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Iron-Regulated Outer Membrane Protein of *Bacteroides fragilis* Involved in Heme Uptake

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Under iron starvation, *Bacteroides fragilis* expresses various iron-regulated outer membrane proteins. In this study, a deferrated minimal medium was used in growth experiments, and the role of one of these iron-regulated outer membrane proteins (a 44-kDa protein) in an iron uptake mechanism which acquires iron from heme compounds was elucidated. When a specific 44-kDa protein antiserum was used in a medium with heme as the only iron source, growth inhibition was observed. These results demonstrate that the 44-kDa outer membrane protein plays an important role in the uptake of heme in *B. fragilis*.

Microorganisms have developed several mechanisms to overcome the iron-withholding capacity of the host. The best-studied system for the uptake of iron by bacteria is the production of iron chelators, called siderophores, which can compete with lactoferrin and transferrin for iron. Iron-repressible outer membrane proteins (IROMPs) usually serve as receptors for iron-siderophore complexes and aid their internalization. Such high-affinity iron transport systems have been detected in many bacterial pathogens (3, 8, 12, 13, 16, 18). Little is known about iron uptake systems in bacteria which do not depend on the synthesis of siderophores. There have been reports of iron acquisition from heme-containing serum proteins (2, 6, 17, 20). The mechanism of a heme-iron uptake system and a possible role for the outer membrane proteins in it is not clear.

Bacteroides fragilis is a clinically important gram-negative, nonsporeforming anaerobic bacterium (1, 9). In animal infection models, the greater virulence of *B. fragilis* compared with that of the more common member of the intestinal flora, *Bacteroides vulgatus*, has been established by several authors (11, 24). In our department, we showed that only virulent *B. fragilis* strains could grow in serum and plasma. The growth inhibition of the other members of the *B. fragilis* group in these media can be abolished by adding iron sulfate (22, 23). IROMPs were found in *B. vulgatus* and *B. fragilis* (15). A 44-kDa outer membrane protein was found only in *B. fragilis*. These results indicated a strong relationship between the virulence of *B. fragilis*, expression of the 44-kDa protein, and ability to grow in serum. We did not find evidence that these bacteria produce iron chelators (15). For these reasons, we have been examining the role of this IROMP in an iron uptake system which obtains iron from heme compounds. In the present study we demonstrate that this IROMP is involved in the uptake of heme by the bacteria.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. fragilis* BE1 is a clinical isolate from the University Hospital of Vrije Universiteit. This strain was isolated from a wound infection and identified with a BBL Minitek Numerical Identification System (Becton Dickinson). We used a minimal culture medium (MM medium) from which the iron was removed by precipitation with $CaCl_2$ (CMM medium) for the growth experiments (15). The concentration of iron in the media used was determined as described by Carter (4). MM, CMM, and deferrated (dialyzed against apotransferrin) CMM media contained 505 ± 25, 155 ± 21, and ≤1 ng of iron per ml, respectively (mean ± standard deviation [SD], n = 5).

Bacteria were grown overnight in MM medium supplemented with hemin (15 μM; BDH); a subculture (1:10) in CMM medium without hemin was incubated for 6 h to obtain mid-log-phase cells. These cells were used to inoculate (1:100) the test vials. Hemin, $FeSO_4 \cdot 6H_2O$ (Merck), protoporphyrin IX (Fluka), human hemoglobin (Calbiochem), and human apotransferrin (Sigma Chemical Co.) were added to these vials at different concentrations (see Results) 5 h before inoculation. The bacterial cells were incubated during all experiments at 37°C in an atmospheric glove box (McCoy) with an atmosphere of 85% N_2–10% H_2–5% CO_2. To prevent iron contamination from glassware, only polystyrene vials, flasks, and other equipment (Sterilin) were used in the growth experiments.

Collodium bag experiments. The collodium bags (SM 13200; Sartorius) were sterilized in a microwave oven for 5 min. For this purpose, the bags were put into the test vials, and both were half-filled with deionized water. The water was discarded after sterilization. Apotransferrin (40 μM) and hemoglobin (1.8 μM) were put into the collodium bags, while CMM medium (5 ml) was added outside the bags. These vials were kept overnight at 4°C. In this method the apotransferrin binds all the free iron in CMM medium, and a completely deferrated CMM medium was obtained. Deferrated 44-kDa protein antiserum (1:4,000) and apotransferrin (10 μM) were added to the CMM medium outside the bags 2 h before inoculation with mid-log-phase cells (1:100). The vials were incubated anaerobically at 37°C during that time.

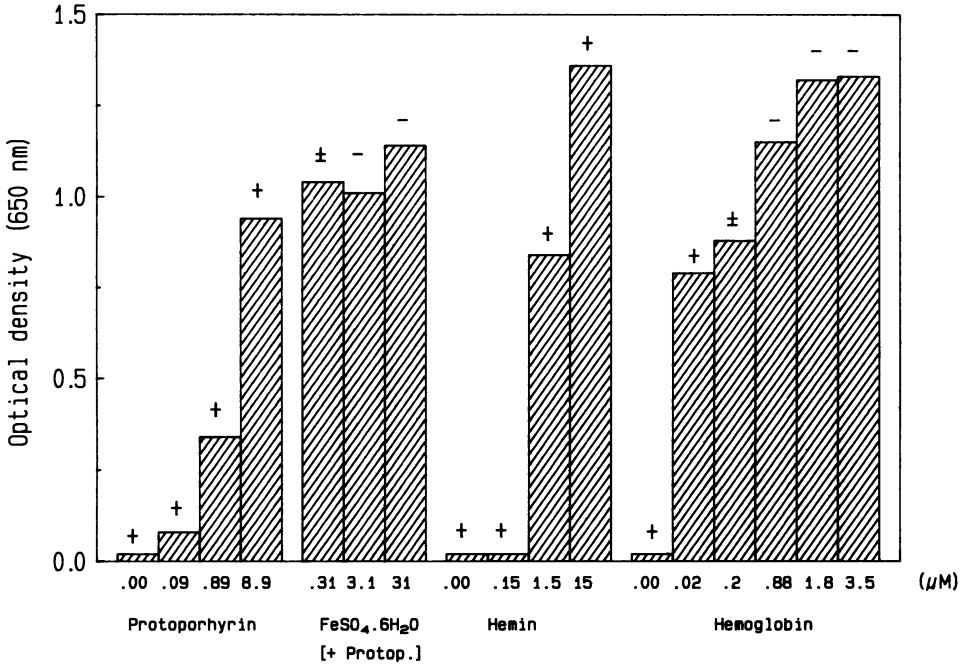

FIG. 1. Comparison at A_{650} of *B. fragilis* cultured in CMM medium supplemented with various iron sources in different concentrations after 20 h of growth. The concentration of PP (Protop.) in cultures supplemented with iron sulfate and PP was 8.9 μM. The symbols above the bars indicate the appearance (+), nonappearance (−), and possible appearance (±) of the 44-kDa protein in the bacterial cells after 20 h of growth, as determined by immunoblotting.

The 44-kDa protein antiserum (600 μl) was deferrated by dialysis against 5 ml of CMM medium containing 60 μM apotransferrin overnight at 4°C. The 44-kDa protein antiserum and deferrated 44-kDa protein antiserum contained 212 ± 13 and ≤1 ng of iron per ml, respectively (mean ± SD, n = 5). The dialysate was filter sterilized. The significance of differences was assessed by two-way analysis of variance (19).

Antiserum. The polyclonal 44-kDa protein antiserum was prepared as follows. A rabbit was immunized four times at weekly intervals with 50 to 300 μg of the 44-kDa protein (purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) which was given first in Freund complete adjuvant subcutaneously and subsequently in Freund incomplete adjuvant intravenously. After a final booster, given 2 weeks after the last injection with 300 μg, the rabbit was bled. The serum was adsorbed with BE1 cells cultured in BMP medium under non-iron-restricted conditions (15). This serum was subsequently used for a second absorption with a cell extract of *B. fragilis* from which the 44-kDa protein had been removed. This cell extract, prepared from bacterial cells cultured in an iron-restricted BMP medium (15), was then electroblotted on nitrocellulose paper (Schleicher & Schuell). Before the second absorption, the 44-kDa band was cut out from the nitrocellulose filter after staining with Ponceau S. Antiserum (100 μl) in 4 ml of antibody buffer (20 mM Tris, 500 mM NaCl, 1% [wt/vol] gelatin, 0.05% [vol/vol] Tween 20 [pH 7.5]) was applied in the second absorption to this filter. After an overnight incubation at room temperature, the antiserum solution was collected and filter sterilized. The specificity of this antiserum was tested by immunoblotting.

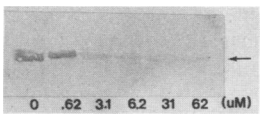

FIG. 2. Detection of the 44-kDa IROMP. Cell extracts of *B. fragilis* cultured in CMM medium with the indicated iron sulfate concentrations were prepared after 48 h of growth. A sodium dodecyl sulfate–11% polyacrylamide gel was loaded with 10 μg of protein per lane. After electrophoresis, the proteins were analyzed by immunoblotting with 44-kDa protein antiserum. The arrow indicates the location of the 44,000-Da polypeptide.

Immunoblotting. Cell extracts from the CMM cultures and cell extracts from bacterial cells cultured in BMP medium under iron stress, for testing the specificity of the antiserum, were prepared as described before (15). The proteins (10 μg per lane) were separated under denaturing conditions (SDS-PAGE) or under nondenaturing conditions (CHAPS-PAGE [14]) and electroblotted onto nitrocellulose paper by the method of Towbin et al. (21). A 1:8,000 dilution of the 44-kDa antiserum was used in all blotting experiments. Goat anti-rabbit immunoglobulins (horseradish peroxidase conjugated; Bio-Rad Laboratories, Richmond, Calif.) were applied as the secondary antiserum in this assay. The filters were stained in 10 ml of blotting buffer (25% [vol/vol] ethanol, 0.18% [wt/vol] $Na_2HPO_4 \cdot 2H_2O$, 1% [wt/vol] citric acid monohydrate [pH 5]) to which 6 mg of tetramethyl benzidine, 20 mg of dioctyl sulfosuccinate in 100 μl of dimethyl sulfoxide, and 6 μl of 30% (vol/vol) H_2O_2 had been added immediately before use.

The antiserum was also tested on intact bacterial cells by means of colony immunoblotting. *B. fragilis* was inoculated on Wilkins-Chalgren anaerobe agar plates (Oxoid).

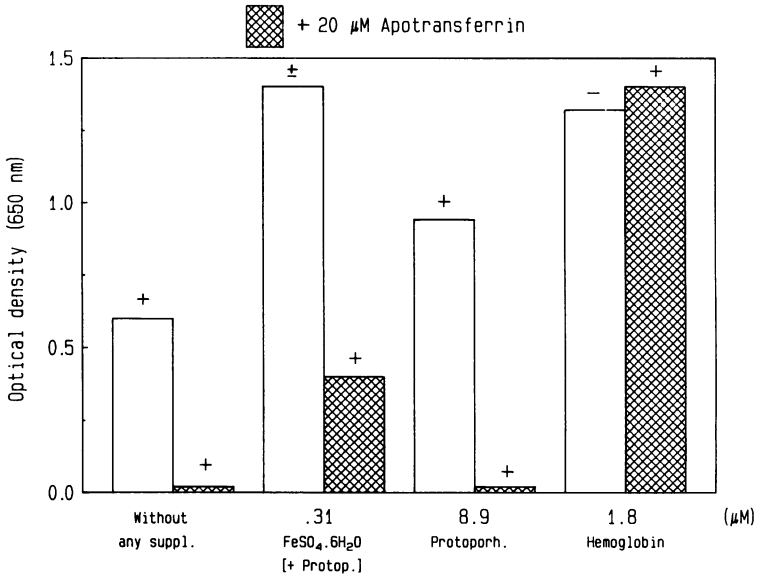

FIG. 3. Comparison at A_{650} of *B. fragilis* cultured in CMM medium supplemented with various iron sources in the presence or absence of apotransferrin (20 μM) after 20 h of growth. The optical cell density of the culture without any supplementation was determined after 48 h of growth. The symbols above the bars indicate the appearance of the 44-kDa protein in the bacterial cells after 20 h of growth (see Fig. 1 legend).

Iron restriction was obtained by supplementation with 125 μM bipyridyl. Nitrocellulose filters were laid on the plates for 1 h. After the colonies were blotted onto the filters, the filters were incubated in 0.5% (vol/vol) H_2O_2 in blocking buffer for 30 min to eliminate the peroxidase activity of the bacterial cells. The procedures for the binding of the primary and the secondary antibodies and for staining were the same as described above.

RESULTS AND DISCUSSION

Growth kinetics of *B. fragilis* in CMM medium. A deferrated minimal growth medium (CMM medium) was used in the growth experiments to elucidate the role of the 44-kDa IROMP in an iron uptake mechanism which acquires iron from heme compounds. The growth kinetics of *B. fragilis* in CMM medium were determined first. At the same time, appearance of the 44-kDa protein at different iron concentrations was determined by means of immunoblotting. The 44-kDa antiserum recognized the denatured and the nondenatured protein. This antiserum also reacted strongly with intact bacterial cells cultured under iron stress and poorly with bacteria from iron-replete cultures (data not shown), which shows that the protein is expressed at the surface of the bacteria.

In CMM medium, the growth of *B. fragilis* was strongly reduced. Without any supplementation, no visible growth was observed after 20 h of incubation, and even after 48 h an A_{650} of only 0.60 was found. Supplementation with various iron sources, such as iron sulfate and various heme compounds, indicated that protoporphyrin (PP) was growth restrictive in CMM medium (Fig. 1). The iron concentration in the medium was low enough to result in expression of the 44-kDa protein. At iron sulfate concentrations greater than 0.62 μm, the 44-kDa IROMP was fully repressed (Fig. 2). This repressive effect was seen at iron concentrations in the CMM medium of about 200 ng of iron per ml. As shown in Fig. 1, iron supplied in increasing amounts in the presence of PP (8.9 μM) resulted in very little growth stimulation. These results demonstrate that PP is needed in CMM medium for growth. The minimal growth of the bacteria after 48 h in CMM medium supplemented with iron sulfate alone is probably the result of an endogenous supply of PP.

An increasing concentration of hemin or hemoglobin in CMM medium also stimulated the growth of the cultures. We had to use much higher concentrations of hemin than of hemoglobin to obtain more or less the same growth kinetics. Comparable concentrations of heme molecules in CMM medium (i.e., 0.15 μM hemin or 0.02 μM hemoglobin ≡ 0.08 μM heme) resulted only in growth enhancement in the hemoglobin culture (A_{650} = 0.79; Fig. 1). Repression of the 44-kDa protein was detected in cultures supplemented with increasing amounts of hemoglobin; however, with hemin this effect was not observed, even at concentrations which were growth stimulating. This repressive effect is probably due to iron contamination of the hemoglobin solutions. Since we were interested mostly in the uptake of heme compounds at concentrations which were relevant for the in vivo situation, we used hemoglobin instead of hemin as a heme source in subsequent experiments.

Further characterization of the role of the 44-kDa protein in the uptake of iron from hemoglobin required a completely iron-free medium. Since *B. fragilis* could not use transferrin iron as an iron source, we used apotransferrin to remove the last traces of iron from the medium. As shown in Fig. 3, this medium (deferrated CMM) resulted in complete inhibition of growth, even after 48 h. In the presence of apotransferrin, the 44-kDa protein was expressed. Growth inhibition was also noticed in cultures supplemented only with PP. In cultures supplemented with $FeSO_4 \cdot 6H_2O$ and

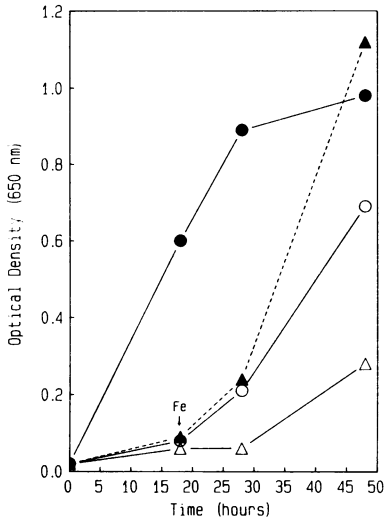

FIG. 4. Growth of *B. fragilis* in CMM medium with hemoglobin as a heme source in the collodium bag growth experiments. Symbols: ●, hemoglobin (1.8 μM) and apotransferrin (40 μM) in collodium bags; ○, extra apotransferrin (10 μM) outside the bags; △, extra apotransferrin and polyclonal 44-kDa protein antiserum (1:4,000) outside the bags; ▲, extra apotransferrin, antiserum, and iron sulfate (31 μM) outside the bags. The iron sulfate was added after 18 h of growth. The differences between the values for extra apotransferrin (○) and extra apotransferrin and antiserum (△) were significant ($P < 0.05$). Each point represents the arithmetic mean of four replicate determinations.

PP, a great decrease in growth was detected. Probably apotransferrin binds most of the added iron sulfate. The growth of the hemoglobin-containing cultures was hardly influenced by apotransferrin. This suggests that the bacterial cells in these cultures used hemoglobin (or heme) as an iron and PP source and that transferrin was not able to compete with the bacteria for the iron in the heme compounds.

Collodium bag experiments. Hemoglobin in solution disintegrates spontaneously into heme, iron, and PP molecules over time. To prevent any contamination of the hemoglobin with iron due to such disintegration, high concentrations of apotransferrin were necessary. Because high concentrations of apotransferrin could damage the bacterial outer membrane and alter the outer membrane permeability (7), we introduced collodium bags to prevent direct interaction between this protein and the bacterial cells. We indeed observed killing of the bacteria with high concentrations of apotransferrin when the collodium bags were omitted (data not shown). In this method, a completely deferrated CMM medium is obtained and only PP and heme will pass the dialysis membrane. However, heme molecules can also disintegrate into PP and iron. For that reason, apotransferrin in a lower, nontoxic concentration was added outside the bags. Under these conditions, heme is the sole iron source for *B. fragilis*. The 44-kDa protein was fully expressed.

The results of these growth experiments are shown in Fig. 4. A substantial difference in growth was detected between the cultures supplemented with hemoglobin and apotransferrin only in a dialysis bag and the cultures with extra apotransferrin outside the bag. This extra apotransferrin binds the iron released from heme outside the dialysis tubing. Now the iron needs for the growth of the bacterial cells depend totally on heme. Addition of a specific antiserum against the 44-kDa protein resulted in growth inhibition of these cultures. This growth inhibition was not due to killing of the cells by this antiserum, because iron-replete cultures showed dramatic growth enhancement. In this case there is enough iron and PP for the growth of the cells. The control experiments with preimmune serum did not show growth inhibition. These results point to an involvement of the 44-kDa protein in the uptake of heme.

The outer membrane of *B. fragilis* is impermeable to heme (M_r 616) because the diffusion pores of this bacterium are small enough (M_r 500) to exclude this compound on the basis of its molecular weight (10). Therefore, this bacterium must possess a heme uptake system. In *Haemophilus influenzae*, an outer membrane protein of 43 kDa has been detected which may play a role in the uptake of heme across the cell envelope (5). It is tempting to speculate that the 44-kDa protein of *B. fragilis* is also a protein which facilitates the passage of heme through the envelope.

In this study we demonstrated that the 44-kDa IROMP plays an important role in a heme uptake system. It would be of interest to determine whether *B. fragilis* can also obtain its heme supply directly from the heme-hemopexin complexes and what role the other IROMPs play in such an iron uptake system in this bacterium. Another important question concerns the contribution of the IROMPs, eventually involved in an iron uptake system, to the virulence of *B. fragilis*. Future studies will focus on acquisition of iron within the heme moiety and the genetics of iron acquisition in relation to virulence in *B. fragilis*.

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