ORIGINAL ARTICLE

Transferrin as a source of iron for *Campylobacter rectus*

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***Background and Objective*:** *Campylobacter rectus* is considered as one of the bacterial species of etiological importance in periodontitis. Iron-containing proteins such as transferrin are found in periodontal sites and may serve as a source of iron for periodontopathogens. The aim of this study was to investigate the capacity of *C. rectus* to assimilate transferrin-bound iron to support its growth.

***Design*:** Growth studies were performed in broth media pretreated with an iron-chelating resin and supplemented with various iron sources. The uptake of iron by *C. rectus* was monitored using ^{55}Fe-transferrin. Transferrin-binding activity was assessed using a microplate assay while the degradation of transferrin and iron removal was evaluated by polyacrylamide gel electrophoresis. A colorimetric assay was used to determine ferric reductase activity.

***Results*:** Holotransferrin (iron-saturated form) but not apotransferrin (iron-free form) was found to support growth of *C. rectus* in an iron-restricted culture medium. Incubation of holotransferrin with cells of *C. rectus* resulted in removal of iron from the protein. A time dependent intracellular uptake of iron by *C. rectus* cells from ^{55}Fe-transferrin was demonstrated. This uptake was significantly increased when bacteria were grown under an iron-limiting condition. Cells of *C. rectus* did not show transferrin-binding activity or proteolytic activity toward transferrin. However, a surface-associated ferric reductase activity was demonstrated.

***Conclusion*:** To survive and multiply in periodontal sites, periodontopathogens must possess efficient iron-scavenging mechanisms. In this study, we showed the capacity of *C. rectus* to assimilate iron from transferrin to support its growth. The uptake of iron appears to be dependent on a ferric reductive pathway.

Keywords: *Periodontitis*; *Campylobacter rectus*; *transferrin*; *iron*

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Periodontitis, a destructive chronic inflammatory disease, results from a polymicrobial infection and is characterized by the destruction of tooth-supporting tissues including the alveolar bone. Although more than 700 bacterial species are found in the oral cavity (1), a group of about 10 bacterial species has been strongly associated with periodontitis (2). There is now a consensus that *Campylobacter rectus* is a member of this group (3). Indeed, the proportions and levels of *C. rectus* are higher in periodontitis sites compared with healthy sites (3, 4). This Gram-negative anaerobic bacterium is also frequently recovered from root canal infections (5). Very recently, Arce et al. (6) demonstrated that *C. rectus* has the ability to translocate *in vivo* from a distant site of infection to the placenta suggesting that it may be an important contributor to adverse pregnancy outcomes associated with periodontal disease.

Iron is a constituent of important metabolic enzymes and is essential for the growth of almost all microorganisms. Consequently, a critical virulence determinant of microorganisms is their ability to obtain iron from their hosts. Although there is an abundance of iron in the extracellular tissue fluids of human, the amount of free ionic iron (10^{-18} M) is far too low to support growth of most bacteria (7, 8). Transferrin is a serum glycoprotein possessing two iron-binding sites and is important *in vivo* for rendering iron unavailable to bacteria (7). Transferrin as well as other iron-containing proteins, including hemoglobin and lactoferrin, are known constituents of gingival crevicular fluid (9). In addition, Goulet et al. (10) reported that gingival crevicular fluid samples obtained from periodontitis patients show the presence of both transferrin and transferrin fragments, which amounts are correlated with the severity of the disease.

Therefore, in the course of periodontitis, transferrin may represent an important source of iron for periodontopathogens.

Different mechanisms by which periodontopathogens can acquire iron from human transferrin to support their growth have been previously described (10–12). *Prevotella nigrescens* and *Prevotella intermedia* possess cell surface receptors with the capacity to bind transferrin (11). *Porphyromonas gingivalis* produces arginine-x-specific and lysine-x-specific gingipains that mediate a proteolytic cleavage of transferrin resulting in disruption of the iron-binding sites with the subsequent release and uptake of free iron (10, 12). The aim of this study was to investigate the capacity of *C. rectus* to assimilate transferrin-bound iron to support its growth.

Materials and methods

Bacteria and growth studies

C. rectus ATCC 33238 was routinely grown in mycoplasma broth base (BBL Microbiology Systems, Cockeysville, MD), which was supplemented with 0.2% glucose, 0.2% sodium formate, and 0.2% sodium fumarate (MBB-GFF). Growth studies were performed using the above medium treated with the chelating resin (3 g/100 ml) Chelex 100 (Bio-Rad Laboratories, Mississauga, Ontario) for 2 h at room temperature with constant agitation. This iron-restricted medium was supplemented with either ferrous sulfate, human apotransferrin (iron-free form), or human holotransferrin (iron-saturated form), all at 20 μM and obtained from Sigma-Aldrich Canada (Oakville, Ontario, Canada). Cultures were incubated at 37°C in an anaerobic chamber (N_2:H_2:CO_2/80:10:10). Bacterial growth was evaluated after 48 h of incubation by measuring the optical density at 660 nm (OD_{660}).

Removal of iron from iron-saturated transferrin

Equal volumes of holotransferrin (0.5 mg/ml) and *C. rectus* cells (OD_{660} = 0.5), treated or not at 60°C for 30 min, were incubated at room temperature for 2 h. Removal of iron from human holotransferrin was determined by urea/borate/EDTA-polyacrylamide gel electrophoresis (PAGE) analysis and Coomassie Blue staining (13). This electrophoretic procedure allows the differentiation of transferrin in the apo- (iron-free) and holo- (iron-saturated) forms.

Uptake of iron from transferrin by C. rectus cells

The ^{55}Fe-transferrin was prepared based on the protocols of Pintor et al. (14) and Simonson et al. (15) using human apotransferrin and [^{55}Fe]$FeCl_3$ (NEN Life Science Products Inc., Boston, MA). Apotransferrin at 1 mg/ml was mixed with 0.0075 μmol of [^{55}Fe]$FeCl_3$ and 0.075 μmol of sodium citrate in 40 mM Tris hydrochloride buffer (pH 7.4) containing 2 mM sodium carbonate. After incubation at room temperature for 30 min, several rounds of dialysis (molecular weight cutoff = 12–14 kDa; 12 h) were performed at 4°C against 40 mM Tris hydrochloride buffer (pH 7.4) containing 2 mM sodium carbonate until no radioactivity was detected in the dialysate using a gamma counter. Thereafter, the final concentration of transferrin was determined by the method of Lowry (16) whereas the iron saturation percentage of transferrin was evaluated by a colorimetric assay (Sigma-Aldrich Canada). By performing these assays, the ^{55}Fe-transferrin preparation was estimated to be 80% iron-saturated. The uptake of ^{55}Fe from ^{55}Fe-transferrin by *C. rectus* cells was determined as follows. The bacteria were grown to late exponential growth phase in MBB-GFF medium. Bacterial cells were harvested by centrifugation (8,000*g* for 15 min) and suspended to an OD_{660} of 1 in MBB-GFF medium treated (iron-restricted condition) or not (normal condition) with the Chelex 100 resin. Heat-inactivated bacteria (60°C for 30 min) were also tested. Equal volumes of ^{55}Fe-transferrin (13 μM) and bacteria were incubated at 37°C under anaerobiosis for 0, 3, and 24 h. Following incubation, cells were harvested by centrifugation (8,000*g* for 15 min), washed twice in PBS, and resuspended in the same buffer to an OD_{660} of 1. The radioactivity associated with the bacteria was quantified using a gamma counter.

Proteolytic degradation of transferrin

Equal volumes of holotransferrin (0.5 mg/ml) and overnight culture of *C. rectus* in MBB-GFF medium were incubated at 37°C for 1, 6, and 24 h. Proteolytic cleavage of holotransferrin was evaluated by sodium dodecyl sulfate (SDS) – PAGE followed by Western immunoblotting using an alkaline phosphatase-conjugated goat anti-human transferrin antibody (1:3,000 dilution). Undegraded transferrin and transferrin fragments were visualized following development in 100 mM carbonate buffer (pH 9.8) containing 0.3 mg/ml nitroblue tetrazolium chloride and 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt. A culture of *P. nigrescens* ATCC 33563 in Todd-Hewitt broth (BBL Microbiology Systems) supplemented with hemin (10 μg/ml) and vitamin K (1 μg/ml) was used as a positive control (11).

Transferrin-binding activity

The transferrin-binding activity of *C. rectus* cells was determined by a microplate assay. Bacterial suspensions (100 μl; OD_{660} = 1 in 50 mM phosphate-buffered saline [PBS]) prepared from an overnight culture in MBB-GFF medium (treated or not with Chelex 100) were applied into wells of a flat-bottomed 96-well microplate, which was then covered and incubated overnight at 37°C. Bacterial suspensions were removed by aspiration and wells were washed with PBS containing 0.5% Tween-20

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(PBST) to remove loosely bound bacteria. Bacterial cells attached on the bottom of wells were fixed with 0.05% glutaraldehyde (1 h) and the wells were further washed three times with PBST. The unreacted sites were then blocked with PBS containing 3% gelatin for 1 h. The solution was discarded and 100 µl of horseradish peroxidase-conjugated transferrin (0.2 µg/ml in PBS; Bio/Can Scientific, Mississauga, Ontario) was added. After a 2-h incubation, wells were washed three times for 5 min with PBST prior to adding 100 µl of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) substrate was added. Following incubation at 37°C for 30 min, the absorbance at 405 nm (A_{405}) was measured using a microplate reader. Cells of *P. nigrescens* ATCC 33563 were used as a positive control (11).

Iron reductase activity

The protocol of Morrissey et al. (17) was used to detect ferric reductase activity in whole cells of *C. rectus* from an overnight culture in MBB-GFF treated with Chelex 100 or with no pretreatment. Cells were suspended in 1 ml of the assay buffer (50 mM sodium citrate, pH 6.5, 5% glucose, 1 mM ferric chloride) to an OD_{660} of 1. Bathophenanthroline disulfonate (BPDS) was then added to a final concentration of 1 mM and samples were incubated at 37°C for 30 and 120 min. Bacterial cells were then removed by centrifugation and the red Fe^{2+}–BPS complex was quantified by recording the absorbance of the assay mixture supernatant at 520 nm (A_{520}). The level of ferrous ions produced was estimated from a reference curve constructed from a solution of known ion concentrations.

Results

Results of growth studies for *C. rectus* cultivated under various iron conditions are presented in Table 1. When the MBB-GFF medium was rendered iron-restricted by treatment with the cation-chelating resin, only a slight growth of *C. rectus* occurred. Adding either ferrous sulfate or holotransferrin stimulated growth of *C. rectus*. Using apotransferrin, the iron-free form of transferrin, instead of holotransferrin did not promote growth of *C. rectus*.

Table 1. Growth of *C. rectus* in MBB-GFF medium supplemented with various iron sources. The medium was rendered iron-restricted by treatment with a cation-chelating resin

	Growth (OD_{660})	
Iron source	Assay 1	Assay 2
None	0.12	0.15
Ferrous sulfate	0.23	0.27
Human holotransferrin	0.30	0.29
Human apotransferrin	0.11	0.09

To correlate the capacity of holotransferrin to support growth of *C. rectus* with the uptake of bound iron, we first demonstrated iron removal from transferrin by urea/borate/EDTA-PAGE analysis. While the *C. rectus* culture supernatant incubated with holotransferrin had no effect on the level of iron saturation (data not shown), cells of *C. rectus* caused a complete removal of iron (Fig. 1). This capacity was lost if bacteria were heat-treated. We then monitored the uptake of iron from ^{55}Fe-transferrin by cells of *C. rectus*. As shown in Fig. 2, *C. rectus* assimilated ^{55}Fe in a time-dependent manner. When bacteria were incubated with ^{55}Fe-transferrin under an iron-restricted condition, a significantly higher capacity to assimilate ^{55}Fe was noted after a 24-h incubation. Treating *C. rectus* cells at 60°C (30 min) completely abolished the uptake of iron thus suggesting the involvement of an active enzymatic process.

To investigate whether proteolytic degradation may be involved in the uptake of iron-bound transferrin, holotransferrin was incubated with a culture of *C. rectus* for various periods of time. As shown in Fig. 3, no breakdown products were detected by SDS-PAGE analysis indicating that proteases active on transferrin are not produced by *C. rectus*. On the contrary, *P. nigrescens* used as a positive control degraded holotransferrin into lower molecular weight fragments (Fig. 3). Since transferrin-binding activity may represent a mechanism of iron acquisition, we analyzed this property in *C. rectus*.

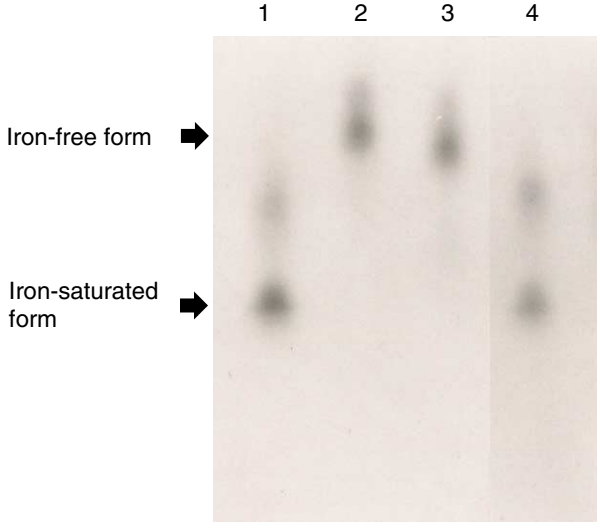

Fig. 1. Removal of iron from holotransferrin by cells of *C. rectus,* as determined by urea/borate/EDTA-PAGE analysis and Coomassie Blue staining. Lane 1, control holotransferrin (iron-saturated form); Lane 2, control apotransferrin (iron-free form); Lane 3, holotransferrin incubated with *C. rectus* cells; Lane 4, holotransferrin incubated with *C. rectus* cells treated at 60°C for 30 min.

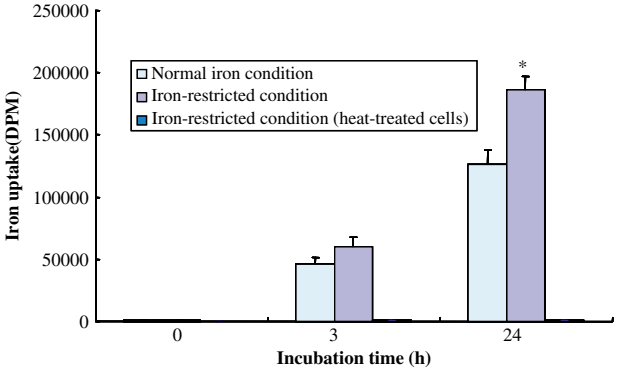

Fig. 2. Intracellular uptake of iron from ^{55}Fe-transferrin by *C. rectus* cells incubated under normal and iron-restricted (treatment with the cation-chelating resin) conditions. Results are expressed as disintegrations per minute (DPM) associated to bacteria following incubation with ^{55}Fe-transferrin. Assays were performed in triplicate and the means ± standard deviations were calculated. *, significantly different between normal and iron-restricted conditions at $p < 0.01$ using a Student's *t*-test.

Results presented in Fig. 4 indicate that *C. rectus* does not possess the ability to bind holotransferrin, on the contrary of *P. nigrescens* used as positive control.

Lastly, the presence of a cell surface ferric reductase activity in *C. rectus* was investigated using a colorimetric assay. As reported in Table 2, ferric reduction was detected after 30 min and increased markedly at 120 min. As observed for the uptake of iron from ^{55}Fe-transferrin, the ferric reductase activity was more pronounced for cells grown under iron-restricted conditions.

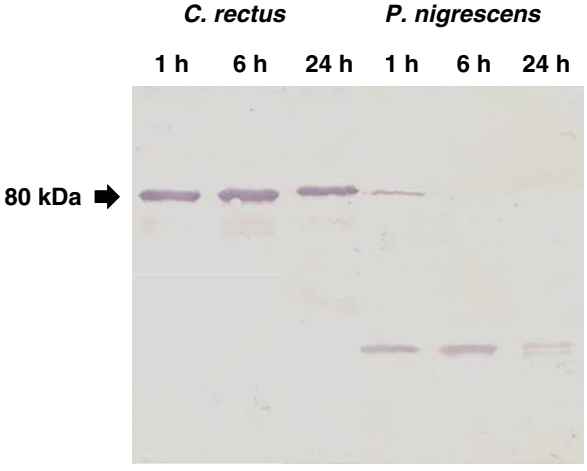

Fig. 3. Susceptibility of holotransferrin to proteolytic degradation by *C. rectus* and *P. nigrescens*. Degradation was monitored after 1, 6, and 24 h of incubation of holotransferrin with bacterial cultures. Samples were analyzed by SDS-PAGE followed by Western immunoblotting using an alkaline phosphatase-conjugated goat antihuman transferrin antibody.

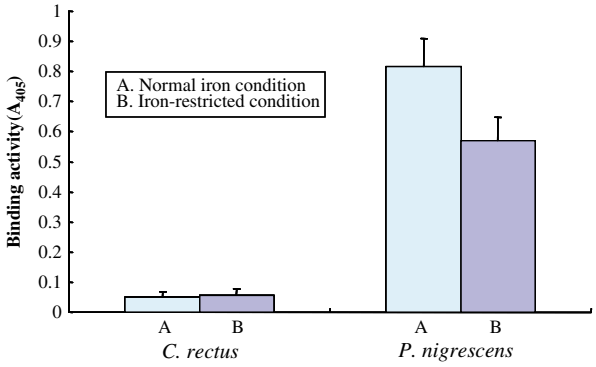

Fig. 4. Transferrin-binding activity of *C. rectus* and *P. nigrescens* cells grown under normal and iron-restricted (treatment with the cation-chelating resin) conditions, as determined by a microplate assay using horseradish peroxidase-conjugated transferrin.

Discussion

Although numerous studies have reported on the mechanisms of iron acquisition by periodontopathogens (10–12, 18–20), there was no data in the literature on *C. rectus*. In this study, we evaluated the ability of *C. rectus* to utilize human transferrin as a source of iron and investigated the mechanism by which iron can be obtained from this plasma protein. Transferrin, whose major physiological roles are the absorption, transport, and exchange of iron in tissues, plays an important role in host defense by rendering the iron unavailable for microorganisms (7). The transferrin concentration in gingival crevicular fluid has been reported to be approximately 70% of that in serum, which is in the range of 20–40 μM (21). It thus represents a potential source of iron in subgingival sites. The *C. rectus* was found to grow in the presence of holotransferrin (iron-loaded form) as the sole source of iron. Using ^{55}Fe-transferrin, we showed that cells of *C. rectus* were able to efficiently remove and assimilate iron bound to transferrin. This iron-uptake was found to be significantly increased when *C. rectus* cells were grown under iron-restricted conditions. Such a capacity to acquire iron from this host protein may be of utmost importance for the ability of *C. rectus* to establish

Table 2. Cell surface-associated ferric reductase activity of *C. rectus*

Iron growth condition	Incubation time (min)	Ferric reductase activity (μmol Fe^{2+} formed)[a]
Normal	30	3.2 ± 1.4
Normal	120	19.4 ± 5.7
Restricted	30	7.2 ± 3.1
Restricted	120	27.7 ± 10.4

[a]Mean ± standard deviation of triplicate assays.

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and multiply in periodontal sites or to cause infections at distant sites. Previous studies have shown that *P. gingivalis* (10, 12, 18), *P. intermedia* (11), and *P. nigrescens* (11) can also use transferrin as a source of iron.

Thereafter, experiments were carried out to attempt to identify the mechanism by which *C. rectus* can sequester iron bound to transferrin. In a previous study (11), we reported that the capacity of *P. nigrescens* to use iron-bound transferrin was related to its capacity to bind the protein on its surface or to proteolytically cleave it. However, *C. rectus* did not show transferrin-binding activity or proteolytic activity toward transferrin. We then investigated the presence of a cell surface ferric reductase activity in *C. rectus* since reduction of ferric to ferrous may favor iron release from transferrin. Our results clearly showed that *C. rectus* is capable of reducing Fe^{3+} to Fe^{2+}. The ferric reductase activity of *C. rectus* was increased when cells were cultivated under iron-restricted conditions, in agreement with our data on the uptake of iron from ^{55}Fe-transferrin by cells of *C. rectus*. Interestingly, a ferric reductase activity has been previously reported in another species of *Campylobacter* (*jejuni*), although its role in iron acquisition from transferrin has not been established (22). Ferric reductase activity as a mechanism of iron acquisition from transferrin has been previously reported in other pathogens including *Candida albicans* (23) and *Histoplasma capsulatum* (24).

In summary, we have demonstrated for the first time the ability of *C. rectus* to use iron-bound transferrin to support its growth. Although *C. rectus* does not appear to possess a specific receptor for transferrin, a surface-associated ferric reductase may physically interact with the substrate to generate reduced ferrous iron that may in turn be captured and transported across the cell envelope by a transporter system that remains to be identified. The iron liberated from transferrin may also enter into cells through outer membrane porins. However, one should not exclude the possibility that *C. rectus* possesses additional mechanisms for iron acquisition from host iron-containing proteins.'

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Conflict of interest and funding

There is no conflict of interest in the present study for any of the authors.

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