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Identification and Purification of Transferrin- and Lactoferrin-Binding Proteins of *Bordetella pertussis* and *Bordetella bronchiseptica*

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***Bordetella pertussis* and *Bordetella bronchiseptica* were both able to grow in iron-deficient medium when supplemented with iron-saturated human lactoferrin or transferrin but not with human apotransferrin. Direct contact between the transferrins and the *Bordetella* cells did not appear to be required for growth but considerably improved the growth of the organisms. Analysis of *B. pertussis* and *B. bronchiseptica* whole-cell lysates from cultures carried out in iron-deficient or iron-replete media revealed iron-repressible proteins (IRPs) of 27 kDa in *B. pertussis* and of 30, 32, 73.5, and 79.5 kDa in *B. bronchiseptica*. Iron-inducible proteins of 16, 23.5, 36.5, and 92.5 kDa and of 17, 23.5, 70, 84, and 91 kDa were also identified in *B. pertussis* and *B. bronchiseptica*, respectively. By use of affinity chromatography with iron-saturated human lactoferrin or transferrin as ligands, the 27- and 32-kDa IRPs from *B. pertussis* and *B. bronchiseptica*, respectively, were specifically isolated. By using iron-chelated affinity columns, we showed that these proteins exhibit an affinity for iron. Cell fractionation experiments indicated that both of these proteins are probably associated with the outer membrane. Growth of the organisms under modulating conditions showed that the production of these IRPs is not under the genetic transcriptional control of *vir* or *bvg*, the general virulence regulon in *Bordetella* spp.**

Most pathogens face the problem of an extremely low availability of free iron in the infected hosts as a result of the sequestering of iron by specific proteins, mainly transferrin and lactoferrin in the extracellular space and ferritin within the cell cytoplasm (40).

Transferrin and lactoferrin are ca. 80-kDa, high-affinity iron-binding glycoproteins, widely distributed and conserved in vertebrates (2). Transferrin is present predominantly in serum, whereas lactoferrin is found mainly in polymorphonuclear leukocytes and mucosal fluids (19). Iron scavenging caused by these proteins reduces the free-iron concentration below 10^{-12} μM (9), which is much lower than the iron concentration required for bacterial growth (0.05 to 0.5 μM).

To overcome this deficiency, bacterial pathogens have developed iron uptake mechanisms. The genes involved in these mechanisms are usually regulated by the availability of iron in that they are repressed in the presence of iron (11). In many bacterial species, these mechanisms are based on the synthesis and secretion of small compounds usually of <1,000 Da, called siderophores, which display high affinity for ferric iron (FeIII). More than 200 natural siderophores have been described. The majority of them are either hydroxamates or catecholates-phenolates. They are capable of removing transferrin- or lactoferrin-bound iron to form ferrisiderophore complexes which in turn are recognized by specific iron-repressible membrane receptors and internalized into the bacterium where the iron is released (11). This iron uptake mechanism has been described in many bacterial species, such as *Vibrio anguillarum* (11), *Escherichia coli* (16), *Klebsiella pneumoniae* (29), and *Pseudomonas aeruginosa* (30).

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Other pathogens, such as *Neisseria gonorrhoeae* (20, 27), *Neisseria meningitidis* (35, 37), *Haemophilus influenzae* type b (26, 34), *Actinobacillus pleuropneumoniae* (12), and *Pasteurella haemolytica* (28), do not secrete detectable siderophores when grown in an iron-deficient environment but produce outer membrane proteins that bind directly and specifically to lactoferrin or transferrin, thereby allowing iron transport into the bacterial cell. The molecular mechanisms which govern this type of iron uptake are not yet elucidated.

Bordetella pertussis, the causative agent of whooping cough, and *Bordetella bronchiseptica*, the etiologic agent of swine atrophic rhinitis and kennel cough, are gram-negative bacilli that adhere to and colonize the epithelium of the upper respiratory tract (13, 38). The capacity of these organisms to colonize the mucosal surface of the upper respiratory tract of vertebrates would imply that they also have developed iron uptake mechanisms allowing growth in the infected host. Redhead et al. (31) have shown that *B. pertussis* was able to grow in vitro, when the only iron available was bound to ovotransferrin, transferrin, or lactoferrin. In their study, neither hydroxamate nor catecholate-phenolate siderophores were detected, raising the possibility that *B. pertussis* is able to acquire iron through direct interaction with iron-saturated transferrins. In contrast, Gorringe et al. (14) and Agiato and Dyer (1) were able to show that *B. pertussis* grown in iron-deficient medium produced hydroxamate siderophores. In addition, the siderophore secretion was found to be abolished when the free-iron-deficient medium was supplemented with iron-saturated human transferrin (14). These results may suggest that *B. pertussis* possesses two mechanisms for iron uptake, one based on hydroxamate siderophore secretion and the other involving a direct interaction with the host iron-binding proteins.

In this study, we show that both *B. pertussis* and *B. bronchiseptica* produce iron-repressible proteins (IRPs), some of which are outer membrane proteins (IROMPs). In addition, we show that one of these IRPs for each species can be purified by affinity chromatography by using immobilized iron-saturated transferrins or iron-chelated Sepharose.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Streptomycin-resistant (Sm^r) *B. pertussis* Tohama I (3) and *B. bronchiseptica* NL1015 (4) were described earlier. Sm^r *B. bronchiseptica* NL1015 was a spontaneous streptomycin-resistant strain, generated by plating *B. bronchiseptica* NL1015 on Bordet-Gengou agar (7) supplemented with 20% defibrinated sheep blood (BG agar) and containing 100 μg of streptomycin per ml (Sigma).

The different *Bordetella* organisms were grown at 36°C for 3 days on BG agar supplemented with 100 μg of streptomycin per ml. A starter culture was initiated by transferring 10 to 20 isolated and hemolytic colonies to 25 ml of Stainer and Scholte (SS) medium (36) containing 10 μg of $FeSO_4 \cdot 7H_2O$ per ml (SS+Fe). When the optical density at 550 nm (OD_{550}) reached 3.0, 5 ml of the starter culture was used to inoculate 500-ml cultures in 2-liter flasks (large scale) or 1 ml was used to inoculate 100 ml in 250-ml flasks (small scale). All cultures were grown either in SS+Fe or in SS−Fe (SS medium without added $FeSO_4 \cdot 7H_2O$), corresponding to iron-replete and iron-deficient growth conditions, respectively. When the growth reached the stationary phase, the cells were harvested by centrifugation and stored at −80°C until use. The iron chelator 2-2′-dipyridyl (2-2′-DPD; Sigma) was used at various concentrations in SS−Fe to further deplete free iron in the iron-deficient culture condition. The 2-2′-DPD stock solution was prepared at 0.5 M in 95% ethanol and kept at 4°C until use. When indicated, iron-free transferrin (apotransferrin), iron-saturated transferrin, or iron-saturated lactoferrin, all of human origin and purchased from Sigma, were added to the small-scale cultures either free in the medium or contained within a dialysis bag (X cellulose casing; Union Carbide) with a molecular mass cutoff of 6 to 8 kDa for proteins. At the completion of the experiments, analysis of the culture medium after removing the dialysis bags showed no detectable transferrins in the medium, indicating that no leakage of the transferrins from the dialysis bags occurred during culturing. When indicated, 5 mM nicotinic acid (Sigma) was added to the culture medium to induce phenotypic modulation (21).

Covalent coupling of transferrins to agarose beads. Transferrins were coupled to Affi-Gel 15 (Bio-Rad Laboratories, Richmond, Calif.) by spontaneous reaction of the ligand primary amino groups with *N*-hydroxysuccinimide ester groups immobilized on the gel essentially as described by Carlsson et al. (10). Briefly, 5 ml of gel was washed with 200 ml of cold, deionized water and resuspended in 10 ml of 100 mM 3-morpholinepropanesulfonic acid (MOPS) (pH 7.5) containing 50 mg of ligand. Coupling was performed overnight at 4°C. The remaining active ester groups were then blocked by the addition of 1 ml of 1 M glycine in 100 mM MOPS (pH 7.5) and the incubation of the gel for 1 h at room temperature. The coupling efficiency ranged between 95 and 100% for all of the ligands used in this study.

Preparation of iron-chelated Sepharose. A 20-ml volume of Chelating Sepharose Fast-Flow (Pharmacia, Uppsala, Sweden) was packed into a 2-cm-diameter column and washed with 300 ml of deionized water. A 100-ml volume of 50 mM $FeCl_3 \cdot 6H_2O$ (FeIII) was circulated overnight through the gel at a flow rate of 1 ml/min. The gel was then washed with 250 ml of deionized water, equilibrated with 150 ml of phosphate-buffered saline (PBS), and stored at 4°C until use.

Affinity chromatography on transferrin-agarose. *B. pertussis* and *B. bronchiseptica* cells from 500-ml cultures were resuspended in 15 ml of sterile, distilled water, and sonicated three times for 3 min at 4°C by using an MSE sonicator delivering a peak-to-peak amplitude of 28 μm. Unlysed cells and cellular debris were removed by centrifugation at 3,000 × *g* for 20 min. A 1-ml volume of DNase I (Sigma) at a concentration of 5 mg/ml was added to the supernatant which was then incubated for 1 h at 37°C. A 3-ml volume of streptomycin at a concentration of 20 mg/ml was added, and the sample volume was adjusted to 500 ml with PBS. The sample was finally applied at a flow rate of 1 ml/min onto a 1.5-cm-diameter column containing 5 ml of transferrin-agarose equilibrated with 150 ml of PBS. The gel was then washed with PBS until the OD_{280}, monitored by a 2510 Uvicord SD (LKB, Bromma, Sweden), reached the baseline. Elution was performed by a stepwise increase of the NaCl molarity. The eluted proteins were collected in 1-ml fractions and stored at −80°C until further analysis.

Immobilized metal affinity chromatography. *B. pertussis* and *B. bronchiseptica* cell extracts from 500-ml cultures were prepared by using the procedure described above. The extracts were then applied at a flow rate of 1 ml/min onto a 1.5-cm-diameter column containing 10 ml of iron-chelated Sepharose equilibrated with 150 ml of PBS. Elution was performed by a stepwise increase of the NaCl concentration. The eluted material was collected in 1-ml fractions and stored at −80°C until use.

SDS-PAGE analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (18) by using a 4% stacking gel and a 12% separating gel. Samples were mixed with one-third volume of solubilization buffer (6% SDS, 15% 2-mercaptoethanol, 30% glycerol, and 0.005% bromophenol blue in 0.18 M Tris-HCl [pH 6.8]) and, in the case of whole cells, submitted to five cycles of freeze-thawing (−80°C, +95°C) before electrophoresis. After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 (Bio-Rad).

Outer membrane preparations. The method described by Schneider and Parker (33) was used for the outer membrane preparations. Briefly, fresh cells from 500-ml cultures were resuspended in 10 ml of 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4) and disrupted by sonication as described above. After removal of unlysed cells and cellular debris by centrifugation, the supernatant was centrifuged at 107,000 × *g* for 1 h at 4°C, and the pellet was carefully resuspended in 20 ml of 50 mM HEPES (pH 7.4) containing 7.5 mM $MgCl_2$ and 2% Triton X-100. The suspension was kept for 30 min at 20°C and then centrifuged at 107,000 × *g* for 1 h at 4°C. The pellet enriched in outer membrane proteins was finally solubilized in bidistilled water and stored in 1-ml fractions at −80°C until further analysis.

Protein assay. Protein concentrations were determined by the method of Bradford (8) by using bovine serum albumin as a standard.

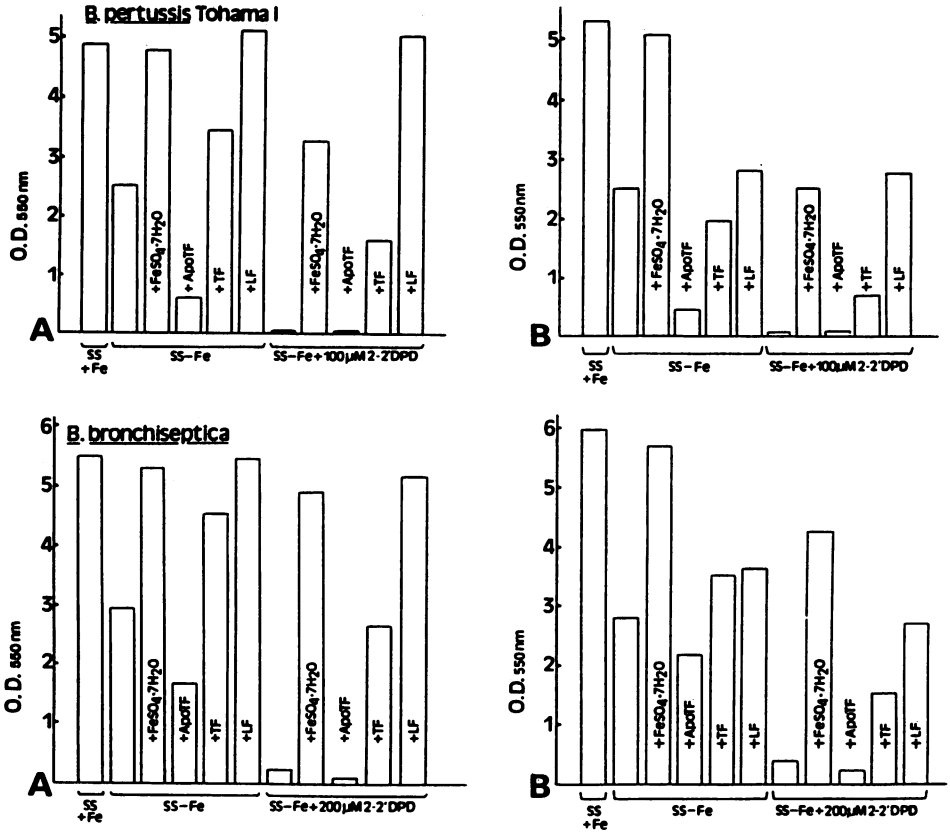

FIG. 1. Growth of *B. pertussis* and *B. bronchiseptica* in SS medium under different conditions of iron limitation. *B. pertussis* Tohama I (upper panels) or *B. bronchiseptica* (lower panels) were grown in SS+Fe, SS−Fe, or SS−Fe + 2-2′-DPD as indicated. Cultures grown in SS−Fe or in SS−Fe + 2-2′-DPD were supplemented with either 10 μg of $FeSO_4 \cdot 7H_2O$, 25 mg of apotransferrin (ApoTF), iron-saturated transferrin (TF), or iron-saturated lactoferrin (LF), as indicated. These iron-binding proteins were either free in the culture medium (panels A) or sequestered in dialysis bags (panels B). Results are expressed as OD_{550} values after 62 h of growth at 36°C in 100 ml of culture medium and are means of duplicate cultures started from independent precultures. Beyond 62 h of incubation, no further growth was detected under any of the growth conditions. Instead, after 62 h, OD values reflected mostly dead cells and cell debris.

RESULTS

Effect of iron restriction on the growth of *B. pertussis* and *B. bronchiseptica*. The effect of iron restriction on the growth of *B. pertussis* and *B. bronchiseptica* was examined by culturing both organisms in SS+Fe or in SS−Fe. For both organisms, growth in SS−Fe was reduced by ca. 50% in comparison to growth in SS+Fe, as determined by OD_{550} measurements at the stationary phase of the cultures (Fig. 1A). To demonstrate that this reduction was due to the limitation of iron availability, $FeSO_4 \cdot 7H_2O$ was added at a final concentration of 10 μg/ml to cultures started in SS−Fe. In these conditions, growth of both organisms was completely restored, indicating that the growth limitation observed in SS−Fe was due to the low iron availability.

Since *Bordetella* organisms are usually not in contact with free iron during natural infection, the capacity of *B. pertussis* and *B. bronchiseptica* to acquire transferrin- or lactoferrin-bound iron was investigated. Cultures grown in SS−Fe in the presence of 250 μg of iron-saturated human transferrin or lactoferrin per ml as a unique source of exogenous iron showed partial growth restoration in the presence of transferrin, whereas lactoferrin induced growth levels comparable to those of the control culture grown in SS+Fe. This suggests that *Bordetella* spp. are able to acquire and use transferrin- and lactoferrin-bound iron. Under the same conditions, apotransferrin failed to restore bacterial growth and had a bacteriostatic effect for both *Bordetella* organisms, since the OD_{550} values observed for the apotransferrin-containing cultures were reduced by 76 and 44% for *B. pertussis* and *B. bronchiseptica*, respectively. This inhibitory effect may be attributed to the chelator and prooxidant activities of apotransferrin (15).

When iron restriction was further increased by the addition of 2-2′-DPD to SS−Fe, growth of *B. pertussis* and *B. bronchiseptica* was completely inhibited by 100 and 200 μM 2-2′-DPD, respectively. This observation suggests that *B. pertussis* presents a higher iron requirement for in vitro growth than *B. bronchiseptica*. Again, iron in the form of $FeSO_4$ or bound to transferrin or lactoferrin was able to restore growth, whereas apotransferrin did not, indicating that the growth inhibition observed in the presence of 2-2′-DPD was due to its capacity to withhold iron from *Bordetella* spp.

To investigate whether direct contact between transferrin or lactoferrin and *Bordetella* cells was necessary for iron acquisition from these iron-transport proteins, cultures were started in 100 ml of SS−Fe containing transferrins, except that the transferrins (0.25 mg/ml) were added within dialysis bags (Fig. 1B). For both bacteria, transferrins in dialysis bags were able to restore growth in comparison with cultures

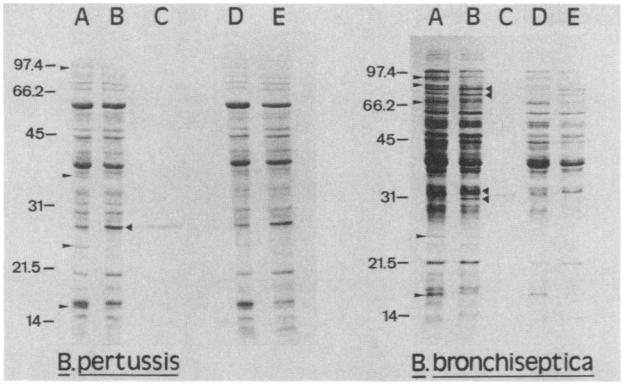

FIG. 2. SDS-PAGE analyses of *B. pertussis* and *B. bronchiseptica* WCLs from organisms grown in SS+Fe (A), SS−Fe (B), SS+Fe + nicotinic acid (D), and SS−Fe + nicotinic acid (E). Lanes C show the *Bordetella* proteins eluted by 0.5 M NaCl from lactoferrin-agarose. Small arrowheads (►) indicate iron-inducible proteins and large arrowheads (▶) indicate iron-repressible proteins. Lanes corresponding to WCLs were loaded with 100 μg of protein, and those corresponding to the purified material were loaded with 1 μg. Molecular mass markers expressed in kilodaltons are given in the margin.

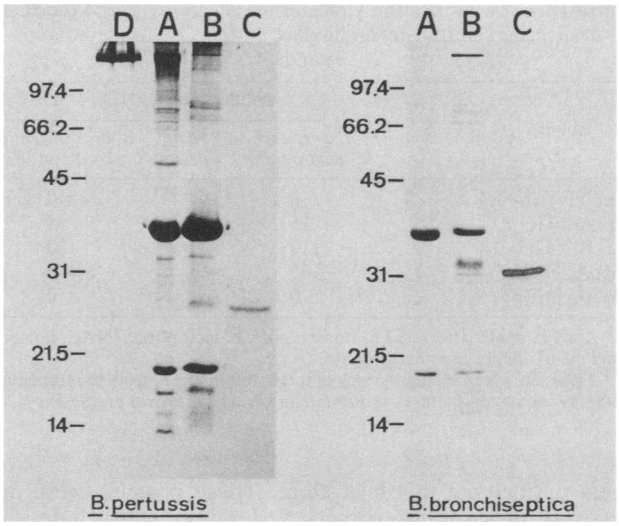

FIG. 3. SDS-PAGE analyses of *B. pertussis* (left panel) and *B. bronchiseptica* (right panel) outer membrane proteins from bacteria grown in SS+Fe (A) or in SS−Fe (B). Lanes C show the proteins eluted by 0.5 M NaCl from FeIII-chelated agarose. Lane D shows *B. pertussis* filamentous hemagglutinin purified on heparin Sepharose (22). Molecular mass markers expressed in kilodaltons are given in the margin.

grown in SS−Fe. However growth was found to be lower under these conditions than in cultures grown in the presence of the transferrins free in the medium. For *B. pertussis* grown in SS−Fe, the lactoferrin- and transferrin-induced growth restorations were reduced by 45 and 43%, respectively, when these proteins were within dialysis bags instead of being free in the culture medium. In the case of *B. bronchiseptica*, growth restoration was reduced by 23 and 33% in the presence of transferrin and lactoferrin, respectively, in dialysis bags. Empty dialysis bags had no effect on the bacterial growth (data not shown).

Similar observations were made in the presence of 2-2′-DPD, suggesting that direct contact between *B. pertussis* or *B. bronchiseptica* and iron-saturated transferrins improves the bacterial growth in iron-deficient medium but is not essential to stimulate growth. The hypothesis that the hydroxamate siderophores have difficulty passing through the dialysis bag to retrieve the iron from the transferrins is unlikely because of the high cutoff (6 to 8 kDa) relative to the size of the siderophores (<1 kDa), although it cannot be ruled out.

Effect of iron restriction on the protein profiles of *B. pertussis* and *B. bronchiseptica*. The responses of *B. pertussis* and *B. bronchiseptica* to iron restriction was studied by SDS-PAGE analysis of whole-cell lysates (WCL), prepared by several cycles of freeze-thawing, and outer membrane preparations from cells grown in SS+Fe and SS−Fe.

The WCL protein profiles of *B. pertussis* grown in these conditions (Fig. 2, left panel, lanes A and B) showed several differences. The production of at least four proteins of 16, 23.5, 36.5, and 92.5 kDa was decreased in iron-deficient medium and hence are called iron-inducible proteins (IIPs), whereas a protein of 27 kDa was produced at higher levels in SS−Fe and is called iron-repressible protein (IRP). When *B. pertussis* was grown in modulating conditions, i.e., in the presence of nicotinic acid (Fig. 2, left panel, lanes D and E), these iron-regulated proteins exhibited the same expression pattern, suggesting that their genes are not under the control of the general virulence regulon.

Analysis of the SDS-PAGE profiles of outer membrane proteins of *B. pertussis* grown in SS+Fe or in SS−Fe (Fig. 3, left panel, lanes A and B) revealed that iron restriction was associated with the overproduction of at least two proteins of 27 and 70.5 kDa and the repression of at least five proteins of 13, 17, 23.5, 47, and 220 kDa. The 220-kDa protein comigrated with the filamentous hemagglutinin (Fig. 3, lane D) purified on heparin Sepharose by methods described earlier (22).

Similar analyses with *B. bronchiseptica* WCL (Fig. 2, right panel, lanes A and B) showed IIPs of 17, 23.5, 70, 84, and 91 kDa and IRPs of 30, 32, 73.5, and 79.5 kDa. The amounts of most of these proteins were not modified when *B. bronchiseptica* was grown in modulating conditions (Fig. 2, right panel, lanes D and E), except for the 30-kDa IRP whose production appeared to be lower in the presence of nicotinic acid. The SDS-PAGE profile of *B. bronchiseptica* outer membrane preparations showed that the 32-kDa IRP comigrated with an outer membrane protein (Fig. 3, right panel, lane B).

Interaction of the *B. pertussis* 27-kDa and the *B. bronchiseptica* 32-kDa IRPs with iron-saturated transferrins. As *B. pertussis* and *B. bronchiseptica* were able to acquire transferrin-bound iron, WCLs of both species were analyzed by chromatography on transferrin- and lactoferrin-agarose in order to detect any specific binding activity. After the samples were loaded onto Affi-Gel-bound transferrins, the gels were washed sequentially with PBS containing 0.1, 0.2, 0.5, or 1 M NaCl. The eluted material was analyzed by SDS-PAGE. This analysis revealed the elution by 0.5 M NaCl of apparently homogeneous 27- and 32-kDa proteins purified from *B. pertussis* and *B. bronchiseptica* WCLs, respectively (Fig. 2, lanes C, and Fig. 3, lanes C). Both proteins appeared pink on Coomassie blue-stained gels. At ionic strengths higher than 0.5 M NaCl and up to 3 M NaCl, no detectable material eluted further from the column.

Table 1 indicates the amounts of transferrin-binding pro-

TABLE 1. Purification yields of the *B. pertussis* and the *B. bronchiseptica* transferrin-binding proteins by using different ligands

Column[a]	Purification yield (μg)[b]	
	B. pertussis 27-kDa protein	*B. bronchiseptica* 32-kDa protein
ApoTF Affi-Gel	0	0
TF Affi-Gel	15	35
LF Affi-Gel	110	120
IMAC FeIII	165	405
IMAC (no Fe)	0	0

[a] ApoTF, apotransferrin; TF, transferrin; LF, lactoferrin; IMAC, immobilized metal affinity chromatography.
[b] Yields are given in micrograms of total protein eluted from the respective columns for 500-ml cultures of *Bordetella* organisms grown in SS−Fe.

teins purified by this procedure. These proteins were not retained on apotransferrin-coupled affinity gels. Under the chromatographic conditions employed, lactoferrin appeared to be a stronger ligand than transferrin for both proteins, since the purification yields of the 27- and 32-kDa proteins were, respectively, 7.3 and 3.4 times higher when lactoferrin was used as the ligand. This may reflect differences in either the immobilization of the ligands to the matrix or in the relative affinity of the 27- and 32-kDa proteins to the transferrins, consistent with the observation that lactoferrin was more efficient than transferrin in restoring *Bordetella* growth in iron-restricted medium.

The electrophoretic mobilities of the purified proteins suggest identity with the 27- and 32-kDa IROMPs of *B. pertussis* and *B. bronchiseptica*. Consistent with this observation, the yield of these proteins purified from *B. pertussis* or *B. bronchiseptica* grown in SS+Fe was substantially lower than that of these organisms grown in SS−Fe (data not shown).

Interaction of the *B. pertussis* and *B. bronchiseptica* transferrin-binding proteins with iron. The affinity chromatography experiments described above indicated that the *B. pertussis* and the *B. bronchiseptica* transferrin-binding proteins were able to bind to the transferrins only when they were iron saturated. To investigate a potential direct role of iron in the interaction between transferrins and the 27- and 32-kDa proteins, iron-chelated Sepharose was used for chromatography of WCLs from *B. pertussis* and *B. bronchiseptica*. After the application of the WCLs and the wash with PBS, the bound material was eluted by sequential application of PBS containing 0.2, 0.5, or 1 M NaCl. The 27- and 32-kDa proteins were again eluted in PBS + 0.5 M NaCl, and SDS-PAGE analysis showed that these proteins were the major components of the PBS + 0.5 M NaCl elution peaks. No proteins were eluted from immobilized metal affinity chromatography columns without iron. The purification yields are reported in Table 1.

To confirm that the 27- and 32-kDa proteins purified on transferrin-agarose were identical to the proteins of the same M_r purified on iron-chelated agarose, sequential chromatography steps were carried out on WCLs of *B. pertussis* and *B. bronchiseptica*. First the WCLs were loaded onto transferrin-agarose. The eluted fractions containing the 27- and 32-kDa proteins were then diluted 10-fold with PBS to decrease the salt concentration and applied onto iron-chelated Sepharose. SDS-PAGE analysis of the fraction eluted by 0.5 M NaCl conclusively showed that the proteins purified by the two methods were indeed identical (Fig. 4). Both

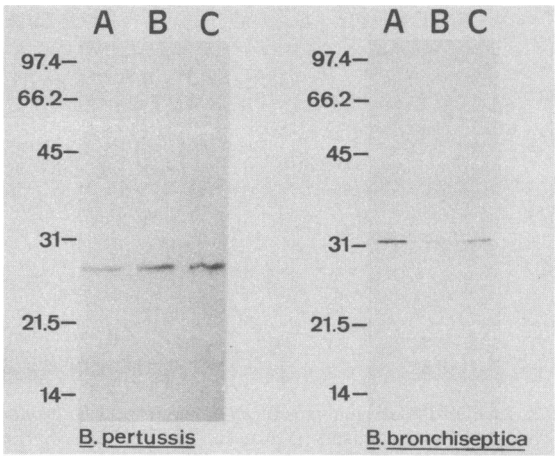

FIG. 4. SDS-PAGE analyses of *B. pertussis* (left panel) and *B. bronchiseptica* (right panel) proteins retained on iron-saturated transferrin (A), FeIII-chelated agarose (B), or iron-saturated transferrin-agarose and FeIII-chelated agarose (C). Molecular mass markers expressed in kilodaltons are given in the margin.

proteins could also be eluted by 50 mM cysteine or 100 mM histidine in PBS (data not shown).

DISCUSSION

To establish infection as respiratory pathogens, *Bordetella* organisms have to acquire iron from iron-sequestering proteins of the mucosal surfaces of the upper respiratory tract. When cultivated in iron-deficient medium containing iron-saturated human lactoferrin or transferrin as a unique source of iron, *B. pertussis* and *B. bronchiseptica* grew as well as they did in iron-replete medium, indicating that both organisms were able to acquire iron from these iron-binding glycoproteins. These findings are in agreement with the observation reported by Redhead and coworkers (31) concerning the ability of *B. pertussis* to grow in vitro under free-iron-restricted conditions but in the presence of ovotransferrin, human transferrin, or human lactoferrin. For both organisms, iron uptake from iron-saturated lactoferrin appeared to be more efficient than that from iron-saturated transferrin, consistent with lactoferrin being the predominant iron-binding protein of mucosal surfaces (2).

Furthermore, direct contact between the transferrins and the *Bordetella* cells, although not essential, significantly stimulated growth, since both lactoferrin and transferrin when sequestered in dialysis bags enhanced growth to a lesser extent than when they were free in the medium. However, the observation that both *B. pertussis* and *B. bronchiseptica* were, nevertheless, able to grow even when the transferrins as a unique iron source were placed in dialysis bags suggests that they were also able to take up iron from these proteins without direct contact. This mechanism could involve hydroxamate siderophores which have been detected in supernatants from iron-restricted cultures of *B. pertussis*, *B. bronchiseptica*, and *Bordetella parapertussis* (1, 14). These observations, therefore, suggest that *B. pertussis* and *B. bronchiseptica* possess two different mechanisms for iron uptake from transferrins, one siderophore mediated and one involving direct contact of the bacteria with transferrins.

Growth of both *B. pertussis* and *B. bronchiseptica* in iron-restricted medium caused increased production of sev-

eral IRPs as well as decreased production of IIPs, indicating that these organisms regulate gene expression as a function of the available free iron. These findings suggest that iron is an important environmental factor capable of modulating gene expression in members of the genus *Bordetella*.

The outer membrane protein profiles of *B. pertussis* and *B. bronchiseptica* grown in the presence or in the absence of added iron showed that some of these IRPs are associated with the outer membrane and hence can be called IROMPs. Such proteins could be involved in iron uptake.

One IRP for each species, i.e., a 27-kDa protein and a 32-kDa protein from *B. pertussis* and *B. bronchiseptica*, respectively, exhibited marked affinity for iron-saturated transferrins. These proteins had molecular masses similar to those of the 27- and 32-kDa IROMPs. Furthermore, they appeared pink in Coomassie blue-stained gels, as did the two IROMPs, suggesting that the purified proteins may be these IROMPs. The development of specific immunological reagents directed against the 27- and 32-kDa proteins should help to resolve this question.

The interaction between the 27- and 32-kDa IRPs and transferrins was observed only with immobilized iron-saturated transferrins and not with apotransferrins, suggesting that iron is important in this molecular recognition. The involvement of iron in this interaction was further supported by the direct binding of the 27- and 32-kDa proteins with ferric iron immobilized on agarose. Moreover, both IRPs could also be eluted from iron-chelated agarose by competition with histidine or cysteine, two amino acids known to be involved in protein-iron interactions (32). Similar elution properties have been reported for the purification of other iron-binding proteins (39).

At present, the physiological role of these transferrin-binding proteins is unknown, but it is tempting to speculate that they may serve as receptors for transferrins in *B. pertussis* and *B. bronchiseptica*. This hypothesis may be further supported by the observation of Redhead and coworkers (31) who showed by immunoblotting experiments that the *B. pertussis* cell surface was able to bind ovotransferrin. However, these authors did not describe the nature of such interactions. *B. pertussis* may thus be able to use various iron-binding proteins as an iron source.

Since the 27- and 32-kDa IRPs bind to both lactoferrin and transferrin, this specificity of interaction appears to be broader than that of, for example, the *A. pleuropneumoniae* (12) or the *P. haemolytica* (28) transferrin receptors, which are specific for porcine and bovine transferrins, respectively. In addition, while such receptors exhibit an M_r of about 70,000 or more, the *Bordetella* transferrin-binding IRPs are of much lower M_r. It is not clear yet whether this difference is the result of proteolytic degradation or whether the *Bordetella* proteins are encoded by shorter open reading frames.

Alternatively, the *Bordetella* IRPs identified in this study could be the functional analog of the *N. gonorrhoeae* iron-binding protein (Fbp), previously called major iron-regulated protein, with an M_r of 37,000 (6). All three proteins stain poorly with Coomassie blue (24). In *Neisseria* spp., this protein is thought to be involved in iron uptake as a central iron-binding protein (23). However, it is not known whether the Fbp can also bind iron-saturated transferrins.

As in other bacterial pathogens, many virulence factors of *Bordetella* organisms are coordinately regulated (25). The expression of the *B. pertussis* virulence genes, such as those coding for pertussis toxin, filamentous hemagglutinin, or hemolysin-adenylate cyclase, is under the control of a central genetic transactivating locus, designated *vir* or *bvg* (5, 41). This transactivation can be modulated by environmental signals, such as temperature, nicotinic acid, and magnesium sulfate (17, 21). The production of the transferrin-binding proteins from *B. pertussis* and *B. bronchiseptica* described in this study do not appear to be regulated by the *vir* locus, since the production of these proteins was unaffected by such modulators. If these proteins are subsequently identified as virulence factors, this may reflect the existence of a second, *vir*-independent genetic regulation of *Bordetella* virulence genes.

Cloning of the structural genes of the *Bordetella* IRPs, currently being carried out in our laboratory, should help to further characterize these proteins and assess their functional roles in iron uptake and virulence.

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