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NOTES

Iron-Repressible Outer Membrane Proteins of *Helicobacter pylori* Involved in Heme Uptake

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***Helicobacter pylori* is known to be a causative agent of gastritis and peptic ulcer disease in humans. The acquisition of iron from the human host may contribute greatly to the virulence of this organism. To study this, *H. pylori* was cultured under iron-restrictive conditions to induce synthesis of possible iron-regulated outer membrane proteins. This was achieved by the addition of 20% (vol/vol) heat-inactivated newborn calf serum, which contains iron-binding proteins like transferrin and albumin, and no free iron. The newborn calf serum was able to bind free ionic iron in brucella broth culture medium. Electrophoretic analysis of outer membrane preparations from *H. pylori* cultured under conditions of iron restriction showed several proteins to be present at elevated levels. These appeared to be iron-repressible outer membrane proteins (IROMPs). In addition, IROMPs with molecular sizes of 77, 50, and 48 kDa were isolated by use of hemin-agarose affinity chromatography. These three heme-binding IROMPs might be involved in the uptake of heme from the host and might therefore be important virulence factors of *H. pylori*.**

Helicobacter pylori is a recently discovered spiral, gram-negative bacterium that colonizes the mucus layer of the human stomach and duodenum (17, 23). Infection is strongly associated with type B antral gastritis and peptic ulcer disease and may be the initiation factor of gastric cancer (5). Several virulence factors of *H. pylori* have been described. The presence of urease, flagella, and adhesins and the production of cytotoxins and hemolysins are accepted as important determinants of pathogenicity (12, 23, 42).

As for all bacteria, iron is an essential growth factor for *H. pylori*. Although there is an abundance of iron in the extracellular body fluids, the free ionic concentration (10^{-18} M) is far too low to support the growth of most microorganisms (14). This is caused by the presence of the iron-binding glycoproteins transferrin, in serum, and lactoferrin, on mucosal surfaces (2, 14, 33). Therefore, for their survival, bacteria require a highly efficient iron acquisition system(s) in vivo which can compete with the iron-withholding system of the host (32, 34).

A common iron acquisition system present in many pathogens is the secretion of low-molecular-mass, high-affinity iron chelators (siderophores) which are able to remove iron from transferrin or lactoferrin (1, 9). Subsequently, specific outer membrane receptor proteins bind the iron-siderophore complex (6, 29). Alternatively, some important human and animal pathogens acquire transferrin- or lactoferrin-bound iron by a siderophore-independent process through direct binding to a specific receptor (25, 28). A large number of bacteria are able to utilize heme compounds as an iron source (22, 35, 37, 40) as a result of the presence of specific receptors which are involved in binding and uptake of heme from hemoglobin or haptoglobin-hemoglobin and hemopexin-heme complexes (8, 24, 31, 38, 43).

Husson et al. were not able to detect any siderophore production by chrome azurol assays with concentrated culture supernatants of 15 *H. pylori* strains, cultured under iron-limited conditions (19). Furthermore, neither human nor bovine transferrin was able to serve as the only iron source (19). *H. pylori* can, however, use human lactoferrin and heme but not bovine lactoferrin as an iron source (19). The lack of siderophore production (19), the need for heme as a growth factor (17), and the hemolytic activity of the bacterium (42), which may increase significantly the availability of heme, suggest the existence of a specific heme uptake system in *H. pylori*. The possession of such an iron-regulated heme uptake system would contribute greatly to the virulence of this microorganism.

In this study, we describe a simple method for the production of an iron-restrictive culture medium for *H. pylori*. We showed that several iron-repressible outer membrane proteins (IROMPs) are present at significantly higher levels under conditions of iron restriction and that three of these IROMPs might be involved in heme binding and/or uptake.

Iron restriction by addition of heat-inactivated serum. To study the production of iron-regulated outer membrane proteins by *H. pylori*, a culturing and determination method with respect to iron-restrictive growth conditions was developed. *H. pylori* ATCC 43504 was precultured for 48 to 72 h on blood agar plates (5% [vol/vol] horse blood) at 37°C under microaerophilic conditions. Liquid culture was performed in an Erlenmeyer flask with brucella broth (BB; Difco Laboratories, Detroit, Mich.) supplemented with 1 g of β-cyclodextrins (Sigma Chemical Co., St. Louis, Mo.) per liter as described by Olivieri et al. (30). The flasks were incubated in a microaerophilic environment on a rotary platform (60 rpm) for 72 h at 37°C. As standard iron chelators 2,2′-dipyridyl (BDH Chemicals) and deferoxamine mesylate (deferoxamine; Sigma) were used. With 2,2′-dipyridyl, we were not able to grow *H. pylori* at

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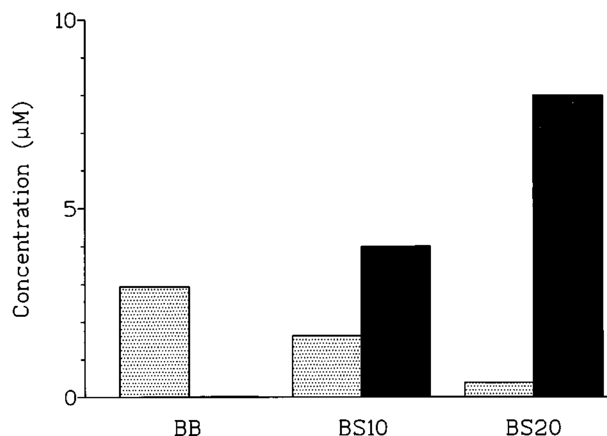

FIG. 1. Concentrations (μM) of free ionic iron (shaded bars) and heme iron (solid bars) in BB, BS10, and BS20.

all, and with deferoxamine, we obtained yields of bacteria that were too low to show a reproducible outer membrane pattern (see Fig. 4). Alternatively, it was inferred that iron restriction could be achieved by the addition of heat-inactivated (60°C) newborn calf serum (NBCS; Gibco Ltd., Paisley, Scotland) to BB. NBCS was used because bovine serum contains iron-binding proteins like transferrin and albumin, which cannot be used by *H. pylori* as a source of iron. For the determination of the amount of free ionic iron (Fe^{3+} and Fe^{2+}) in the media, a minor modification of the method described by Carter was used (7). Total proteins were precipitated with an equal volume of ice-cold acetone. The Fe^{3+} ions left in the supernatant are reduced to Fe^{2+} by the addition of 0.02% (wt/vol) ascorbic acid. The Fe^{2+} ions react with ferrozine to form a complex whose absorbance can be measured at 562 nm.

We could not detect any free iron in 100% NBCS. BB contained significant amounts of free ionic iron (2.94 μM) (Fig. 1). When NBCS was added to the medium, the amount of free ionic iron was reduced significantly. The free-iron concentration in BB with 20% (vol/vol) NBCS (BS20) was almost 10 times reduced compared with that in BB (0.39 μM). In BB with 10% (vol/vol) NBCS (BS10), we measured an ionic iron concentration of 1.63 μM. The free-iron concentration in BS20 was less than 1 μM, which is the minimum concentration most enteric bacteria require for their growth (33). It was therefore concluded that the BS20 medium might serve as an iron-restrictive growth medium for *H. pylori.*

Additionally, we determined the amount of heme compounds by a modified cyanomethemoglobin method (27). An equal volume of Drabkin's reagent (Sigma) was added to the culture medium, and the A_{540} was measured. Significant amounts of heme compounds could not be detected in BB. However, BS10 and BS20 contained significant amounts of these heme compounds, from which we calculated the total amounts of iron due to the presence of these heme molecules to be 4 and 8 μM, respectively (Fig. 1). Probably, this heme iron alone is available as an iron source for *H. pylori*, since in BS20 culture medium, only very low amounts of free ionic iron are present. Moreover, *H. pylori* is unable to use the transferrin-bound iron, present in NBCS, as an iron source (19).

Analysis of iron-repressible outer membrane proteins (IROMPs). To examine whether *H. pylori* synthesizes IROMPs, outer membrane proteins of cells cultured for 72 h in the iron-rich BB and iron-restricted BS20 media were isolated in the following way. Cells from 100-ml liquid cultures were harvested by centrifugation at 5,000 × *g*, washed twice in phosphate-buffered saline (PBS), and transferred to Eppendorf tubes. The cells were incubated with 1% (wt/vol) CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate} (Pierce Chemical Co., Rockford, Ill.)–12% (wt/vol) sucrose–50 mM sodium carbonate at 4°C for 30 min. Subsequently, 10% (wt/vol) sodium lauryl sarcosinate (Sarkosyl; CIBA-GEIGY Pharmaceutical Co.) was added to a final concentration of 5% (wt/vol), and the solubilized cells were incubated for another 30 min at room temperature. The tubes were centrifuged at 70,000 × *g* for 2 h, the outer membrane pellet was air dried and solubilized in 100 μl of sodium dodecyl sulfate (SDS) sample buffer (0.0625 M Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 10% [vol/vol] glycerol, 0.001% [wt/vol] bromophenol blue, 5% [vol/vol] β-mercaptoethanol). The level of contamination of the outer membrane preparation with inner membrane proteins was found to be less than 10% on the basis of succinate dehydrogenase activity determination (26). An SDS–12% polyacrylamide gel was loaded with 5 μl of solubilized outer membrane proteins (approximately 1 μg) per lane. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by use of the discontinuous buffer system of Laemmli (21), and the gels were stained with Coomassie brilliant blue.

Several outer membrane proteins with molecular sizes of approximately 89, 77, 50, 48, 37, 33, and 27 kDa were present at higher levels when isolated from bacteria cultured for 72 h

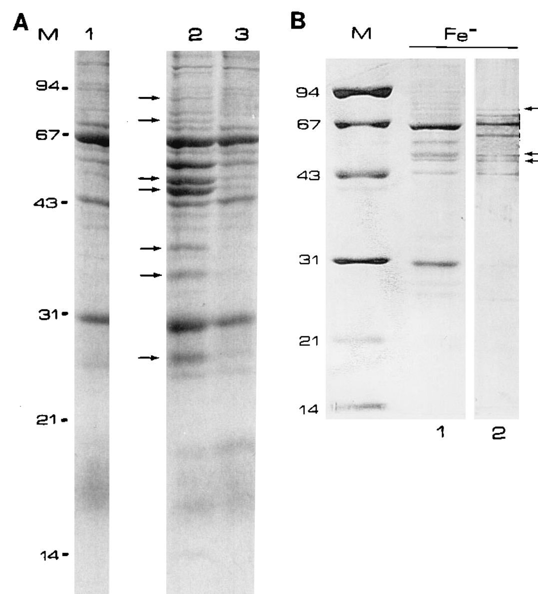

FIG. 2. (A) SDS-PAGE (12%) of outer membrane preparations of *H. pylori* ATCC 43504 grown for 72 h in iron-rich BB (lane 1), iron-restrictive BS20 (lane 2), and iron-replete BS20 with 10 μM Fe citrate (lane 3). Outer membrane material was loaded in equal amounts of 1 μg per lane. Sizes of molecular mass markers in kilodaltons are given on the left. Arrows indicate the positions of IROMPs. (B) SDS–12% PAGE of *H. pylori* proteins eluted with 1.5 M guanidine-HCl from hemin-agarose (lane 2). *H. pylori* cells were cultured under iron-restrictive conditions (BS20) and incubated microaerophilically with hemin-agarose for 3 h. Proteins were solubilized with 1% CHAPS, and the hemin-agarose was washed extensively. Elution was carried out with 0, 0.5, 1.0, 1.5, and 2.0 M guanidine-HCl. Only the 1.5 M guanidine-HCl fraction was found to contain protein. Lane 1 represents an outer membrane protein profile of *H. pylori* cultured in the iron-restrictive BS20 medium, in which synthesis of IROMPs is induced. Three IROMPs (77, 50, and 48 kDa) shown in lane 1 (and Fig. 2A, lane 2) appear to have molecular sizes identical to those of three hemin-binding proteins in lane 3 (indicated with arrows). Molecular size markers (M) in kilodaltons are shown on the left.

in iron-restrictive BS20 medium than when isolated from bacteria cultured in iron-rich BB medium (Fig. 2A, lanes 1 and 2). When an excess of Fe^{3+} (10 μM Fe citrate) was added to the BS20 cultures (iron repletion), these proteins were completely or partially repressed (Fig. 2A, lane 3). From these results, we concluded that production of these outer membrane proteins is iron repressible.

Although the yield of bacteria grown with deferoxamine was too low to be suitable for a reproducible outer membrane preparation (see Fig. 4), we have indications that the outer membrane profile from bacteria grown with deferoxamine is the same as that from bacteria cultured in BS20 (data not shown). The higher yield of bacteria and IROMPs was an important advantage for our future experiments, so we decided to use only BS20 as an iron-restrictive culture medium for *H. pylori.*

To determine whether the IROMPs of *H. pylori* shown in Fig. 2A, lane 2, are involved in binding and/or uptake of heme, hemin-agarose affinity chromatography was used. *H. pylori* was cultured in 50-ml portions of BS20 for 72 h at 37°C. The cells were washed twice in PBS, and the pellets were suspended in 0.5 ml of fresh BB medium. One hundred fifty microliters of hemin-agarose (Sigma) was washed twice with 1 ml of washing buffer (50 mM Tris-HCl, 100 mM NaCl [pH 8.0]). The cell suspension was incubated microaerophilically with the hemin-agarose at 37°C for 3 h. After three washes, the cells were solubilized by the addition of 150 μl of 1% CHAPS–50 mM sodium carbonate–12% sucrose and incubation at 4°C for 1 h. The hemin-agarose was again washed three times with 1 ml of washing buffer. Bound proteins were eluted batchwise with 0.5, 1.0, 1.5, and 2 M guanidine-HCl in 10-μl fractions and dialyzed. Subsequently, the fractions were analyzed by SDS-PAGE and silver staining. It appeared that proteins were eluted only with 1.5 M guanidine-HCl. This indicates that the heme-binding proteins, which might operate in a heme-binding complex, have a high affinity for heme. With SDS-PAGE and silver staining, polypeptides with molecular sizes of 77, 71, 66, 60, 50, 48, 44, 30, and 26 kDa were found in the 1.5 M guanidine-HCl-eluted fraction (Fig. 2B, lane 2). Three of these proteins had the same molecular sizes as three IROMPs of 77, 50, and 48 kDa (Fig. 2B, lane 1, and Fig. 2A, lane 2). These results suggest that these three IROMPs could be involved in the binding and uptake of heme as an alternative iron source.

To investigate the importance of expression of heme-binding IROMPs for *H. pylori* during infection, Western blots (immunoblots) of *H. pylori* IROMPs were incubated with sera from patients infected with *H. pylori*. Preliminary results show that the 48-, 50-, and 77-kDa heme-binding IROMPs are immunogenic and expressed in vivo (data not shown).

Growth of *H. pylori* in iron-restrictive media. We measured optical densities of *H. pylori* bacteria cultured for 72 h in BB with different concentrations of NBCS (Fig. 3). We also isolated outer membranes from these cultures to study IROMP expression. BB with 50% (vol/vol) NBCS (BS50), which is the most iron-restrictive medium, gave the lowest 72-h growth levels but the highest levels of IROMPs. The BS20 medium caused higher growth levels at 72 h, and the IROMP levels were slightly less than those in BS50. When 10% (vol/vol) NBCS was added (i.e., BS10), the bacteria showed the highest growth levels but the IROMP levels were moderate (Fig. 3). The better growth obtained in BS10 than in BS20 is probably caused by a lower iron retention in BS10. However, in BS10, IROMPs are present at moderate levels in spite of this higher concentration of free iron. The reason for this might be that the free-iron concentration (1.63 μM) (Fig. 1) exceeds the critical free-iron concentration for induction of IROMP synthesis. This suggests a similarity between *H. pylori* and most enteric bacteria, for which this critical free-iron concentration is 1 μM (33). The free-iron concentration in BS20 is below this critical value, so synthesis of IROMPs, necessary for iron uptake, is required by the microorganism. We concluded that the addition of 10% NBCS to BB results in a free-iron concentration which is roughly equal to the critical iron concentration required for expression of IROMPs in *H. pylori.*

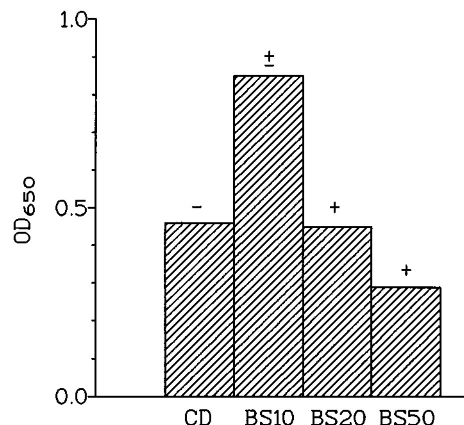

FIG. 3. Growth levels of *H. pylori* ATCC 43504 cultured for 72 h in BB supplemented with 0.1% (wt/vol) cyclodextrins (non-iron-restrictive control [CD]), in BS10, in BS20, or in BS50. Symbols above bars: +, high levels of IROMPs; ±, moderate levels of IROMPs; −, no IROMPs present (as determined after SDS-PAGE of outer membrane preparations). OD_{650}, optical density at 650 nm.

The higher growth rates obtained in BS10 than in BB are probably caused by the presence of additional growth factors from the serum added to BS10. Furthermore, in BS10, the synthesis of IROMPs by *H. pylori* could play a role in the uptake of heme or heme compounds from NBCS as an alternative iron source and as a growth factor. The growth-stimulating effect of heme and growth factors from NBCS in BS20 is probably sufficient to compensate for the growth inhibition

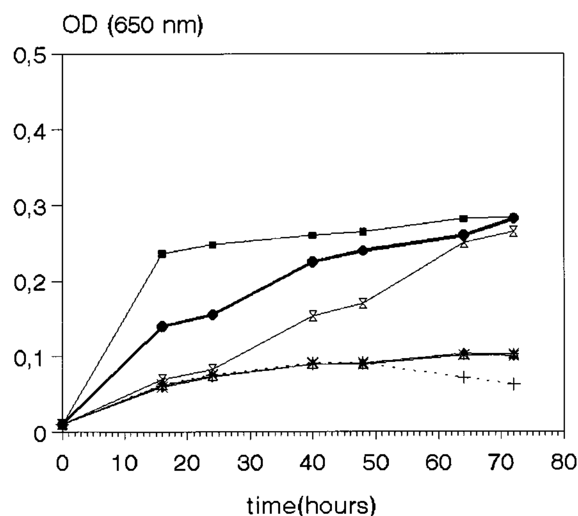

FIG. 4. Growth curves of *H. pylori* ATCC 43504 in BB supplemented with 75 μM deferoxamine (*), 300 μM deferoxamine (×), 750 μM deferoxamine (+), 300 μM deferoxamine plus 10 μM Fe citrate (△), 300 μM deferoxamine plus 10 μM Fe nitrate (◆), or 300 μM deferoxamine plus 10 μM hemoglobin (■) or unsupplemental (●) or in BS20 medium (⧖). OD, optical density.

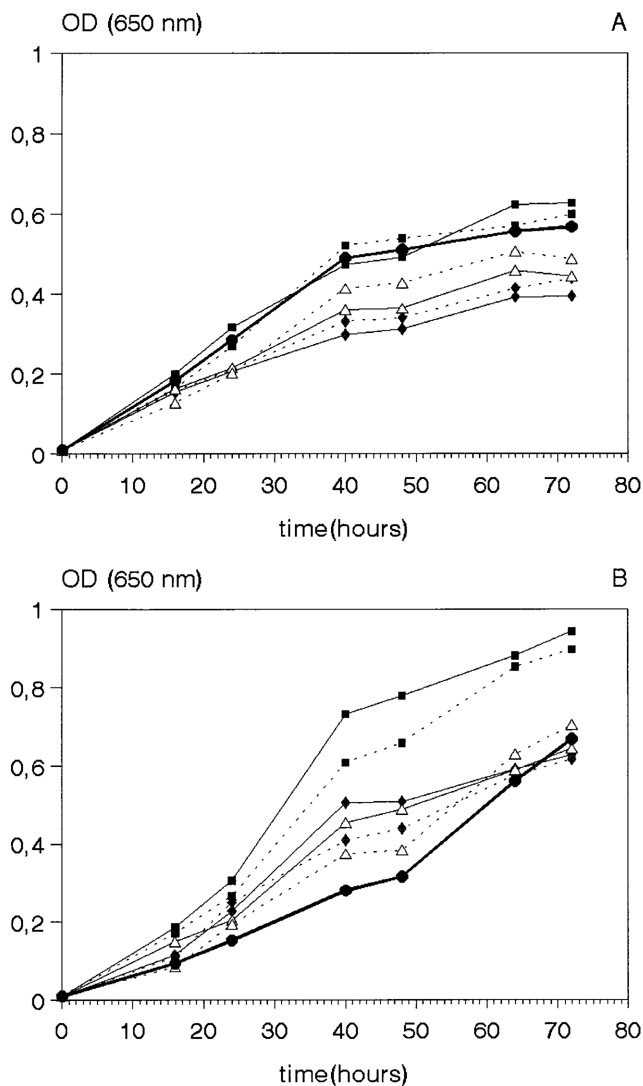

FIG. 5. Growth curves of *H. pylori* ATCC 43504 in non-iron-restrictive BB (A) and iron-restrictive BS20 (B). Media were either unsupplemented (●) or supplemented with 1 μM Fe citrate (– – △ – –), 10 μM Fe citrate (—△—), 1 μM Fe nitrate (– – ◆ – –), 10 μM Fe nitrate (—◆—), 1 μM hemoglobin (– – ■ – –), or 10 μM hemoglobin (—■—). OD, optical density. The maximally achieved growth levels of *H. pylori* cultured in BB-cyclodextrins and BS20 in the experiments shown in this figure differ from those of the experiments shown in Fig. 3 and 4. This can be explained by the fact that different experiments, in which the inocula and the initial conditions of the cells are variable, were performed. However, in all experiments, the same tendencies of growth rates were observed.

caused by iron restriction by that serum. As a result of this, the growth rate in BS20 remains as high as that in BB.

Very low growth levels compared with those in BB and BS20 were obtained with three concentrations of deferoxamine (Fig. 4). Supplementation of BB containing 300 μM deferoxamine with Fe citrate or Fe nitrate did not result in growth stimulation. However, supplementation with human hemoglobin showed a significant growth stimulation, especially during the first 16 h (Fig. 4). The reason for this could be that the supplemented free-iron molecules from Fe citrate and Fe nitrate are not available for *H. pylori* because of the strong chelating activity of deferoxamine. Deferoxamine cannot bind the supplemented hemoglobin, so hemoglobin can be used as an alternative iron source for *H. pylori* and will give rise to growth stimulation. The optical densities which are maximally achieved are the same as those achieved in BB and BS20.

There were no significant growth differences observed between unsupplemented BB medium and BB medium supplemented with Fe citrate, Fe nitrate, or hemoglobin at 1 and 10 μM concentrations (Fig. 5A). Apparently, sufficient iron is available from BB medium alone. The slightly lower optical densities caused by the iron complexes could be due to the formation of toxic oxygen radicals by the excess of Fe^{3+}.

Supplementation of the iron-restrictive BS20 culture medium with 10 μM Fe citrate or 10 μM Fe nitrate resulted in growth stimulation during the first 40 h (Fig. 5B). However, the optical densities measured at the 72-h time point were about the same as those for bacteria cultured in unsupplemented BS20 medium. It could be that only in these first 40 h is enough free ionic iron available to leak easily into the cell by passive diffusion (33) and stimulate growth. After this time point, the bacteria have probably used all of the available free iron. This results in a recovery of the initially achieved iron-restrictive environment. The iron of the 1 μM Fe citrate and Fe nitrate supplements is probably not sufficient to give rise to an initial growth stimulation such as that demonstrated by the 10 μM concentration. From the results shown in Fig. 1, it can already be seen that 10 μM free iron is enough to overcome the iron restriction due to the serum addition, which is about 3 μM. We used Fe citrate as an iron source because it has been shown already that Fe citrate can be used as the only iron source by *H. pylori* (19). To determine the influence of citrate on *H. pylori* growth, we also used Fe nitrate as an iron-containing supplement. There was no significant difference between these compounds regarding growth inhibition or stimulation. We conclude that the effects on growth are due completely to the iron.

The initial growth stimulation when the BS20 medium is supplemented with hemoglobin is even higher than that resulting from supplementation with Fe citrate or Fe nitrate. Interestingly, we also found that the addition of high amounts of hemoglobin did not result in a repression of IROMPs, whereas supplementation with Fe citrate and Fe nitrate did. Therefore, the expression of these proteins is only iron regulated and not heme regulated. *H. pylori* might use its IROMPs for uptake of heme iron from the medium itself (serum) and the supplemented hemoglobin. The uptake of heme iron with the IROMPs (iron restrictive) might be more efficient than the uptake of free iron without the IROMPs (non-iron restrictive). Also, uptake of the whole heme molecule under iron restriction could be more beneficial to the bacterium than uptake of free iron without iron restriction.

A heme uptake system could have great advantages for bacteria growing on mucosal surfaces like *H. pylori*. First, large amounts of heme compounds are present as a result of the desquamation of the epithelial cells (14). Second, heme-scavenging systems of the host mucosal cells are not very efficient (32). Last, *H. pylori* congregrates at the intercellular junctions of epithelial cells. By the production of hemolysins, heme compounds from erythrocytes in the bloodstream could be easily accessible because of leakage through these junctions (17, 41). In addition, the presence of cytotoxins and the high production of ammonia due to catalysis of urea from the stomach by urease (13) may cause tissue damage as a result of which leakage of heme compounds from the bloodstream increases and intracellular heme compounds are released.

Recently, genes encoding heme-binding and/or heme uptake proteins have been characterized for *Vibrio cholerae*, *Plesiomonas shigelloides*, *Haemophilus influenzae*, and *Yersinia enterocolitica* (10, 15, 16, 18, 38, 39). It was shown that heme uptake in *Y. enterocolitica* is a TonB-dependent process in which the

hemin receptor interacts with the TonB protein (20, 38). Also, a putative TonB-dependent heme receptor of 77 kDa was described in *V. cholerae* (18). To date, several iron-regulated, TonB-dependent receptors from different gram-negative bacteria have been described (3, 6). These receptors, which bind mainly iron-siderophore complexes, show some regions of high homology and molecular sizes between 70 and 85 kDa (3, 4, 36). The *H. pylori* 77-kDa heme-binding IROMP might also be such a TonB-dependent heme receptor when heme uptake in *H. pylori* is a TonB-dependent process. The 50- and 48-kDa IROMPs could be iron stress-related porins, since 51- and 48-kDa outer membrane proteins of *H. pylori* which are heat modifiable and show porin characteristics have been identified (11). Alternatively, these IROMPs could be part of a more specific heme uptake system and be involved in further uptake of the heme molecule.

The results of this study strongly suggest the presence of a heme uptake system in *H. pylori*. Some IROMPs are involved in the binding of heme. The 77-kDa IROMP, which is a potential candidate for the initial binding of heme or heme compounds, is currently under detailed investigation.

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