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Siderophore Production and Membrane Alterations by *Bordetella pertussis* in Response to Iron Starvation

LISA-ANNE AGIATO* AND DAVID W. DYER

Department of Microbiology, State University of New York at Buffalo, Buffalo, New York 14214

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Bordetella pertussis **was grown in iron (Fe)-free defined medium to limit the growth of the organism. Doubling times of the Fe-starved organism increased by approximately 1 h, and a 40% reduction in the final extent of growth in Fe-depleted medium was observed. Under these conditions, a hydroxamate siderophore named bordetellin was secreted by *B. pertussis*. Lactoferrin and transferrin supported growth of *B. pertussis* even when the protein was sequestered inside dialysis tubing. This suggested that binding of lactoferrin and transferrin to *B. pertussis* was not essential and that bordetellin production plays a major role in Fe uptake. Solid-phase dot blot assays indicated weak binding of lactoferrin to the cell surface, consistent with previous reports of a lactoferrin receptor. Three new proteins of 97, 77, and 63 kDa were synthesized in response to Fe starvation. Fe-inducible proteins of 103, 72, 24, 21, and 18 kDa were also observed. The synthesis of lipopolysaccharide was also altered by Fe availability.**

Bordetella pertussis is the causative agent of whooping cough. This gram-negative coccobacillus is transmitted through the inhalation of infected droplets. The organism establishes infection by colonizing the upper respiratory mucosa. This initial stage of infection is termed the catarrhal phase and lasts approximately 2 weeks. As the infection progresses, the organism moves down the respiratory tree, causing inflammation. It is during this paroxysmal stage of infection that the characteristic cough associated with the disease is present.

Iron (Fe) is a growth requirement for virtually all microbes (36). In animals, most Fe is found intracellularly, sequestered in ferritin or bound in heme compounds. In the human body, extracellular Fe^{3+} is sequestered from microbes by binding to proteins such as lactoferrin (LF) and transferrin (TF). These mechanisms keep the level of free Fe below the minimum levels (0.4 to 1 μM) required for the growth of most microbes (36). These nonspecific mechanisms for restricting bacterial growth have been termed nutritional immunity (36). Pathogenic bacteria must be capable of removing the Fe from host Fe-binding compounds if they are to initiate and sustain infection. Several mechanisms exist by which bacteria remove Fe from host compounds. Many microorganisms such as *Escherichia coli*, *Pseudomonas* spp., and *Shigella* spp. secrete low-molecular-weight Fe-chelating compounds termed siderophores (20). Siderophores typically bind ferric iron; the siderophores produced by pathogenic bacteria are commonly able to remove Fe^{3+} from LF and TF (19). The ferri-siderophore complex is then bound by a bacterial surface receptor and processed to remove the Fe^{3+} ion for use by the organism (19).

Other microbes, such as the pathogenic *Neisseria* spp., possess receptors for host Fe-binding proteins (27, 35). These pathogens bind TF and LF to separate outer membrane receptors, and the Fe is then removed from the protein. *Haemophilus influenzae* has also been reported to acquire Fe from TF by a siderophore-independent mechanism and the acquisition of Fe from the protein required cell-to-protein contact (18).

Few reports have appeared in the literature concerning the Fe uptake system of *B. pertussis*. Redhead et al. (24) suggested that *B. pertussis* synthesized a receptor for host Fe-binding proteins in response to Fe starvation. These researchers concluded that a single cell surface receptor bound both human TF and LF, unlike the pathogenic *Neisseria* spp. which have separate receptors. In these studies, siderophore production was not observed (24). Gorringe et al. (9) reported that *B. pertussis* secreted a hydroxamate siderophore in response to low Fe availability. This report concluded that *B. pertussis* utilized a dual mechanism of Fe uptake, making use of both the hydroxamate siderophore and the receptor described by Redhead et al. (24). More recently, Redhead and Hill reported the removal of Fe^{3+} from TF by a receptor-mediated mechanism, but the putative receptor was not identified (23).

This study provides new information on the Fe sources capable of supporting growth of the organism. We have collected evidence that a siderophore-mediated Fe uptake system plays a major role in Fe^{3+} acquisition from LF and TF. We examined the membrane protein and lipopolysaccharide (LPS) profiles of cells grown under high- and low-Fe conditions and report the changes observed in synthesis due to Fe availability.

MATERIALS AND METHODS

Bacterial strains and growth media. For most experiments, *B. pertussis* DBP2, a spontaneous streptomycin-resistant derivative of strain Tohama I, was used. In certain instances, *B. pertussis* 18323 and BB114 were also examined. Bacterial strains were maintained on Stainer-Scholte (30) agar plates containing 10% defibrinated sheep blood (Crane Laboratory, Syracuse, N.Y.). Hemolytic (*vir*$^+$) bacteria were used in all experiments. Plates were incubated in a 37°C and 5% CO_2 environment in a Napco model 6100 CO_2 incubator (Napco Scientific Co., Tualatin, Oreg.). Broth cultures were grown in Stainer-Scholte medium (SSM) or in Chelex-treated defined medium (CDM) (37). Broth cultures

* Corresponding author.

were shaken at 250 rpm at 37°C in a Series 25 incubator shaker (New Brunswick Scientific, Edison, N.J.). In certain experiments, streptomycin was included in the medium at a concentration of 200 μg/ml in broth and 400 μg/ml in blood plates. All reagents were purchased from Sigma Chemical Co., St. Louis, Mo., unless otherwise noted.

Iron depletion of growth media. All glassware was soaked overnight in 3 N nitric acid and rinsed extensively with distilled, deionized water. Disposable plasticware was used whenever possible to eliminate Fe contamination. All media were depleted of Fe by treatment with the cation exchange resin Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.) as previously described by West and Sparling (37). All water used for preparing Fe-free media had a resistivity of at least 15 MΩ. The amount of residual Fe in Chelex-treated SSM (CSSM) was determined with a ferrozine reagent (31). The average Fe concentration from several batches of CSSM was calculated to be 0.3 μM. The Fe concentration was also measured by atomic absorption with a Perkin-Elmer 1100B atomic absorption spectrophotometer equipped with an HGA 700 graphite furnace and used according to the manufacturer's recommendations (Perkin-Elmer, Oak Brook, Ill.). $Fe(NO_3)_3$ (0.2 μg/ml) in 1% HNO_3 was used as the standard. By this method, the Fe content of CSSM was determined to be 0.5 μM. This amount of Fe is the minimum level required for growth of most gram-negative organisms (36).

Growth of *B. pertussis* with nonprotein iron. Strain DBP2 was grown overnight in CSSM without added Fe. The cells were harvested by centrifugation at 4,000 × *g* for 15 min at room temperature and washed twice in fresh CSSM, and the cells were suspended in 20 ml of CSSM. A 5% inoculum was added to fresh CSSM, and various Fe sources were added to a final concentration of 10 μM Fe. Growth was measured by optical density in a Klett-Summerson colorimeter equipped with a green filter (Klett Mfg. Co., Long Island City, N.Y.).

Heme was dissolved in 10 mM NaOH, filter sterilized, and used at a final concentration of 10 μM. Ferric dicitrate and ferric pyrophosphate were made by combining sodium citrate or sodium pyrophosphate and ferric nitrate in a 10:1 molar ratio (citrate or pyrophosphate to Fe). All Fe sources were prepared immediately before use, filter sterilized, and added to the medium at a final concentration of 10 μM Fe.

Growth with TF and LF. Growth curves in CDM were performed to determine whether human LF and TF would support the growth of *B. pertussis*. LF and TF were dissolved at 16 mg/ml in a solution of 50 mM Tris (pH 7.5), 150 mM NaCl, and 20 mM $NaHCO_3$ to give a final protein concentration of 200 μM. The proteins were 30% saturated with Fe by the addition of 120 μM ferric dicitrate and then incubated at 37°C for 1 h. Citrate and unbound Fe were removed by overnight dialysis against 4 liters of a solution of 50 mM Tris (pH 7.5), 150 mM NaCl, and 20 mM $NaHCO_3$ with two changes of buffer. The proteins were then filter sterilized. The protein concentration was determined by using Bio-Rad protein dye concentrate with bovine serum albumin as the reference. The amount of Fe bound to the proteins was measured spectrophotometrically using the extinction coefficients of the Fe-saturated forms of the proteins: human LF, $\varepsilon^{1\%}_{470} = 0.51$; human TF, $\varepsilon^{1\%}_{470} = 0.6$ (1). $NaHCO_3$ (10 mM) was added to the medium containing TF and LF to maintain strong binding of Fe^{3+} to the protein (10). The proteins were added to the medium to a final concentration of 10 μM Fe.

In some experiments, TF and LF were labeled with ^{125}I using Iodo-beads (Pierce, Rockford, Ill.) according to the manufacturer's specifications. NaI (50 mM) was added to 5 ml of TF or LF (at a concentration of 16 mg/ml) and incubated for 30 min at 37°C. This step was added to suppress nonspecific binding of ^{125}I to these proteins. The protein was then dialyzed against 4 liters of a solution of 50 mM Tris (pH 7.5), 150 mM NaCl, and 20 mM $NaHCO_3$ overnight to remove any unbound NaI. Two Iodo-beads were washed in 5 ml of a solution of 50 mM Tris (pH 7.5), 150 mM NaCl, and 20 mM $NaHCO_3$. The beads were then incubated in 500 μl of buffer containing 100 μCi of ^{125}I for 15 min at room temperature. TF or LF was then added to Iodo-beads and allowed to incubate at room temperature for 15 min. The reaction was stopped by removing the protein from the beads. The protein was dialyzed as described above with additional dialysis against 2 liters of CDM overnight with two changes of buffer. To ensure that the protein had not been damaged during the iodination procedure, the labeled protein was run on 7.5% polyacrylamide gels (13) and silver stained (38). The gel was dried and used to expose Kodak XAR film (Kodak, Rochester, N.Y.) for 16 h.

In order to determine whether cell-to-protein contact was necessary for growth with TF and LF, the radiolabeled protein was sequestered inside Spectra/Por 1 dialysis tubing with a molecular mass cutoff of 6,000 to 8,000 Da (Spectrum Medical Industries, Los Angeles, Calif.). The tubing was long enough so that both ends were closed with a single Spectra/Por closure (Spectrum Medical Industries) at the mouth of the flask. Neither end of the dialysis tubing was immersed in the culture medium. This ensured that the protein contained in the dialysis bag would not leak into the medium from an incompletely closed dialysis clip. Protein was pushed to the middle of the tubing which ran along the bottom of the flask and was completely immersed in CDM. Control flasks containing sterile CDM in the tubing were included to ensure that Fe was not introduced on the dialysis tubing and that the tubing itself did not interfere with growth.

Siderophore assays. *B. pertussis* was grown to late log phase in CSSM and in CSSM plus 10 μM added Fe. Cells were removed by centrifugation, and the supernatants were filter sterilized through a 0.45-μm-pore-size nylon membrane (Nalgene Co., Rochester, N.Y.). The chrome azurol S (CAS) assay of Schwyn and Neilands (28) was performed essentially as described by these researchers using Desferal mesylate (CIBA Pharmaceuticals, Summit, N.J.) as a reference Fe-binding compound. The Csaky assay (7), which detects the presence of hydroxamic acids, was carried out by using hydroxylamine to generate a standard curve. In this assay, we used a 30-min acid hydrolysis step instead of the 4-h step originally suggested (7). Longer hydrolysis times significantly decreased detection of the hydroxylamine standard and any hydroxamate present in the samples. Culture supernatants were also tested for the presence of phenolates using the Arnow assay (2).

The Fe-binding ability of an excreted product in the culture supernatant was also assayed spectrophotometrically. Ferric nitrate (final concentration, 100 μM) was added to filter-sterilized culture supernatant from cells grown in CSSM and in CSSM plus 10 μM $FeSO_4$. A difference absorption scan was performed on the supernatant from the Fe-starved cultures using the Fe-replete culture supernatant as the reference. A Perkin-Elmer Lambda 3 UV-Vis spectrophotometer equipped with a chart recorder was used to scan the supernatant from 600 to 390 nm.

Receptor assay for human LF and TF. Dot blot assays were performed as described previously (27) using LF and TF conjugated to alkaline phosphatase (AP) (Jackson Immuno-

chemicals, Avondale, Pa.). Briefly, cells were grown to late log phase in CDM and CDM plus 10 μM Fe. A 1.0-ml aliquot was removed from the culture and adjusted to an optical density of 100 Klett units. A 100-μl aliquot was filtered onto BA85 nitrocellulose (Schleicher and Schuell, Keene, N.H.), using a Mini-fold II apparatus (Schleicher and Schuell). The dots were allowed to air dry. Nonspecific protein binding sites on the nitrocellulose were blocked with Tris-buffered saline (50 mM Tris [pH 7.5], 150 mM NaCl) containing 0.5% skim milk. Cells were probed with 2 μg of TF-AP per ml in fresh blocking buffer; LF-AP (2 μg/ml) was diluted in blocking buffer in which the NaCl concentration had been increased to 500 mM to suppress nonspecific binding (4). Phosphatase activity was detected by using a Bio-Rad Alkaline Phosphatase Conjugate Substrate Kit. *Neisseria meningitidis*, which has been shown to possess TF and LF receptors (27, 35), was used as a positive control for receptor activity. Control dots not incubated with the conjugate were included to ensure that no false-positive reactions occurred as a result of endogenous AP activity.

Membrane alterations in response to iron availability. Strain DBP2 was grown to late log phase in CSSM and CSSM plus Fe. Cells were collected by centrifugation for 10 min at 10,000 × *g*, and membranes were prepared essentially as described by Redhead (22). After centrifugation, the cell pellet was suspended in 5 ml of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0), and the cells were disrupted by passage through a French pressure cell (SLM Instruments, Urbana, Ill.) at 16,000 lb/in^2. Unbroken cells were removed by centrifugation at 10,000 × *g* for 10 min. Crude membrane fractions were collected by ultracentrifugation in a Beckman 70.1 Ti rotor at 100,000 × *g* for 1 h. These crude membranes were washed in 10 mM HEPES (pH 8.0) and pelleted by ultracentrifugation as described above. Enrichment for outer membranes was achieved by extraction with 2% Triton X-100 in 10 mM HEPES (pH 8.0), followed by ultracentrifugation for 1 h at 100,000 × *g*. The Triton X-100-insoluble pellet was collected and resuspended in 10 mM HEPES (pH 8.0) (22). Membrane protein concentrations were determined by a modified Lowry method (15). Membrane proteins were separated by electrophoresis through 7.5 and 15% polyacrylamide-sodium dodecyl sulfate (SDS) gels (13) using gels (13 by 16 cm) in a Hoeffer Scientific gel box. Proteins were visualized by silver staining (38).

B. pertussis LPS was also examined. Membrane samples were digested with proteinase K (20 μg/mg of membrane protein) for 1 h at 37°C (12). The digested samples were electrophoresed through 14% acrylamide gels containing 18% urea (14). LPS was stained by silver staining with periodic acid oxidation (34). LPS from *Salmonella minnesota* LPS mutants R_a, R_c, and R_e were used as molecular size markers. The molecular masses (in daltons) are as follows: 4,243 for R_a, 3,130 for R_c, and 2,584 for R_e (26).

Western blots (immunoblots) were performed as described by Towbin et al. (33). BPE3, a monoclonal antibody against pertactin (69-kDa outer membrane protein), was kindly provided by Roberta Shahin of the Center for Biologics Evaluation and Research, Food and Drug Administration.

RESULTS

Growth with various iron sources. A 40% average reduction in the final extent of growth was observed when *B. pertussis* DBP2 was grown in CSSM without the addition of Fe. Doubling times of the organism increased from 5 h in Fe-replete medium to 6 h in Fe-depleted CSSM. *B. pertussis* was capable of utilizing $FeSO_4$, ferric dicitrate, and heme as the sole source of Fe (Fig. 1). DBP2 also grew well in the presence of ferric pyrophosphate (data not shown). Similar results were observed when the organism was grown in CDM (data not shown). Increasing the concentration of added Fe to 300 μM Fe did not enhance the rate or the final extent of growth (data not shown).

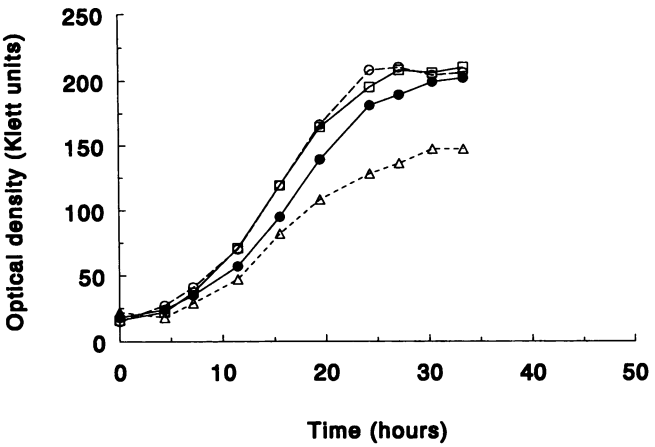

FIG. 1. Iron-dependent growth of DBP2 in CSSM. Symbols: △, no Fe added; □, 10 μM $FeSO_4$; ○, 10 μM ferric dicitrate; ●, 10 μM hemin.

In preliminary experiments, we observed that LF and TF precipitated in sterile CSSM after overnight incubation at 37°C. The reasons for this are unknown. The LF and TF growth curves were done with CDM, which has been used extensively to study the growth of pathogenic *Neisseria* spp. with LF and TF (4, 16, 35, 37). *B. pertussis* grew with the same doubling times in CSSM and CDM, but to a slightly higher final extent in CDM (data not shown). In these experiments, we added 10 mM $NaHCO_3$ to the medium to ensure that the Fe^{3+} atom remained tightly bound to LF and TF (10). We used LF or TF labeled with ^{125}I in these experiments so that leakage of the protein from the dialysis tubing could be monitored. Since LF may undergo oxidative damage as a result of radioiodination (25), we examined iodinated LF by SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. LF labeled with Iodo-beads was not damaged by this method (data not shown). Figure 2 shows the results of a typical growth curve with ^{125}I-labeled LF. DBP2 grew with a doubling time of approximately 7.5 h when the ^{125}I-labeled LF was free in the medium. *B. pertussis* was capable of using LF and TF as the Fe sources, even when the protein was sequestered within dialysis tubing (Fig. 2 and data not shown). A longer lag period was commonly observed when cell-to-protein contact was blocked, but the doubling time of the culture and the final extent of growth remained the same. These data suggested that growth may be enhanced by cell-to-protein contact, but this contact was not essential for the utilization of Fe^{3+} from LF (Fig. 2) and TF (data not shown). To ensure that the dialysis bag had not leaked during the experiment, samples from the ^{125}I-labeled LF cultures were counted in a Beckman 5500 gamma counter (Beckman Instruments, Irvine, Calif.). In each of three experiments, no appreciable radioactivity was detected in the medium when the LF was sequestered inside dialysis tubing. A control culture grown with CDM inside dialysis tubing grew at the same rate as did

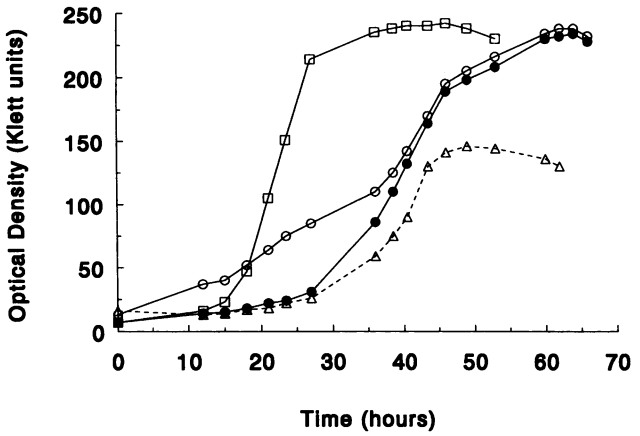

FIG. 2. Growth of DBP2 with LF as the sole Fe source in CDM. Symbols: △, dialysis membrane control (no added Fe or LF); □, 10 μM $FeSO_4$; ○, LF free in the medium; ●, LF sequestered inside dialysis membrane.

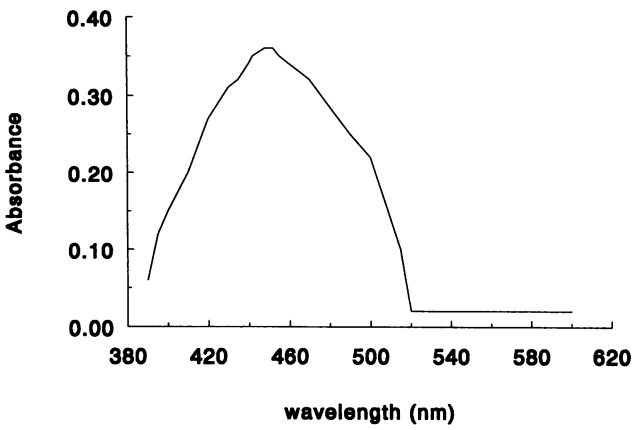

FIG. 3. Difference absorbance scan of spent culture supernatant at pH 7.9 from Fe-stressed bacteria after the addition of 100 μM $Fe(NO_3)_3$. Spent culture supernatant of Fe-replete bacteria was used as a reference.

cells grown in CDM without the addition of Fe, indicating that exogenous Fe was not introduced on the dialysis tubing and that the dialysis tubing was not inhibiting growth in any way (Fig. 2). This suggested that a siderophore-mediated Fe acquisition system may play a major role in Fe uptake from host Fe-binding proteins.

Siderophore assays. The CAS assay of Schwyn and Neilands (28) detected 12 μM (Desferal equivalents) of Fe-binding material in the supernatant of *B. pertussis* grown under Fe-depleted conditions. This Fe-binding activity appeared in the medium in response to Fe stress and not under Fe-replete conditions, which is a common characteristic of siderophore biosynthesis (20). However, the CAS reagent will also detect low-affinity Fe chelates (such as phosphate) that are not siderophores (28). The results of the Csaky assay indicated that *B. pertussis* DBP2 secreted 28 μM hydroxamic acid in response to Fe deprivation. No phenolate compound was detected using the Arnow assay (2). The Csaky assay (7) was also used to examine the supernatants of strains BB114 and 18323 grown in CSSM. In one typical experiment, BB114 excreted 80 μM hydroxamate and 18323 excreted 70 μM hydroxamate.

The ability of the hydroxamate excreted by strain DBP2 to bind Fe^{3+} was tested by examining the difference absorption spectra of culture supernatants after the addition of Fe^{3+}. Culture supernatant from the culture with Fe was used as the reference in this experiment. Fe-binding compounds typically have an absorption maximum in the range of 400 to 500 nm (19). At pH 7.6 (the pH to which SSM is buffered [30]), the difference absorption spectrum of Fe-starved culture supernatants [containing 100 μM $Fe(NO_3)_3$] had a broad peak between 400 and 500 nm, with the maximum absorption (A_{max}) at 450 nm (Fig. 3). Visually, culture supernatants from Fe-starved DBP2 turned a faint red color when ferric nitrate was added. These observations are characteristic of microbial siderophores (19). We also examined the response of this absorbance spectrum to changes in pH. The pH of filter-sterilized culture supernatant from cells grown at pH 7.6 (final pH of the culture was 7.9) was lowered in increments by the addition of 0.5 M HCl. At several pH values, a difference absorption spectrum was determined. Figure 4 illustrates the effect of pH on the A_{max} of the ferri-hydroxamate complex in crude supernatant. The absorption peak of 450 nm remained constant between pH values of 7.9 and 5.0. This peak shifted to 460 nm as the pH was lowered to 4.5 and 4.0. At pH 3.5, the A_{max} shifted to 490 nm, and at pH 3.0 and below, the absorption peak had shifted to 500 nm. This shift in absorption maximum as the pH of the medium is lowered is a property of several hydroxamate siderophores (3, 6, 29). We have named this hydroxamate siderophore bordetellin.

TF and LF receptor assay. Since we had observed siderophore production, we were interested in determining whether *B. pertussis* bound TF or LF, as suggested by Redhead et al. (24). Dot blot assays were performed to test for LF and TF receptor activity. By using this assay, binding of the LF-AP conjugate to strain DBP2 was detected (Fig. 5). Similar results were obtained with TF-AP (data not shown). This suggested that the organism may possess a LF or TF receptor, as suggested by Redhead et al. (24). Although this dot blot assay is a useful crude measure of receptor activity, the dot blot assay does not faithfully reproduce the kinetics of binding of TF to the *N. meningitidis* TF receptor (35).

Membrane alterations in response to iron availability. Several alterations in the membrane protein profile were consistently noted in response to Fe availability. Fe-repressible proteins (FeRPs) synthesized in response to Fe starvation

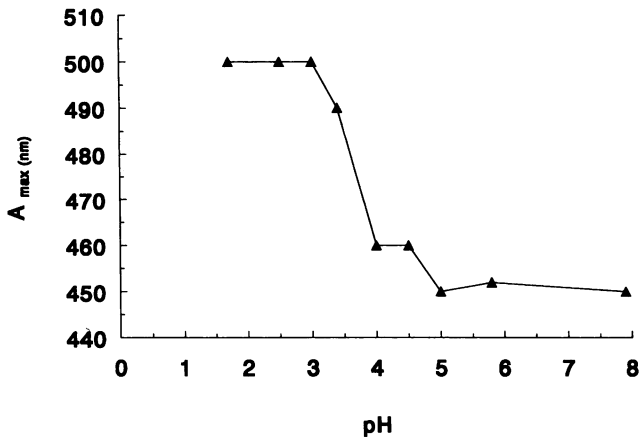

FIG. 4. Effect of pH on the A_{max} of bordetellin in spent culture supernatants.

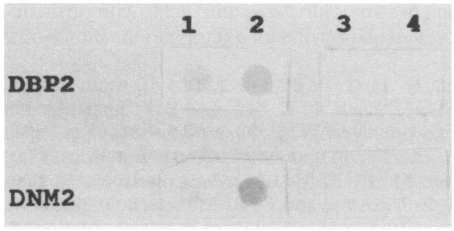

FIG. 5. Dot blot assay for LF receptor. For dots 1 and 3, cells were grown in CDM and 10 μM $FeSO_4$; for dots 2 and 4, cells were grown in CDM with no added Fe. Dots 1 and 2 were incubated with LF-AP, while dots 3 and 4 were incubated with AP substrate. DNM2 is a *N. meningitidis* strain.

were seen at 93, 77, and 63 kDa (Fig. 6). Fe-inducible proteins (FeIPs), seen only in membranes from cells grown under high Fe concentrations, were observed at 103, 72, 24, 21, and 18 kDa (Fig. 6). Occasionally, we observed changes in other membrane proteins in response to Fe availability, but these changes were not reproducible. Since the 72-kDa FeIP was near the molecular size of pertactin, the 69-kDa outer membrane adhesin of *B. pertussis* (5), we performed a Western blot using monoclonal antibody BPE3 (5) to determine whether this FeIP was pertactin. No difference in the levels of pertactin with respect to Fe availability was detected by using this assay (data not shown). The ferric dicitrate Fe transport system in *E. coli* is inducible by citrate (8). For this reason, we examined the membrane protein profiles of *B. pertussis* for citrate-inducible proteins. We also checked for heme-inducible proteins, because cells provided with heme as the sole Fe source seemed to have a longer lag

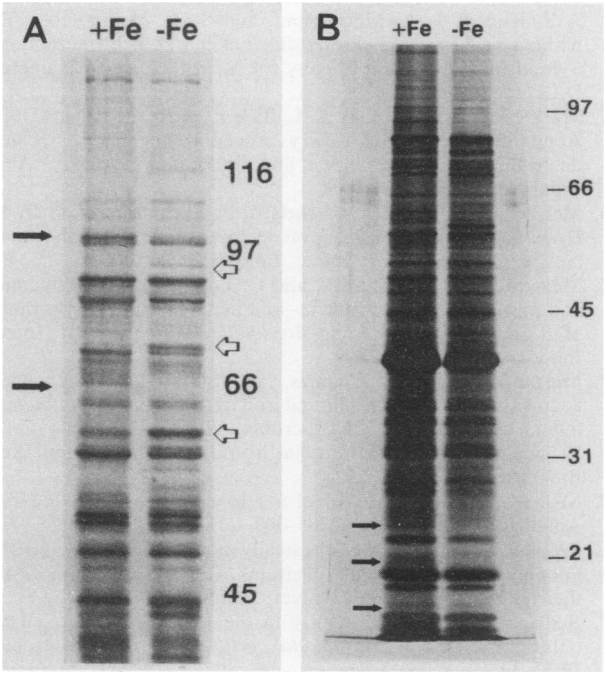

FIG. 6. SDS-PAGE analysis of Triton X-100-insoluble membrane proteins from DBP2 cells grown in CSSM with or without 10 μM $FeSO_4$. Gels containing SDS and 7.5% (A) and 15% (B) polyacrylamide were used. Solid arrows indicate FeIPs, while open arrows indicate FeRPs. M_w standards are indicated on the right side of each gel.

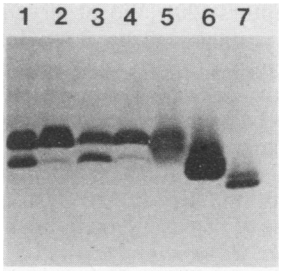

FIG. 7. Urea-SDS-PAGE of DBP2 membranes. Lanes 1 and 3 are membranes from cells grown in CSSM plus 10 μM $FeSO_4$; lanes 2 and 4 are membranes from cells grown in CSSM with no added Fe. Lanes 1 and 2 contain intact membranes, while lanes 3 and 4 contain membranes digested with proteinase K. Lanes 5 to 7 contain LPS from *S. minnesota* mutants R_a (lane 5), R_c (lane 6), and R_e (lane 7).

phase than cells provided with other Fe sources, suggesting that the heme uptake system may be inducible. Neither citrate- nor heme-inducible proteins were observed (data not shown).

We observed changes in the LPS in response to Fe starvation. *B. pertussis* synthesizes two types of LPS (21). LPS type II has a higher phosphate content and migrates faster in SDS-PAGE than LPS type I. The observed level of type II LPS was severely reduced in organisms that were stressed for Fe (Fig. 7). This alteration was not due to association of LPS with membrane proteins, since migration was identical regardless of proteinase K digestion. This suggests that LPS phosphorylation may be inhibited by low Fe availability.

DISCUSSION

B. pertussis is capable of growth with a variety of Fe sources. Ferrous sulfate is the Fe source incorporated into the original formulation of SSM (30). Ferric dicitrate, ferric pyrophosphate, and heme were capable of supporting the growth of *B. pertussis*. The organism secretes a hydroxamate siderophore, which we named bordetellin, in response to Fe deprivation. Bordetellin is probably responsible for removing Fe^{3+} from LF and TF.

The ability of *B. pertussis* to use LF and TF as Fe sources was not dependent upon cell-to-protein contact, as demonstrated by dialysis bag experiments in which LF or TF was physically separated from the organisms. A longer lag phase was observed when direct contact between the cell and LF was prevented, although the bacteria ultimately reached the same final cell density. This suggests that LF binding to the cell may facilitate the removal of Fe^{3+} from the protein by bordetellin. Such a mechanism would presumably enhance the efficiency of a siderophore. Recently, Menozzi et al. (17) described the purification of a 28-kDa LF-binding protein that may participate in enhancing the removal of Fe from LF. Redhead et al. (24) suggested that *B. pertussis* possessed a single receptor which bound LF and TF; this may be the LF-binding protein identified by Menozzi et al. (17). However, equilibrium binding assays to demonstrate saturable, concentration-dependent binding of ^{125}I-labeled LF and TF are necessary to demonstrate the presence of a TF or LF receptor.

We have reported the presence of several Fe-regulated proteins. It is probable that some of these FeRPs play a role in the Fe uptake system. The receptor for the ferri-bordetellin complex is most likely Fe regulated, as are other

siderophore receptors (20). We observed several proteins whose synthesis is enhanced by Fe-replete conditions. Although the function of these proteins is unknown, one possible role may be in Fe storage. The Fe-dependent alterations in the LPS of the organism may also play some role in the pathogenesis of pertussis.

From the results of this study, we can propose a model for the functioning of the Fe uptake system of *B. pertussis* during the process of disease in the human host. In the early stages of infection, *B. pertussis* grows on the upper respiratory mucosa. We infer that sufficient heme may be present on this mucosal surface to support the growth of *B. pertussis*, since *H. influenzae* is often found at this site. *H. influenzae* requires heme (or at least porphyrin) for growth (11). However, the concentration of heme present in these mucosal secretions is probably low, and its origin is unknown. Porphyrin may be released by other organisms growing in the mixed microbial environment of the upper respiratory tract. LF is also present in these respiratory mucosal secretions and maintains free Fe at low levels. Therefore, it is likely that *B. pertussis* uses both heme and LF as Fe sources during the early part of infection. We observed that organisms grown with heme as the sole Fe source had the same doubling time as cells grown with $FeSO_4$ and Fe dicitrate. When LF was the only Fe source, however, the doubling times increased by 2.5 h. This suggests that heme may be more readily used as an Fe source on the respiratory mucosum, and the contribution of LF-Fe to the growth of the organism may be somewhat less. As the infection progresses, *B. pertussis* descends into the lower respiratory tract. This is accompanied by significant inflammation in the lower airways. In patients with chronic bronchitis, the LF levels in bronchiolavage fluids were 10-fold higher than normal (32). TF levels increased fourfold in these lavage fluids as a result of transudation of plasma components (32). We suggest that during the latter stages of infection *B. pertussis* is growing in an Fe-rich environment in which LF and TF are the primary Fe sources. Paroxysmal-stage inflammation will also lower the pH of the respiratory mucosal secretions. This will tend to increase even further the availability of Fe from LF and TF, because the Fe on these proteins becomes labile at lower pH (1). These factors may increase Fe availability dramatically during the latter stages of the disease. We observed that certain membrane proteins are produced by *B. pertussis* under high Fe availability. It is possible that these FeIPs may represent proteins that are important for the pathogenesis of the latter stages of infection by *B. pertussis*.

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