INFECTION AND IMMUNITY, June 1993, p. 2694–2697
0019-9567/93/062694-04$02.00/0

Vol. 61, No. 6

NOTES

Iron Acquisition by *Helicobacter pylori*: Importance of Human Lactoferrin

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Received 28 December 1992/Accepted 22 March 1993

***Helicobacter pylori* is known to be an etiologic agent of gastritis and peptic ulcer disease in humans. However, the mechanism by which this organism acquires iron has not been studied. For this investigation, *H. pylori* was grown in iron-restricted medium. Siderophore production was not detected by chemical assays, and the strains were unable to use enterochelin and pyochelin for growth in low-iron media. Human lactoferrin supported full growth of the bacteria in media lacking other iron sources, but neither human transferrin, bovine lactoferrin, nor hen ovotransferrin served as a source for iron. Since lactoferrin was found in significant amounts in human stomach resections with superficial or atrophic gastritis, the iron acquisition system of *H. pylori* by the human lactoferrin receptor system may play a major role in the virulence of *H. pylori* infection.**

Helicobacter pylori is a curved or spiral gram-negative microaerophilic bacterium which was first isolated from a human gastric biopsy specimen in 1983 (29). Since the first isolation, it has become apparent that this organism is an etiologic agent of gastritis and peptic ulcer disease in humans (1, 3, 6). A number of putative virulence factors, including hemagglutinins, flagella, cytotoxins, and urease, are thought to enable the survival of the organism in the gastric mucosa (1, 12, 23). Studies of various bacterial pathogens have established that the ability to acquire iron in vivo is also an important virulence factor (4, 30).

In the human body, extracellular Fe^{3+} is sequestered from microbes by binding to proteins such as lactoferrin and transferrin (30). Two major mechanisms by which bacteria remove iron from host compounds are described. The most thoroughly studied type of system is a siderophore-mediated iron acquisition system. This is commonly able to compete with lactoferrin and transferrin for iron. The iron-siderophore complex is then bound to a bacterial surface receptor and processed to remove the Fe^{3+} ion for use by the organism. This siderophore-mediated system has been described for *Escherichia coli*, *Pseudomonas aeruginosa*, and other bacteria (11, 20). Other microbes such as the pathogenic *Neisseria* spp. or *Haemophilus influenzae* possess iron acquisition systems utilizing surface receptors for host Fe-binding proteins (11, 13, 21). These pathogens bind transferrin and lactoferrin to distinct outer membrane receptors, and the iron is then removed from the protein.

This study was initiated to define the mechanism by which *H. pylori* acquires iron.

The reference strain *H. pylori* ATCC 43504 and 15 isolates from patients with histological gastritis were tested. Strains were maintained as stock cultures by freezing them at −70°C in brain heart infusion (BHI) broth (Difco Laboratories) containing 10% (vol/vol) glycerol. Cultures were grown on brain heart agar supplemented with 5% (vol/vol) fetal calf serum and incubated for 2 to 3 days at 37°C under microaerophilic conditions.

Iron-restricted growth was achieved by growing bacteria in BHI broth supplemented with the iron chelator ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDHA; Sigma Chemical Co., St. Louis, Mo.) to a final concentration of 150 μM, or in some experiments, the BHI broth was passed five or six times through a column of Chelex 100 ion-exchange resin (Bio-Rad Laboratories). In both cases, a growth factor supplement similar in composition to IsoVitaleX (BBL Microbiology Systems) was added, except that glucose and $Fe(NO_3)_3$ were omitted. The amount of residual Fe in Chelex 100-treated BHI broth (CBHI broth) was measured by atomic absorption with an atomic absorption spectrophotometer and was found to be lower than 0.1 μM. Disposable plasticware was used whenever possible to eliminate Fe contamination. All glassware was soaked overnight in 0.5% EDTA and rinsed extensively with distilled deionized water.

The method used to screen for siderophore production was described by Schwyn and Neilands (27). Cultures were incubated for 72 h at 37°C in a shaking incubator under a microaerophilic atmosphere. Uninoculated medium controls were also incubated. Concentrated culture supernatants were prepared by centrifugation at 12,000 × *g*, filtration through a 0.45-μm-pore-size membrane filter, and lyophilization. These supernatants were suspended in deionized water to give a 10-fold concentration and tested for the presence of siderophore. Desferal (desferrioxamine mesylate; CIBA-GEIGY Pharmaceutical Co.) was used as a positive control.

Pure human and bovine lactotransferrins were obtained in the native form (30% iron-saturated proteins) from pooled lactosera by ion-exchange chromatography by the methods of Spik et al. (28) and Chéron et al. (5), respectively. Human serum transferrin in the iron-free form was purchased from Behring (Marburg, Germany), and hen ovotransferrin (OTF) type IV from chicken egg white was purchased from Sigma.

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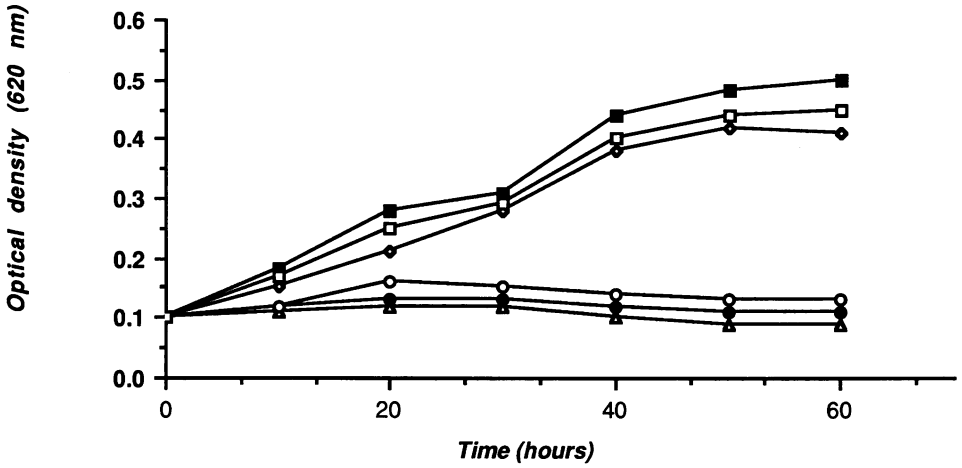

FIG. 1. Growth stimulation of iron-starved cells in CBHI broth by 10 μM 30% Fe-saturated HLF (□), 10 μM 30% Fe-saturated BLF (○), 10 μM 30% Fe-saturated HTF (●), 8 μM hemin (◇), 10 μM 30% ferric dicitrate (■), and no Fe (control) (△).

Both human transferrin (HTF) and OTF were saturated with 30% iron by adding a sufficient amount of 0.07 M $FeCl_3$ to the proteins in a solution of 0.1 M sodium citrate–0.1 M bicarbonate buffer, pH 8.2 (17). The excess of reagent was removed by gel filtration through a Sephadex G-25 column (20 by 3 cm). The protein concentrations were determined as described by Lowry et al. (15) with bovine serum albumin as the standard, and the amounts of iron were measured with sulfobathophenanthroline reagent (Biopack-fer; Biotrol, Paris, France).

Growth cultures in CBHI broth were prepared to determine which iron sources would support the growth of *H. pylori*. The reference strain and two medical strains were grown for 48 h on supplemented BHI broth. The cells were harvested by centrifugation at 4,000 × *g* for 15 min at room temperature and washed twice in fresh CBHI broth. They were then suspended in 20 ml of CBHI broth. A 5% inoculum was added to fresh CBHI broth, and various iron sources were added up to a final concentration of 10 μM Fe as follows: ferric dicitrate, 30% Fe-saturated human lactoferrin (HLF), 30% Fe-saturated HTF, 30% Fe-saturated bovine lactoferrin (BLF), 30% Fe-saturated OTF, and hemin. Growth was measured by optical density at 620 nm. Ferric dicitrate was made by combining sodium citrate and ferric chloride in a 10:1 molar ratio. All Fe sources were prepared immediately before use, filter sterilized, and added to the medium at a final concentration of 10 μM iron. In order to determine whether cell-to-protein contact was necessary for growth with HLF, HTF, or BLF, these proteins were sequestered inside Spectra/Por 1 dialysis tubing with a molecular mass cutoff of 6,000 to 8,000 Da. The tubing was sterilized by heating and closed; one end was sterilized and immersed in the culture medium, and the other was placed outside the medium at the mouth of the flask. Growth at 37°C in a rotary shaker under a microaerophilic atmosphere was monitored as described above. Growth stimulation of iron-starved cells was also measured with purified siderophores (enterochelin and pyochelin) at concentrations of 10 μM each.

H. pylori cells were fractionated to prepare outer membranes as previously described (2). After 72 h of incubation, cells were harvested in 20 mM Tris hydrochloride (pH 7.4) at 4°C and centrifuged twice at 10,000 × *g* for 10 min each time. They were sonicated on ice four times for 30 s each time with 20-s intervals by using a Branson Sonifier. The preparation was then centrifuged two times at 5,000 × *g* for 20 min each time to remove whole cells, and the supernatant was centrifuged for 1 h at 100,000 × *g* at 4°C. The resulting pellet was suspended in 3 ml of sterile distilled water, added to 20 ml of 1% sodium lauryl sarcosinate (Sarkosyl; CIBA-GEIGY) in 7 mM EDTA for a 20-min incubation at 37°C, and then recentrifuged at 100,000 × *g* for 2 h at 4°C. The pellet was suspended in this buffer and recentrifuged at 100,000 × *g* for 2 h, and the resultant Sarkosyl-insoluble pellet was suspended in 1.0 ml of sterile water and stored at 4°C. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using the discontinuous buffer system of Laemmli (14). The gels were stained with Coomassie brilliant blue.

To determine whether strains of *H. pylori* possessed siderophore-mediated iron acquisition systems, a simple screening assay for siderophore production was performed. Fifteen strains were grown in iron-limited broth medium, and supernatants were collected. None of these strains was able to produce siderophores, as revealed by chrome azurol assays; they never contained more siderophore activity than the uninoculated medium. Some bacterial species which fail to produce their own siderophores can use compounds secreted by other microbes. Therefore, growth stimulation of *H. pylori* in low-iron medium supplemented with enterochelin and pyochelin was tested. The presence of either enterochelin or pyochelin in the medium did not affect the growth of *H. pylori* (data not shown).

Since the assays did not reveal the presence of a siderophore-mediated iron transport system, other methods of iron acquisition were investigated. *H. pylori* was grown in iron-limited medium, and an important reduction in the final extent of growth was observed when *H. pylori* strains were grown in CBHI broth without addition of Fe. This growth limitation was reversible when ferric dicitrate or heme was added as a source of Fe (Fig. 1). The abilities of different lactoferrins and transferrins to support the growth of three iron-starved strains were also studied. The results were the same for all three strains. In the experiment with the reference strain (Fig. 1), in which the HLF-complexed iron-to-protein ratio was 0.30, the iron-deficient medium supported the growth of iron-starved *H. pylori* cells nearly as well as the medium containing an excess of ferric citrate did.

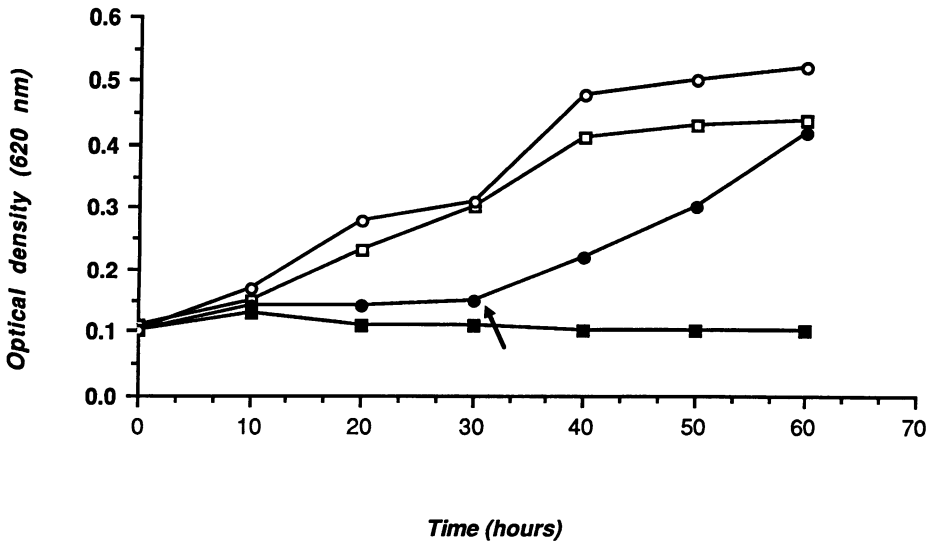

FIG. 2. Growth stimulation of iron-starved *H. pylori* cells in CBHI broth with HLF as the sole Fe source. Symbols: ■, dialysis membrane control (no added HLF or Fe); □, 10 μM 30% Fe-saturated HLF free in medium; ●, 10 μM 30% Fe-saturated HLF sequestered inside dialysis membrane; ○, 10 mM 30% ferric dicitrate. The arrow shows the time of release of Fe-saturated HLF from the dialysis bag by a puncture with a sterile needle.

Iron-starved cells provided with 30% iron-saturated BLF, HTF, or OTF did not show any increase in growth. The ability of *H. pylori* to use HLF as an Fe source was dependent on cell-to-protein contact, as demonstrated by dialysis bag experiments in which HLF was physically separated from the organisms. Growth of *H. pylori* was not observed under these experimental conditions but was observed after the dialysis bag was cut (Fig. 2). Thus, there were no inhibitory substances in the HLF or the dialysis bag which could cause growth limitation. Hemin could also enhance *H. pylori* growth, but heme compounds are normally not available for infecting microorganisms because of their localization. Therefore, pathogenic bacteria can utilize the iron in heme compounds only after it is made available by some form of tissue damage which releases intracellular material.

All of these results suggest that the mechanism by which *H. pylori* takes up iron from iron-binding proteins is very specific and resembles the receptor-mediated lactoferrin or transferrin iron acquisition mechanisms in *Neisseria meningitidis* (25, 26) and *N. gonorrhoeae* (18, 19). The iron uptake mechanisms of *N. meningitidis* and *N. gonorrhoeae* are dependent on HLF and HTF receptors which are highly specific for the human protein (24), suggesting that this property is partially responsible for conferring the human host specificity of these microorganisms. In this study, we showed the same specificity for *H. pylori*; that is, it takes up iron only from HLF. These results might explain the human host specificity of this microorganism described above and why *H. pylori* does not colonize many animal species that other microorganisms colonize (9).

HLF is present both intracellularly (in neutrophils) and in various secretions such as saliva, tears, nasal secretions, and intestinal secretions (11). Recently, the expression of this Fe-binding protein in normal human gastric tissues and tissues displaying benign, hyperplastic, or malignant histologic symptoms by a single 2.5-kb mRNA was studied (16). Stomach resections with superficial or atrophic gastritis had a high frequency of expression, while 85 and 86% of tumors

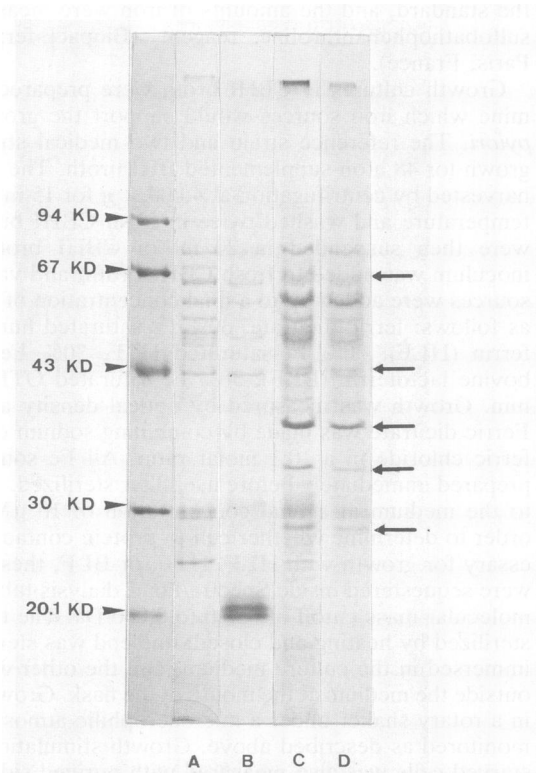

FIG. 3. SDS-PAGE of Coomassie blue-stained outer membrane proteins of *H. pylori* ATCC 43504 (lanes A and C) and a medical strain of *H. pylori* (lanes B and D) under iron-sufficient and iron-deficient conditions. Molecular size markers are in the left lane. Lanes: A and B, iron-sufficient conditions (cells grown in CBHI broth with additional ferric dicitrate); C and D, iron-deficient conditions (cells grown in CBHI broth without additional iron). Iron-repressible proteins are indicated by arrows.

and normal resections, respectively, were negative. Immunocytochemistry showed localization of HLF in cells of both antral and body glands. HLF retains its iron-binding properties in acidic conditions (it is stable at pHs of ≥4). Histological examinations of gastric biopsy tissue showed that *H. pylori* was consistently seen in the intercellular junctions of gastric epithelial and gastric mucus-secreting glands (12). All of these observations suggested that iron acquisition by the HLF receptor system of *H. pylori* might explain stomach colonization by *H. pylori* and play a major role in the virulence of *H. pylori* infection.

The effect of iron starvation on the outer membrane protein compositions of two strains of *H. pylori* (the reference strain and a medical strain) was determined by comparing the SDS-PAGE profiles obtained when cells were grown with iron and those obtained when the cells were grown without iron (Fig. 3). Cells were harvested after 48 h of incubation at 37°C. In each of the four profiles, many major outer membrane proteins were present, such as those with apparent molecular masses of 62, 57, 52, 48, 44, and 30 kDa. These were described by other investigators (7, 22). Fe-repressible proteins synthesized in response to Fe starvation were seen at 67, 45, 37, 33, and 29 kDa. In iron-deficient broth, this culture was more difficult, and the Fe-regulated proteins might be induced as a result of poor growth conditions. However, they appear different from the heat shock proteins of *H. pylori*, which are produced by stress such as heat shock or carbon starvation and which have been described elsewhere (8, 10). These heat shock proteins have molecular masses of 54 and 62 kDa. Some of the Fe-regulated proteins that we describe here may play a role in HLF binding and in the release of iron from this protein. Their identities should be further investigated.

This work was supported in part by the Direction de la Recherche et des Études Doctorales, Ministère de l'Éducation Nationale, the Université des Sciences et Technologie de Lille, and the Centre National de la Recherche Scientifique (Unité Mixte de Recherche du CNRS no. 111).

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