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Identification, Characterization, and Immunogenicity of the Lactoferrin-Binding Protein from *Helicobacter pylori*

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Iron acquisition plays an important role in bacterial virulence. Different studies have been initiated to define the mechanism by which *Helicobacter pylori* acquires iron. We had previously demonstrated that human lactoferrin (HLf) supported full growth of the bacteria in media lacking other iron sources. The ability of *H. pylori* to use HLf as an iron source had been found to be dependent on cell-to-protein contact. Since lactoferrin has been found in significant amounts in human stomach resection specimens from patients with superficial or atrophic gastritis, the iron uptake of *H. pylori* via a specific HLf receptor may play a major role in the virulence of *H. pylori* infection. In this study, by using affinity chromatography with biotinylated HLf and streptavidin-agarose, we identified a 70-kDa lactoferrin-binding protein (Lbp) from outer membrane proteins of *H. pylori*. This Lbp was only present when *H. pylori* was grown in an iron-starved medium, suggesting that it serves in iron uptake. Direct binding assays with increasing concentrations of biotinylated HLf demonstrated that the lactoferrin interaction with the outer membrane of *H. pylori* grown in iron-restricted medium was saturable. Competitive binding experiments with bovine and human lactoferrin and with transferrin of horse, bovine, and human origin indicated that this Lbp appeared highly specific for HLf. A number of other studies have focused on the importance of transferrin and lactoferrin receptors in pathogenic bacteria and their specificity with the host species. This observation might explain the very strict human specificity of *H. pylori*.

There is now considerable evidence suggesting that the ability of human pathogens to cause diseases is directly related to their ability to obtain iron. Therefore, efficient iron acquisition is thought to be an important virulence factor (11, 33). Iron, an essential nutrient for bacterial growth (5), is not readily available in the extracellular compartment of the human host. It is sequestered by transferrin in the systemic circulation and extracellular compartments and by lactoferrin on the mucosal surfaces (7). Pathogenic bacteria have to adapt to this iron-limiting environment by developing highly specific and effective iron assimilation systems. Most of them synthesize and secrete siderophores, low-molecular-weight compounds which compete with the host iron-binding proteins and shuttle iron back to the bacterial cells (24). Other bacteria are able to sequester iron directly from transferrin and lactoferrin through direct binding to a specific receptor on the outer membrane (26). These include the pathogenic species of the genus *Neisseria* (17, 23), *Haemophilus influenzae* (29), *Pasteurella haemolytica* (25), *Actinobacillus pleuropneumoniae* (12), and *Bordetella pertussis* (22). The surface receptors for transferrin and lactoferrin of human pathogens such as *Neisseria meningitidis* and *Neisseria gonorrhoeae* have a strong specificity for the human forms of these proteins (17, 31). This phenomenon occurs with other pathogens that possess iron uptake systems utilizing surface receptors for transferrin in other host species; thus, the porcine pathogens *A. pleuropneumoniae* and *Actinobacillus suis* only interact with porcine transferrin and *P. haemolytica*, a representative bovine pathogen, only binds bovine transferrin (30).

Helicobacter pylori, a gram-negative spiral bacterium, specifically colonizes the gastric epithelium of humans. The reasons for this tissue tropism are unclear; the tropism may be due to the attachment to the host epithelium by the bacterium. *H. pylori* is now recognized as the major etiologic agent of chronic active gastritis and is generally accepted as having a causative role in the pathogenesis of gastric and duodenal ulcers, gastric adenocarcinoma, and lymphoma (21, 27). For all pathogenic bacterial infections, four distinct events are essential for successful colonization: transmission to the new host, targeting to the preferred site of colonization, survival at the selected site, and efficient metabolism and division. For *H. pylori*, each of these events is associated with a series of environmental obstacles that are unique to the gastric mucosal surface. The gastric epithelium is bathed in mucus, which is constantly flowing, mechanically cleaning the tissue surface. The passage of food will increase this mechanical disturbance. To overcome these shearing actions, *H. pylori* crosses the mucus layer with the help of three to seven flagella (9) and then adheres to the underlying tissue (3, 10). To survive in the acidic microenvironment of the stomach, *H. pylori* has urease activity, considered the major virulence factor (8).

Iron is particularly depleted at the mucosal surfaces, and, as for all bacteria, iron is an essential growth factor for *H. pylori*. Few reports have appeared in the literature concerning the iron uptake system of *H. pylori*. We reported that human lactoferrin and heme supported full growth of *H. pylori* in media lacking other iron sources and that siderophore production by this bacterium was not detected by chrome azurol S assays (14). In contrast, Illingworth et al. detected siderophore production in response to low iron availability by chrome azurol S assays (15). However, the siderophore(s) has not yet been identified. In another report, Worst et al. showed that *H. pylori* synthesized three iron-repressive outer membrane proteins (IROMPs) (77, 50, and 48 kDa) which might be involved in heme binding and/or uptake (34). In each of these three reports, several IROMPs were described but only certain features were shown to be definitely identical since the procedures were different.

In this paper, we demonstrate the presence of a human

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lactoferrin-binding protein (Lbp) in *H. pylori* and characterize its ligand specificities, properties that may contribute to the host specificity of this bacterium, and its immunogenicity in *H. pylori*-infected patients.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Six different strains were studied: *H. pylori* NCTC 11637^T, *H. pylori* CIP 104086, and four clinical strains of *H. pylori*. The organisms were grown on Columbia agar plates (Difco Laboratories, Detroit, Mich.) supplemented with 10% horse blood in a microaerobic atmosphere at 37°C. Freshly grown cells were used to inoculate liquid brain heart infusion (BHI) supplemented with 7% fetal calf serum, with BHI plus 25 μM desferrioxamine (desferrioxamine mesylate; Ciba-GEIGY Pharmaceutical Co.), or with BHI plus 25 μM desferrioxamine and 60 μM $FeSO_4$ to a starting A_{590} of 0.2. Bacteria were incubated with shaking for 72 h prior to harvest.

Chemicals. Human lactoferrin and transferrin and bovine lactoferrin and transferrin were a gift from G. Spik (Laboratoire de Chimie-Biologie, Faculté des Sciences, Villeneuve d'Ascq, France). Horse transferrin was obtained from Sigma Chemical Co., St. Louis, Mo. Horseradish peroxidase-conjugated streptavidin (HRP-streptavidin), streptavidin-agarose, and biotin-X-NHS [sulfosuccinimidyl-6-(biotamido)hexanoate] were obtained from Pierce Immunotechnology (Rockford, Ill.). Peroxidase-conjugated rabbit anti-human immunoglobulin G was obtained from Bio-Rad (Paris, France).

Sera. Serum samples were collected from patients examined at the gastroenterology unit of the Claude Huriez Hospital, Lille, France. Ten sera from patients with *H. pylori*-positive cultures and three from patients with *H. pylori*-negative cultures were studied. Sera were controlled by a qualitative enzyme-linked immunosorbent assay method against major *H. pylori* antigens immobilized on a strip (Helicoblot kit; Genelabs Diagnostic, Singapore).

Biotinylation of HLf. Human lactoferrin (HLf) was biotinylated according to the manufacturer's instructions. Briefly, 20 mg of HLf was dissolved in 1 ml of sodium bicarbonate buffer, pH 8.5. An aliquot of 0.4 mg of biotin-X-NHS was directly added to the protein, and the mixture was incubated for 2 h on ice and then was incubated for 30 min at room temperature. The unbound biotin was removed by ultrafiltration with a Centricon-30 microconcentrator (Amicon Corporation, Danvers, Mass.). The biotinylated HLf was divided into aliquots and stored at −20°C.

Preparation of OMPs. Bacterial cells were harvested by centrifugation and washed three times in phosphate-buffered saline (PBS), pH 7.4. The pellet was suspended in ice-cold PBS containing 5% (wt/vol) of CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate) (Sigma Chemical Co.). Bacteria were eliminated by centrifugation, and the supernatant containing the OMPs was ultracentrifuged at 100,000 × *g* for 1 h. The pellet was air dried, solubilized in sample buffer, divided into aliquots, and frozen at −20°C. The level of contamination of OMPs with inner membrane proteins was determined, along with the succinate dehydrogenase activity (32).

Direct binding assays. Ten microliters of OMP suspension (1 mg/ml) was directly applied to nitrocellulose paper (0.45-μm-pore-size paper; Amersham, Cleveland, Ohio) with a slot blot apparatus and was blocked after air drying with 5% bovine serum albumin and 0.1% Tween 20 in PBS (pH 7.4) at 37°C for 2 h. The membrane was washed with buffer, placed in the slot blot apparatus, and then incubated with different concentrations of biotinylated HLf at 37°C. After three new washings, HRP-streptavidin, diluted 1/5,000 in blocking solution, was added to the blot. After a 1-h incubation at room temperature, the blot was revealed with an enhanced chemiluminescence kit (Amersham). The results were converted to optical density at 570 nm with a densitometer (Sebia, Paris, France).

Competitive binding assays. Iron-limited OMPs were immobilized on nitrocellulose paper as described above. Competitive binding assays were performed with mixtures containing biotinylated HLf (1 μg/ml) and different unconjugated iron-binding proteins (500 μg/ml). The binding mixture was incubated at 37°C for 1 h. The paper was washed three times with PBS. The blocking and revelation procedures were identical to those described above.

Batch affinity isolation of HLf-binding protein. Biotinylated HLf (20 μg) was mixed with 0.2 mg of OMPs in 1 ml of 50 mM Tris hydrochloride–100 mM NaCl (pH 8.0) buffer and incubated with gentle agitation for 1 h at 37°C. Proteins were pelleted by centrifugation at 16,000 × *g* for 30 min in an Eppendorf microcentrifuge. They were resuspended in 1 ml of buffer and incubated with 100 μl of a 1/2 dilution of streptavidin-agarose for 1 h at room temperature. The mixture was centrifuged at 750 × *g* for 3 min, and the supernatant was removed. The affinity pellet was washed three times with buffer supplemented with 100 mM guanidine-HCl then twice with sample buffer before being suspended in 50 μl of solubilization buffer (16) and boiled for 5 min. Ten microliters was used in sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (SDS–7.5% PAGE).

SDS-PAGE and Western blotting. Electrophoresis of proteins was routinely performed by SDS-PAGE with a discontinuous gel system using a Tris-HCl–glycine buffer system (16). The gels were stained with silver nitrate. To study immunological properties or lactoferrin-binding capacity, proteins were electrophoretically transferred onto nitrocellulose paper using a Bio-Rad Transblot apparatus at 100 V for 1 h at 4°C in 25 mM Tris-HCl buffer (pH 8.3) containing 0.2 M glycine and 20% (vol/vol) methanol. The blocking solution consisted of PBS (pH 7.4) supplemented with 5% bovine serum albumin and 0.01% Tween 20. Blots were incubated in serum (1/100 dilution in PBS) from *H. pylori*-infected or -noninfected patients. After three washings, the paper was incubated for 1 h with peroxidase-conjugated anti-human immunoglobulin G (1/5,000 dilution). Alternatively, blots were incubated with biotinylated HLf and then with HRP-streptavidin (1/1,000 dilution). These papers were then developed with 0.4% (wt/vol) diaminobenzidine and 1% hydrogen peroxide for 10 min.

Protein concentrations were determined by the method of Lowry et al. (19), with bovine serum albumin as standard.

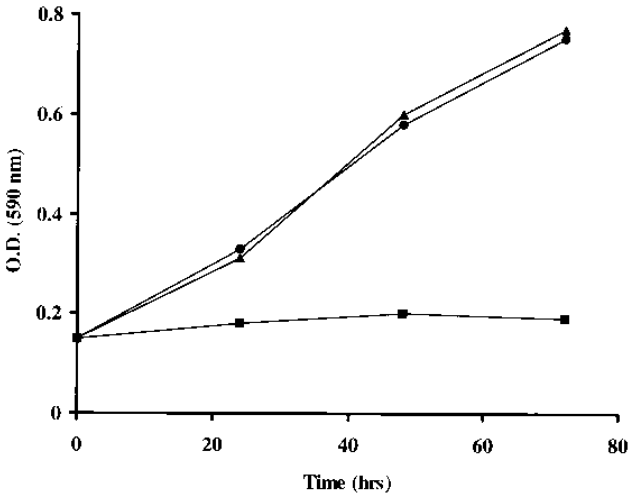

FIG. 1. Growth of *H. pylori* in iron-sufficient and iron-deficient conditions. A 48-h culture of *H. pylori* strain NCTC 11637^T was used to inoculate BHI (▲), BHI containing 25 μM desferrioxamine (■), or BHI containing 25 μM desferrioxamine plus 60 μM $FeSO_4$ (●). O.D., optical density.

RESULTS

Bacterial growth and IROMPs. As shown in Fig. 1, *H. pylori* did not grow in the presence of 25 μM desferrioxamine and growth was restored when 60 μM $FeSO_4$ was added. This data demonstrated that iron was the limiting factor. IROMPs were expressed in response to iron starvation at 129, 70, 56, 48, 41, 37, and 31 kDa as shown by SDS-PAGE analysis (Fig. 2, lane a).

Detection, regulation, and specificity of HLf-binding activity. The exclusive use of HLf for growth of *H. pylori* (14) suggested that iron acquisition would be mediated by a surface Lbp. We thus used a binding assay that required the labelling of HLf with biotin. The biotinylated HLf binding was detected with HRP-streptavidin. To determine whether the HLf-binding activity was iron regulated, *H. pylori* was grown in iron-restricted BHI containing 25 μM desferrioxamine. Ten micrograms of OMPs from *H. pylori* grown in iron-depleted or -repleted medium was spotted on nitrocellulose sheets and incubated with concentrations of biotinylated HLf ranging from 0.156 to 2.5 μg/ml. As shown in Fig. 3, growth in medium containing desferrioxamine resulted in significant levels of detectable HLf-binding activity. When an excess of $FeSO_4$ (60 μM) was added to the chelator to obtain iron-sufficient conditions, HLf binding was highly reduced; this result demonstrated that the induction of HLf-binding protein expression was due to the effective withholding of iron. The HLf binding on the OMPs was saturable since HLf binding on OMPs prepared from cells grown in iron-starved medium increased with the concentrations of biotinylated ligand and reached a maximum at 1.25 μg/ml (Fig. 3).

In order to assess the specificity of the HLf-binding protein, a competitive binding assay with various iron-binding proteins

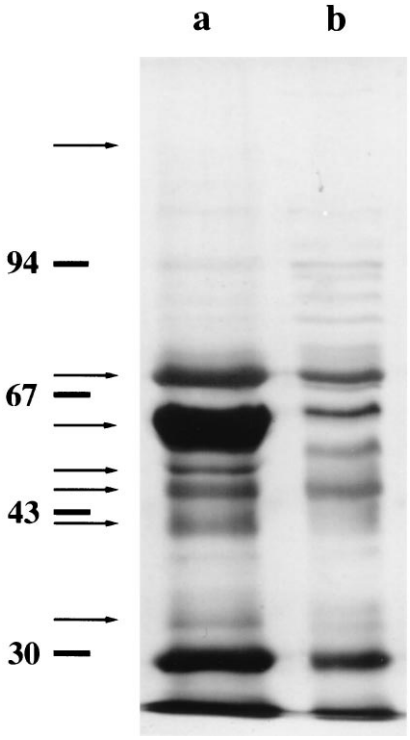

FIG. 2. IROMPs of *H. pylori* strain NCTC 11637^T. Lane a, membrane extract from cells grown in iron-deficient BHI containing 25 μM desferrioxamine; lane b, membrane extract from cells grown in iron-sufficient BHI containing 25 μM desferrioxamine and 60 μM $FeSO_4$. Proteins were extracted as described in Materials and Methods, separated by SDS–7.5% PAGE, and stained with silver nitrate. Numbers refer to the molecular masses (kDa) of the standard proteins, and arrows indicate the positions of the IROMPs.

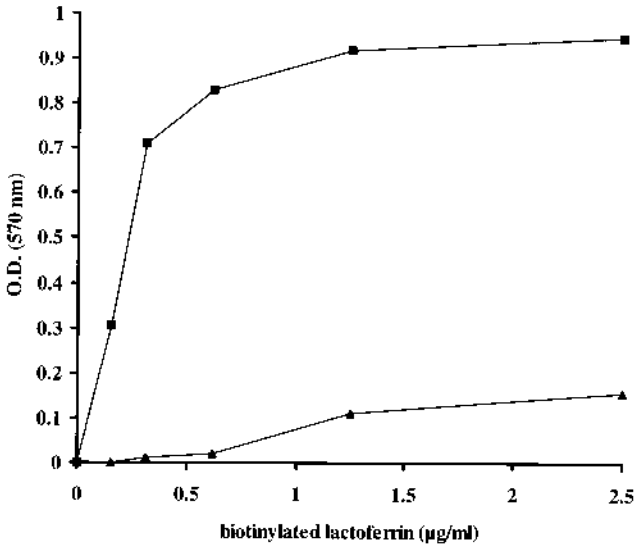

FIG. 3. Saturability of the HLf-binding protein in *H. pylori* NCTC 11637^T. Iron-starved (■) and -sufficient (▲) OMPs were spotted onto nitrocellulose and exposed to 200 μl of biotinylated HLf (0.15, 0.31, 0.62, 1.25, and 2.5 μg/ml) in the slot blot apparatus. The paper was washed and incubated with HRP-streptavidin. Then, the blot was developed with the chemiluminescent HRP substrate. The results were converted to optical densities (O.D.) at 570 nm with a densitometer.

was performed; the proteins included human and bovine lactoferrin, human and bovine transferrin, and horse transferrin. An analysis of the results revealed that the *H. pylori* Lbp was highly specific for HLf. Only HLf was able to compete with human biotinylated lactoferrin. Inhibition of the biotinylated HLf with unconjugated HLf was found to be 92%. A partial inhibition by bovine lactoferrin was observed. This protein inhibited the HLf interaction by 35%. No such inhibition could be detected when human, bovine, and horse transferrins were used. These results were reproducible and statistically significant since they were means of three experiments.

Identification of the HLf-binding protein. To identify the protein(s) involved in the specific binding of HLf to iron-deficient OMPs, affinity chromatography with biotinylated HLf and streptavidin-agarose was used. The results obtained with *H. pylori* NCTC 11637^T by the affinity purification procedure are illustrated in Fig. 4A. When biotinylated HLf was used with OMPs from iron-deficient cells (Fig. 4, lane a), a 70-kDa protein was isolated. In comparison with SDS-PAGE analysis of iron-deficient and iron-sufficient OMPs (Fig. 1), it appeared that this isolated protein was an IROMP. This was supported by the low quantities of the protein in SDS-PAGE gels from iron-sufficient OMPs and the absence of the protein after the affinity chromatography procedure (Fig. 4, lane b). When biotinylated HLf was omitted from the affinity purification mixture (data not shown), the protein could not be isolated, indicating that specific binding to HLf was involved.

This 70-kDa Lbp was not detected in electroblots of OMPs exposed to biotinylated HLf and HRP-streptavidin (data not shown).

To compare Lbps among different *H. pylori* strains, the reference strains NCTC 11637^T and CIP 104086 and four clinical isolates were subjected to affinity isolation. The 70-kDa Lbp was isolated from all strains.

Immunogenicity of the Lbp. The immunogenicity of the Lbp was determined by immunoblotting (Fig. 4B). The 70-kDa Lbp obtained by affinity chromatography was subjected to SDS–7.5% PAGE, and the blot was transferred onto nitrocellulose membrane. After being blocked, the blot was incubated with different sera (1/100 dilution). Sera were collected from 10 *H. pylori*-infected patients (2 with gastritis, 7 with duodenal ulcers, and 1 with a gastric adenocarcinoma) and from 3 noninfected patients used as controls. All *H. pylori*-positive sera gave the same reactivity against the 70-kDa protein (Fig. 4, lane c). This protein reacted only slightly with the three *H. pylori*-negative sera (Fig. 4, lane d). This data demonstrated that the Lbp from *H. pylori* was expressed in vivo.

DISCUSSION

Different studies have been initiated to define the mechanism by which *H. pylori* acquires iron. We had previously demonstrated that heme or HLf supported full growth of the bacteria in media lacking other iron sources (14). This mechanism is common to several pathogens that are able to sequester iron directly from heme, hemoglobin, transferrin, or lactoferrin. The uptake is thought to take place via specific receptors. Worst et al. (34) described three heme-binding IROMPs of 77, 50, and 48 kDa that might be involved in the uptake of heme. Here, we identified an Lbp of 70 kDa. This Lbp was expressed more when *H. pylori* was grown in iron-starved medium (Fig. 2), suggesting its involvement in iron acquisition. The 70-kDa Lbp was not detected from electroblots of OMPs exposed to biotinylated HLf and HRP-streptavidin (data not shown). It is probable that this protein lost its lactoferrin-binding properties when exposed to denaturing conditions, such as those used for SDS-PAGE. This phenomenon was also described for the transferrin-binding protein (Tbp) TbpA from *Neisseriaceae* (2,

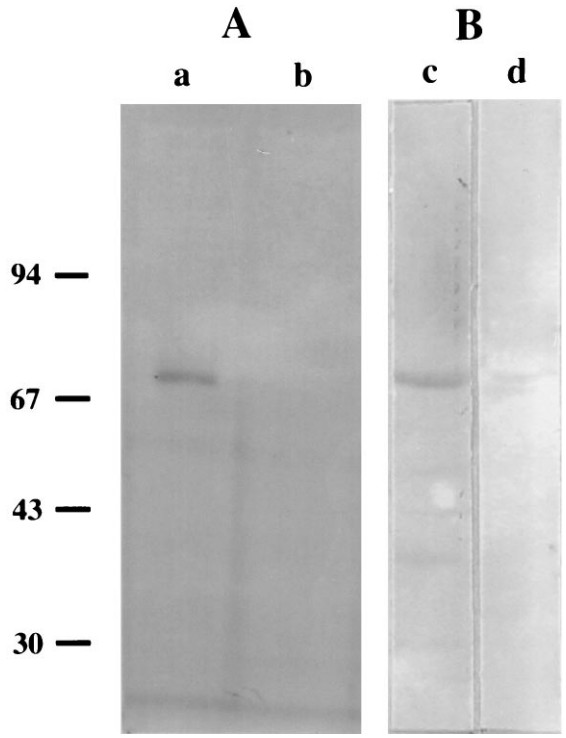

FIG. 4. Identification and immunogenicity of the HLf-binding protein in *H. pylori* NCTC 11637^T. (A) The HLf-binding protein was isolated by affinity chromatography of OMPs on biotinylated HLf from iron-starved (lane a) and -sufficient (lane b) *H. pylori* cultures. Proteins were analyzed by SDS-7.5% PAGE followed by silver staining of the gel. (B) Immunoblots of the Lbp obtained as described above. Lane c, infected patient serum; lane d, noninfected patient serum. Numbers refer to the molecular masses (kDa) of the standard proteins.

4). No direct binding of transferrin and lactoferrin was demonstrated by Illingworth et al. (15) in electroblots of OMPs from iron-starved cells exposed to HRP-conjugated glycoproteins. We demonstrated that in response to iron limitation by desferrioxamine, *H. pylori* expressed seven proteins with molecular masses of 129, 70, 56, 48, 45, 42, and 31 kDa (Fig. 2). Among these, three IROMPs with molecular masses of 48, 37, and 31 kDa were described by Worst et al. (34), who used as iron-restrictive conditions a medium supplemented by the addition of 20% (vol/vol) heat-inactivated newborn calf serum which contained iron-binding proteins such as transferrin, albumin, and no free iron. The 41-kDa protein was also described by Illingworth et al. (15).

Bacterial Lbp mediating iron uptake from lactoferrin were identified in a number of different human pathogens: *N. meningitidis* (4, 23, 31), *N. gonorrhoeae* (17), *H. influenzae* (29), *B. pertussis* (22), and *Moraxella catarrhalis* (4). Among these, only *B. pertussis* was able to produce siderophores (1). For *N. meningitidis*, *N. gonorrhoeae*, and *B. pertussis*, HLf serves as a physiological source of iron: like *H. pylori* they grew as well in iron-deficient medium containing iron-saturated lactoferrin as a unique source of iron as they did in iron-replete medium. Single Lbps were described for *N. gonorrhoeae* (31), *H. influenzae* (29), and *B. pertussis* (22) with molecular masses of 101, 105, and 27 kDa, respectively, while two Lbps, 100- to 105-kDa LbpA and 65- to 85-kDa LbpB were identified for *M. catarrhalis* and *N. meningitidis* (4). The loss of the 105-kDa Lbp in *N. meningitidis* was correlated with the inability of the bacterium to bind lactoferrin used as an iron source (28). Gray-Owen and Schryvers (13) postulated that LbpB might facilitate iron removal from lactoferrin by altering the glycoprotein conformation. The bacterial binding proteins of high molecular weight for lactoferrin and transferrin show extensive homologies and also some homologies to heme receptors and to iron siderophore receptors of *Escherichia coli* (6, 18). The 70-kDa Lbp isolated from *H. pylori* resembles low-molecular-weight LbpBs described for *N. meningitidis*, *M. catarrhalis*, and *Moraxella bovis* (4). First, they are very similar in apparent molecular weight on the basis of SDS-PAGE analysis; and second, as in *Neisseria* species (2, 4), the 70-kDa Lbp could not bind human lactoferrin after SDS-PAGE and electroblotting. Further investigations may provide insight on homologies between the Lbp from *H. pylori* described here and low-molecular-weight LbpBs described for other pathogens.

From direct binding assays with increasing concentrations of biotinylated HLf, we demonstrated that the lactoferrin interaction with OMPs of *H. pylori* was saturable in high concentrations (Fig. 3). This property is a characteristic of a ligand-receptor interaction. To study the specificity of this interaction for HLf, competitive binding assays were performed against iron-starved OMPs. The binding of HLf was specific for this ligand since it was inhibited by itself and not by human, bovine, or horse transferrin. A partial inhibition was observed with bovine lactoferrin, although this protein was not used as an iron source by *H. pylori* (14). Such a discrepancy between the lactoferrin-binding activity of bacteria and their ability to obtain iron from lactoferrin has been described for other pathogens (17). Conformational homologies between human and bovine lactoferrin might explain this partial inhibition. The specificity of the Lbp for human glycoprotein might explain the very strict host specificity of this pathogen. Numerous studies have focused on the importance of Tbps in pathogenic bacteria and their specificity for the host species. Using the representative pathogens *N. meningitidis* (human), *P. haemolytica* (bovine), and *A. pleuropneumoniae* (porcine), they demonstrated that these pathogens possess Tbp specific for human, bovine, and porcine transferrin, respectively. These bacteria can only effectively use the transferrin from the host as a source of iron for growth (30). Lbps in *N. meningitidis* and *N. gonorrhoaea* are also specific for human lactoferrin (17).

IROMPs expressed in pathogens have attracted considerable attention over recent years. The acquisition of iron from transferrin and/or lactoferrin is probably essential for the establishment and dissemination of most bacterial infections. It was demonstrated that the meningococcal Tbps, TbpA and TbpB, had vaccine potential (2). Like these Tbps, the Lbp of *H. pylori* identified in this study has a number of features that make it an ideal candidate for vaccine development. First, our study suggests that *H. pylori* might be dependent upon Lbp for iron uptake in vivo. In the course of the pathogenesis, *H. pylori* first colonizes the gastric mucosal epithelium; this is followed by invasion across the mucosal barrier. Lactoferrin is thought to be the primary iron source on the mucosal surface, where it can retain its iron-binding properties under extreme conditions of pH (it is stable at pHs of $\geq$4). Second, there is evidence for the in vivo production of a 70-kDa protein which is immunogenic.

Since lactoferrin has been found in significant amounts in human stomach resection specimens from patients with superficial or atrophic gastritis (20), the iron uptake of *H. pylori* via a specific HLf-binding protein may play a major role in the pathogenicity of *H. pylori* infection. Cloning the *H. pylori* Lbp gene, currently being carried out in our laboratory, should help to further characterize this protein and assess its functional roles in iron uptake and in virulence.

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