuged to remove the cells. The supernatants were further filtered through a 0.22 μm pore size nylon membrane (Nalgene Co., Rochester, NY) and the chrome azurol S (CAS) assay was performed as described by Schwyn & Neilands (1987).

Sera and antibodies

Pooled sera from 15 pertussis patients with high titers against *B. pertussis*, as measured by enzyme-linked immunosorbent assay (ELISA) (Nagel *et al.*, 1985), and pooled sera from Dutch 4-year-old children participating in a trial to compare the efficacy of different boosters for pertussis vaccination (Berbers *et al.*, 1999) were used in this study. Children were vaccinated within the regular National Immunisation Program at the ages of 2, 3, 4, and 11 months with the Dutch WCV. Serum samples obtained 1 month after booster vaccination with WCV from 15 children with high titers against *B. pertussis*, as measured by ELISA (Nagel *et al.*, 1985), were pooled and used in this study.

Monoclonal antibody 4-37F3 (IgG1) against *B. pertussis* FHA (Poolman *et al.*, 1990) was kindly provided by the Netherlands Vaccine Institute, Bilthoven, the Netherlands.

Lectins

The fluorescein isothiocyanate-labeled lectins (FITC-lectin) from *Dioclea violácea* (DvL), *Dioclea grandiflora* (DgL), *Cratylia floribunda* (CfL), *Bryohtamnium triquetrum* (BtL), and *Lonchocarpus sericeus* (LsL) (Dam *et al.*, 1998; Barbosa *et al.*, 2001; Neves *et al.*, 2001; Alencar *et al.*, 2005) were kindly provided by Dr Holanda Teixeira, Universidade Federal do Ceará, Fortaleza, Brazil.

Cells and growth conditions

The human alveolar epithelial cell line A549 (human lung type II pneumocyte) (American Type Culture Collection CCL185, Rockville, MD) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U mL⁻¹ penicillin, and 100 μg mL⁻¹ of streptomycin (Sigma, St Louis, MO). Routine subcultures for A549 pneumocytes were performed at 1 : 3 split ratios by incubation with 0.05% trypsin – 0.02% EDTA for 5 min at 37°C . Cell cultures were incubated at 37°C in 5% CO_2 . For attachment assay, epithelial cells were cultured in tissue culture trays to 80% confluence.

Lectin-binding studies

The lectin-binding capacity of *B. pertussis* grown in either an iron-replete or in an iron-depleted medium or A549 cells

was investigated as described by Moreira *et al.* (2003) with minor modifications. Briefly, a monolayer of A549 cells grown on a glass coverslip, or 15 μL of a bacterial suspension containing 3×10^7 bacteria mL^{-1} , placed on glass slides, were air dried and further fixed in methanol at 22 °C for 20 min. After washing with phosphate-buffered saline (PBS)–5% bovine serum albumin (BSA), the bacteria were incubated with 15 μL of increasing concentrations of FITC-lectin in PBS–5% BSA (concentration range: 0 to 750 $\mu\text{L mL}^{-1}$) at 22 °C for 30 min. The slides were then washed three times with PBS supplemented with 5% BSA, mounted in PBS–glycerol (50%), and observed by epifluorescence microscopy using a DMLB microscope coupled to a DC 100 camera (Leica Microscopy Systems Ltd, Heerbrugg, Switzerland).

Mucin-binding studies

The mucin-binding capacity of *B. pertussis* grown in either an iron-replete or in an iron-depleted medium was investigated as described by Belcher *et al.* (2000). Briefly, 2.5 μg of mucin type I from bovine submaxillary glands (Sigma) was desiccated onto a microtiter plate at 37 °C. Mucin-treated and -untreated plates were then washed with PBS and blocked with 5% dried skim milk in PBS for 1 h at 37 °C and again washed with PBS. GFP-labeled *B. pertussis* grown in either an iron replete or in an iron-depleted medium were added to the plates (2×10^8 bacteria well^{-1}) and incubated at 37 °C for 1 h. Nonadherent bacteria were removed by three washing steps with PBS. Fluorescence in each well was determined by a 96-well fluorimeter (Berthold Technology Twinkle LB970). Wells coated with mucin or blocking agent alone were used as the control of background fluorescence.

Preparation of OM protein fraction

Fractions containing OM proteins from *B. pertussis* grown either in iron-depleted SS or in iron-replete SS media were obtained as described previously by Molloy *et al.* (2000). Briefly, the cells were disrupted in an Aminco French press with two cycles at 14 000 psi, and unbroken cells were removed by centrifugation at 8000 g for 10 min at 4 °C. The supernatant was diluted with ice-cold 0.1 M sodium carbonate (pH 11) to a final volume of 60 mL and stirred slowly on ice for 1 h. The carbonate-treated samples were submitted to ultra centrifugation in a Beckman 55.2 Ti rotor at 115 000 g for 1 h at 4 °C. The supernatant was discarded and the pellet (OM protein-enriched fraction) was suspended and washed in 2 mL of 50 mM Tris/HCl, pH 7.5. The washed membrane fractions were collected by centrifugation, suspended in distilled water, and the protein concentration was determined.

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 before incubation with *B. pertussis* at a multiplicity of infection (MOI) of 100 bacteria per cell (unless otherwise stated) in the presence or the absence of 500 $\mu\text{g mL}^{-1}$ of sialic acid (Sigma, St Louis, MO), 500 $\mu\text{g mL}^{-1}$ of LsL, 500 $\mu\text{g mL}^{-1}$ of BtL, 500 $\mu\text{g mL}^{-1}$ of D-manosa (Sigma), 500 $\mu\text{g mL}^{-1}$ of mucin type I from bovine submaxillary glands (Sigma), or 500 $\mu\text{g mL}^{-1}$ of BtL plus 500 $\mu\text{g mL}^{-1}$ of mucin type I. In selected experiments, bacteria were incubated with either 500 $\mu\text{g mL}^{-1}$ of mucin type I during 30 min at 37 °C, or 100 $\mu\text{g mL}^{-1}$ of proteinase K (Sigma) for 30 min at 37 °C, followed by the addition of PMSF (1 mM) to stop the proteolytic activity. In both cases, bacteria were washed with DMEM plus 10% of FCS after treatment, and evaluated in their capacity to attach to A549 cells as described above. Finally, attachment assays were also performed in the presence of 2.5% v/v of pooled sera from either infected or vaccinated donors exhibiting high anti-*B. pertussis* antibody titers. Attachment assays in the presence of 2.5 v/v of nonimmune sera with an undetectable level of antibodies against *B. pertussis* were run in parallel.

Bacterial inoculates were quantified by plating appropriate dilutions onto BG plates. 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% CO_2). No changes in the number of viable A549 cells were detected upon infection. Experiments were stopped by washing the monolayer with DMEM medium supplemented with 10% FCS, before fixation with methanol. Bacterial adherence was quantified by microscopy after crystal violet staining. Adherent bacteria were counted by microscopic examination of 20 randomly selected fields showing a minimum of seven epithelial cells per field. All experiments were conducted at least three times in duplicate.

Immunoblot analysis

Bacterial cells and OM fractions were run in sodium dodecyl sulfate-polyacryl amide gel electrophoresis (SDS-PAGE) 10% and 12.5% (Laemmli, 1970), respectively. Proteins were transferred onto polyvinylidene fluoride (Immobilon, PVDF, Millipore) sheets (Towbin *et al.*, 1992), and further incubated with mouse monoclonal anti-FHA antibodies, pooled sera from pertussis patients, or pertussis vaccinee. The immunochemical detection was performed using alkaline phosphatase-conjugated goat anti-mouse IgG or

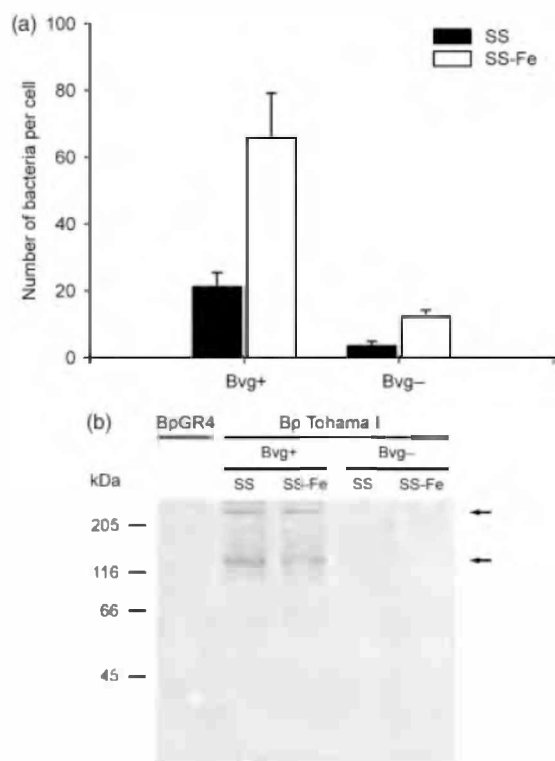


Fig. 1. Effect of nutritional iron conditions on *Bordetella pertussis* attachment to human respiratory epithelial cells, A549. (a) Virulent (Bvg+) and modulated (Bvg-) *B. pertussis* grown either in an iron-replete (SS) or in an iron-depleted medium (SS-Fe) were allowed to adhere to A549 cells at a multiplicity of infection (MOI) of 100. Data represent mean \pm SD of at least three independent experiments. (b) Western blot analysis of whole-cell lysates of virulent (Bvg+) and modulated (Bvg-) *B. pertussis* tohama I grown either in an iron-replete (SS) or in an iron-depleted medium (SS-Fe), and *B. pertussis* mutant strain lacking the expression of FHA (BpGR4). Membranes were probed with mouse anti-FHA antibodies. The migration of molecular mass markers (kDa) is indicated on the left.

alkaline phosphatase-conjugated goat anti-human IgG (Jackson Immuno Research, Baltimore Pike).

Statistical analysis

Student's test (confidence level: 95%) or ANOVA was used for statistical data evaluation. The significance of the differences between the mean values of the data evaluated by ANOVA was determined with the least significant difference (LSD) test at a confidence level of 95%. Results are shown as means and SD.

Results and discussion

Iron is a scarce but essential nutrient for bacterial cell growth. Bacterial proteins endowed with iron-scavenging properties are important virulence factors for many human pathogens. Previous studies have demonstrated that iron

limitation induces the synthesis of several *B. pertussis* OM proteins and other factors, which are often involved in iron acquisition (Redhead *et al.*, 1987; Redhead & Hill, 1991; Agiato & Dyer, 1992; Brickman & Armstrong, 1999; Brickman *et al.*, 2006), and well-known *B. pertussis* virulence factors, including PT (Thalen *et al.*, 2006). In this study, it was found that *B. pertussis* grown under iron limitation attached more efficiently to A549 cells than bacteria cultured under normal conditions (Fig. 1). This could not be explained by an increase in the expression of the major adhesin of *B. pertussis*, i.e. FHA, as detected by Western blot (Fig. 1). This was corroborated by additional experiments, using bacteria lacking the expression of FHA, which showed a threefold increase in bacterial attachment ratios after growth under iron limitation, suggesting that iron starvation induces expression of iron-dependent adhesins (Fig. 1). Proteinase K treatment of *B. pertussis* grown in either iron-replete or in iron-depleted media before incubation with epithelial cell monolayers significantly reduced bacterial attachment ratios, indicating that increased adhesion in response to iron stress is mediated by OM proteins or proteins associated with the cell surface (Fig. 2).

Bacteria often use host membrane carbohydrates as ligands for attachment. Therefore, a panel of lectins with different specificities was used in order to identify potential carbohydrate residues that might mediate the iron-dependent increase in bacterial attachment. Most lectins, including DvL, DgL, or CfL, did not interfere with bacterial attachment. Addition of LsL reduced the increase in bacterial attachment observed after growth under iron limitation. Interestingly, attachment of bacteria grown in iron-replete

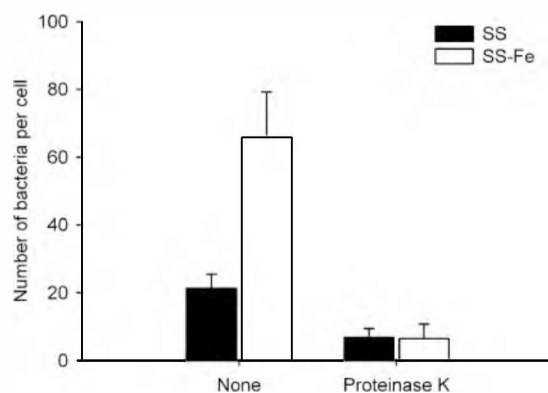


Fig. 2. Attachment of *Bordetella pertussis* grown under different iron nutritional conditions after proteinase K treatment. Virulent *B. pertussis* grown either in an iron-replete (SS) or in an iron-depleted medium (SS-Fe) were incubated with 100 µg mL⁻¹ proteinase K during 30 min at 37 °C, followed by the addition of phenylmethyl sulfonyl fluoride (1 mM) to stop the proteolytic activity, and further incubated with A549 cells (MOI: 100). Attachment of virulent *B. pertussis* grown in either SS or in SS-Fe, without proteinase K treatment (none), was evaluated in parallel. Data represent mean \pm SD of three independent experiments.

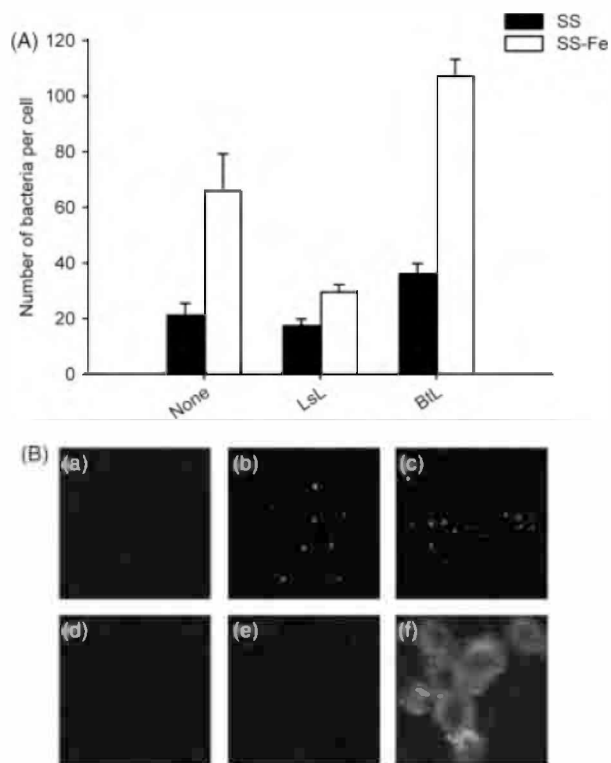


Fig. 3. Effect of lectins on *Bordetella pertussis* attachment to A549 cells. (A) Attachment of virulent *B. pertussis* grown in either an iron-replete (SS) or in an iron-depleted medium (SS-Fe) to human respiratory epithelial cells A549 (at an MOI of 100) in the absence or in the presence of LsL ($500 \mu\text{g mL}^{-1}$) or BtL ($500 \mu\text{g mL}^{-1}$). Data represent mean \pm SD of three independent experiments. (B) BtL and LsL binding to *B. pertussis* and A549 cells. Fifteen microliters of a bacterial suspension containing 3×10^7 bacteria mL^{-1} was placed on glass slides, air dried, fixed in methanol, and further incubated with (a) PBS (control), (b) FITC-LsL, or (c) FITC-BtL. Monolayers of A549 cells grown on glass coverslips were air dried, fixed in methanol, and further incubated with (d) PBS (control), (e) FITC-LsL, or (f) FITC-BtL. Fluorescence microscopy was performed with a $\times 100/1.25$ objective under oil immersion using a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany). Representative panels of one out of four independent experiments are shown.

media was also slightly but significantly ($P < 0.05$) decreased by the presence of LsL. Ligands of this lectin are still poorly characterized. The present results suggest that *B. pertussis* constitutively expresses such ligands, and that their expression is up-regulated when bacteria are exposed to iron limitation (Fig. 3a). The presence of BtL enhanced the attachment of *B. pertussis* grown both in an iron-replete and in an iron-depleted medium. After incubation of lectins with either A549 cells or *B. pertussis*, both BtL and LsL could be detected on bacteria cultured under either iron-replete or under iron-depleted conditions, but only BtL could be detected on epithelial cells. Representative results are shown in Fig. 3b. To clarify this interaction, inhibition studies using the two sorts of known BtL ligands, i.e. simple and complex

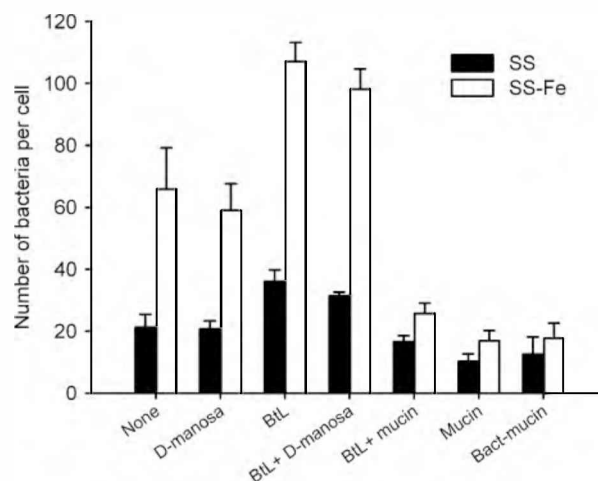


Fig. 4. Increase of iron-stressed *Bordetella pertussis* attachment to A549 cells involves carbohydrate residues. Attachment of virulent *B. pertussis* grown in either an iron-replete (SS) or in an iron-depleted medium (SS-Fe) to human respiratory epithelial cells A549 (at an MOI of 100) in the absence or in the presence of D-mannosa ($500 \mu\text{g mL}^{-1}$), BtL ($500 \mu\text{g mL}^{-1}$), BtL plus D-mannosa (BtL+D-mannosa) ($500 \mu\text{g mL}^{-1}$ of each); BtL plus mucin (BtL+mucin) ($500 \mu\text{g mL}^{-1}$ of each); and mucin alone (mucin) ($500 \mu\text{g mL}^{-1}$). Additionally, virulent *B. pertussis* grown either in SS or in SS-Fe were incubated with mucin ($500 \mu\text{g mL}^{-1}$), followed by washings steps, before the incubation with A549 cells at an MOI of 100 (Bact-mucin). Data represent mean \pm SD of three independent experiments.

carbohydrates, were performed. The simultaneous addition of BtL and D-mannose, the only simple carbohydrate recognized by this lectin, during attachment experiments did not alter the attachment ratios (Fig. 4). However, the addition of mucin, a complex carbohydrate and a known BtL ligand, not only abolished the BtL-mediated increase of bacterial attachment but also resulted in a level of bacterial attachment lower than the respective controls (Fig. 4). These results might be consistent with an inhibitory effect on the interaction of BtL and A549 cells, or bacteria and A549 cells.

Because *B. pertussis* was previously reported to bind mucin (Belcher *et al.*, 2000), it was next investigated whether the increased attachment of iron-deprived *B. pertussis* to A549 cells was associated with an increase in bacterial mucin binding upon iron limitation. Pretreatment of *B. pertussis* with mucin before incubation with A549 cells reduced attachment to A549 cells, irrespective of bacterial culture conditions (Fig. 4). However, the magnitude of the decrease was higher for bacteria cultured under iron limitation abolishing the increase observed in the attachment level of iron-starved bacteria. As shown in Fig. 5a, the presence of mucin inhibits bacterial attachment in a dose-dependent manner. The inhibition curve for iron-stressed bacteria shows a sharper slope suggesting that these bacteria are more sensitive to the inhibitory effect of mucin. The higher

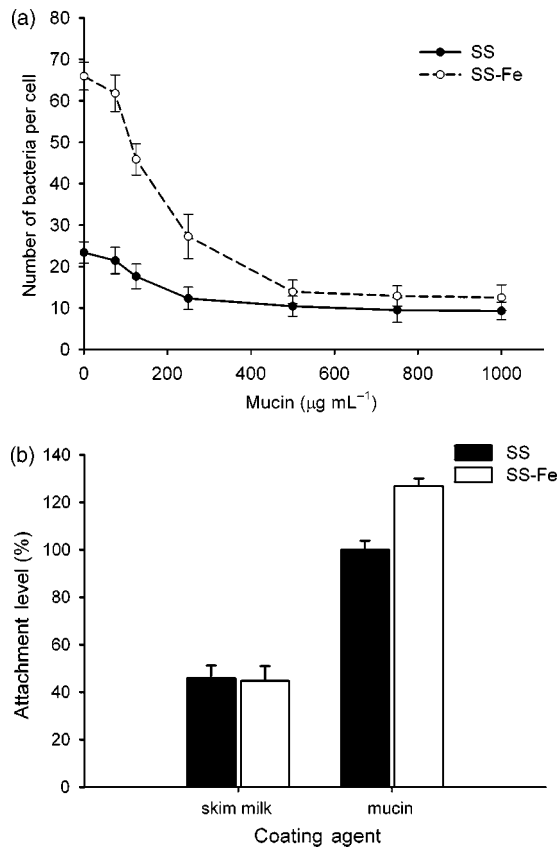


Fig. 5. Iron starvation increases the mucin-binding activity of *B. pertussis*. (a) Attachment of virulent *Bordetella pertussis* grown in either an iron-replete (SS) or in an iron-depleted medium (SS-Fe) to human respiratory epithelial cells A549 (at an MOI of 100) in the absence or in the presence of increasing concentration of mucin (0–1000 $\mu\text{g mL}^{-1}$). (b) Binding of virulent *Bordetella pertussis* grown in either an iron-replete (SS) or in an iron-depleted medium (SS-Fe) to mucin-coated plates. Background fluorescence of the mucin and skim milk well-coated were subtracted. Fluorescence due to the attachment of bacteria grown under iron-replete (SS) conditions was set as 100% of the attachment level. Data represent mean \pm SD of three independent experiments.

mucin-binding activity of iron-starved *B. pertussis* was further confirmed by assessing bacterial adhesion to micro-titer plates coated with bovine salivary mucin (Fig. 5b). Taken together, these results seem to indicate that iron limitation induces a phenotype that facilitates mucin-binding activity and bacterial attachment to A549 cells through a similar mechanism.

Mucin binding by *B. pertussis* requires sialic acid residues on glycoproteins (Belcher *et al.*, 2000). To confirm the hypothesis that facilitation of bacterial attachment through iron limitation depends on the presence of sialic acid glycoproteins, sialic acid was used in competition assays. As can be seen in Fig. 6, the presence of sialic acid mimicked the effect of mucin on bacterial attachment levels.

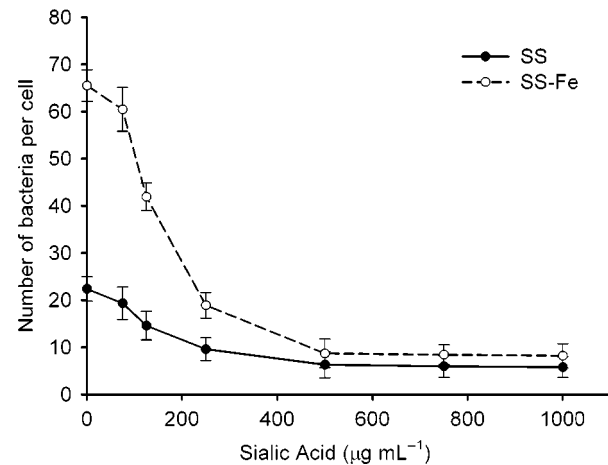


Fig. 6. *Bordetella pertussis* attachment to A549 cells is inhibited by sialic acid. Attachment of virulent *B. pertussis* grown either in an iron-replete (SS) or in an iron-depleted medium (SS-Fe) to human respiratory epithelial cells A549 (at an MOI of 100) in the absence or in the presence of increasing concentration of sialic acid (0–1000 $\mu\text{g mL}^{-1}$). Data represent mean \pm SD of three independent experiments.

Taken together, these results indicate that iron limitation, which mimics *in vivo* conditions encountered by *B. pertussis*, enhances bacterial mucin binding and facilitates attachment to epithelial cells through a similar mechanism. Iron limitation was also shown to induce the expression of mucin-binding proteins in another human respiratory pathogen, *Pseudomonas aeruginosa*, and this proved to be a crucial factor for host colonization (Scharfman *et al.*, 1996). Mucus poses a challenge to infecting bacteria. *Bordetella pertussis* infection was previously shown to induce mucin production in host cells (Belcher *et al.*, 2000; McNamara & Basbaum, 2001). Most of its virulence factors contribute to the production of viscous mucus during infection (Belcher *et al.*, 2000). Therefore, the mucin-binding activity of *B. pertussis* has been thought to represent a bacterial counter-defensive mechanism (Belcher *et al.*, 2000). The present results seem to support this hypothesis, because physiological conditions such as iron limitation induce a bacterial phenotype characterized by increased mucin binding and bacterial attachment to epithelial cells.

To investigate whether iron-dependent surface-associated proteins are immunogenic and elicit protective antibodies, attachment of bacteria grown under iron-deficient and -sufficient conditions were tested in the presence of sera from individuals who had either been infected with *B. pertussis* or had been vaccinated with a whole-cell vaccine. These sera displayed similar titers of *Bordetella*-specific IgG, as determined by ELISA (1300 and 1340, respectively). However, Western Blot analysis of OM-enriched fractions showed that sera from vaccinated and infected individuals contained different *B. pertussis*-specific antibodies (Fig. 7).

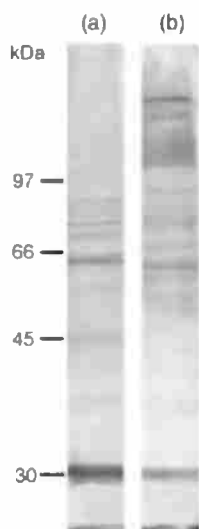


Fig. 7. Western blot analysis of OM fractions of virulent *Bordetella pertussis* grown under iron starvation. OM-enriched fractions of *B. pertussis* grown in an iron-depleted medium were run in SDS-PAGE, transferred onto PVDF membranes, and further probed with pooled sera from either (a) vaccinated or (b) infected individuals. Migration of molecular mass markers (kDa) is indicated on the left.

Table 1. Effect of serum from infected or vaccinated donors on A549 cells attachment of *Bordetella pertussis* grown under different iron conditions

| Growth medium/treatment | Attachment (bacteria per cell) \pm SD |
|----------------------------------|---|
| SS | 19.5 \pm 1.39 |
| SS/nonimmune sera* | 20.3 \pm 2.03 |
| SS/vaccinee sera [†] | 8.3 \pm 1.14 |
| SS/infected sera [‡] | 6.7 \pm 0.47 |
| SS-Fe | 65.1 \pm 1.7 |
| SS-Fe/nonimmune sera* | 67.2 \pm 1.1 |
| SS-Fe/vaccinee sera [†] | 19.5 \pm 3.24 |
| SS-Fe/infected sera [‡] | 4.07 \pm 0.64 |

*Adhesion assays were performed in presence of 2.5% v v⁻¹ nonimmune sera.

[†]Adhesion assays were performed in presence of 2.5% v v⁻¹ pooled sera from vaccinated donors.

[‡]Adhesion assays were performed in presence of 2.5% v v⁻¹ pooled sera from infected donors.

Values represent the mean \pm SD of three independent experiments.

These differences in specificity paralleled differences in the biological activity of these antibodies in attachment assays. The results in Table 1 show that convalescent sera from individuals with pertussis decreased attachment levels of iron-starved bacteria to a higher extent. Conversely, in the presence of sera from vaccinated individuals, which are expected to lack antibodies specific for proteins induced under iron limitation, the relative reduction in attachment was similar for bacteria cultured under iron-replete and

iron-depleted conditions (60–70%). Importantly, the presence of nonimmune sera did not modify the attachment.

Taken together, these results suggest that iron limitation induces expression of *B. pertussis* surface-associated proteins that facilitate mucin binding and bacterial attachment through a sialic-acid-dependent mechanism. Iron limitation-induced surface-associated proteins may represent important virulence factors, and are immunogenic. Antibodies against proteins induced by iron limitation seem to interfere with bacterial attachment to epithelial cells. Identification of these proteins may eventually help the development of new vaccines that can prevent bacterial colonization.

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Statement

Written informed consent was obtained from a parent, guardian or adult before enrollment. This study was approved by the institutional review board of the National Institute of Public Health and the Environment, the Netherlands.

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