Human transferrin as a source of iron for

*Streptococcus intermedius*

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Abstract

*Streptococcus intermedius* is well known to produce severe infections in various areas of the body. In this study, we evaluated the ability of *S. intermedius* to utilise human transferrin as a source of iron and investigated the mechanism by which iron can be obtained from this plasma protein. Adding either ferrous sulfate or holotransferrin to an iron-deficient culture medium allowed growth of *S. intermedius*. Cultivation of *S. intermedius* under an iron-poor condition was associated with the overexpression of a 58 kDa cell surface protein. Neither siderophore activity nor reductase activity could be detected. Moreover, cells of *S. intermedius* did not show transferrin-binding activity or proteolytic activity toward transferrin. It was found that *S. intermedius* could rapidly decrease the pH of the medium during cell growth, resulting in a release of iron from holotransferrin. When the buffering capacity of the culture medium was significantly increased, the holotransferrin could not support growth of *S. intermedius*. It is suggested that under certain circumstances, *S. intermedius* may migrate from its normal niche (oral cavity), reach a particular site and create a localised environment where the pH can be lowered with the subsequent release of iron from transferrin. This would allow bacterial growth and initiation of the infectious process.

Keywords: Human transferrin; Iron acquisition; Bacterial virulence; *Streptococcus intermedius*

1. Introduction

*Streptococcus intermedius*, a microaerophilic to anaerobic Gram-positive bacterium, is part of the normal flora of the human oral cavity, upper respiratory tract, urogenital tract and gastrointestinal tract [1,2]. It has been found in recent years that *S. intermedius* as well as the other members of the *Streptococcus milleri*-group (SMG), *Streptococcus constellatus* and *Streptococcus anginosus*, may be ‘unrecognised pathogens’ that can produce severe infections in various areas of the body [3,4]. Indeed, studies that used reliable sampling methods and an appropriate identification scheme revealed that *S. intermedius* is often isolated from abscesses in the brain, lung and liver [2,3,5,6]. It may also account for up to 15% of all cases of community-acquired infective endocarditis [1].

Iron is a constituent of important metabolic enzymes and is essential for the growth of almost all
microorganisms. 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 \((10^{-18} \text{ M})\) is far too low to support growth of most bacteria [7,8]. Transferrins, which are serum glycoproteins possessing two iron-binding sites, are important in vivo for rendering the iron unavailable to bacteria. However, several different mechanisms by which pathogenic bacteria can acquire iron from human transferrin have been described [7–9]. Extracellular low molecular mass iron-chelating agents, also called siderophores, can sequester the iron bound to transferrin and transport it to a specific membrane receptor. Some bacterial species can also obtain iron from transferrin via a siderophore-independent system. This may be accomplished by: (1) production of cell surface receptors highly specific for transferrin; (2) proteolytic cleavage of transferrin that results in disruption of the iron-binding sites with the subsequent release of free iron; and (3) reduction of exogenous \(\text{Fe}^{3+}\) and the consequent release of \(\text{Fe}^{2+}\).

Although there are some data available on the virulence factors produced by \(S. \text{intermedius}\) [1], nothing is known concerning the ability of this bacterium to acquire iron from host proteins. We propose that \(S. \text{intermedius}\) may migrate from their normal niches, such as the oral cavity, via the bloodstream and cause serious infections in various organs of humans. This implies that \(S. \text{intermedius}\) should be able to resist the bacteriostatic action of serum and use plasma proteins as a source of iron. In this study, we demonstrated the capacity of human transferrin to serve as a source of iron for the growth of \(S. \text{intermedius}\). The mechanism by which iron is obtained from transferrin was also investigated.

2. Materials and methods

2.1. Bacterial strains and growth conditions

\(S. \text{intermedius}\) ATCC 27335 and strain 41 were used in this study. Bacteria were routinely grown in \(\text{Mycoplasma}\) broth base (MBB) (BBL Microbiology Systems, Cockeysville, MD) which was supplemented with 0.2% glucose. Growth studies were performed using the above medium treated to sequester residual iron. This was done by adding 200 \(\mu\text{M} 2,2'\text{-dipryridyl or treating the medium with Chelex 100 (Sigma, St. Louis, MO), a cation-chelating resin (3 g/100 ml), for 2 h at room temperature with constant agitation. These iron-poor media were supplemented with either 50 \(\mu\text{M ferrous sulfate, 0.5 mg per ml human apotransferrin (iron-free form) or 0.5 mg per ml human holotransferrin (iron-saturated form). In one experiment, \(S. \text{intermedius}\) ATCC 27335 was cultivated in active human serum. Media were inoculated (1% inoculum) with an overnight culture of \(S. \text{intermedius}\). All cultures were incubated at 37\(^\circ\)C in an anaerobic chamber (\(\text{N}_2/\text{H}_2/\text{CO}_2\), 80:10:10). Growth under the various iron conditions was evaluated by measuring the optical density at 660 nm after 12, 24, 36 and 48 h of incubation. All growth assays were carried out in triplicate to ensure reproducibility.

2.2. Electrophoretic analyses of transferrin

Removal of iron from human holotransferrin following growth (36 h) of \(S. \text{intermedius}\) ATCC 27335 and strain 41 in MBB-glucose containing 2,2'-dipyridyl and holotransferrin was determined by urea/borate/EDTA-polyacrylamide gel electrophoresis (PAGE) analysis [10] of the culture supernatants. This electrophoretic procedure allows to distinguish transferrin as apotransferrin (iron-free form), holotransferrin (diferric form) or in the monomeric form in which the iron is associated with either the N- or C-domain binding site. Proteolytic cleavage of transferrin and production of lower molecular weight fragments following growth of \(S. \text{intermedius}\) ATCC 27335 was evaluated by sodium dodecyl sulfate (SDS)-12.5% PAGE analysis of the culture supernatant, according to the procedure of Laemmli [11]. Gels were stained for proteins with Coomassie brilliant blue.

2.3. Analysis of cell surface proteins

Cells of \(S. \text{intermedius}\) ATCC 27335 (250-ml culture) grown (48 h) in either MBB-glucose (normal iron condition) or MBB-glucose containing 2,2'-dipyridyl (iron-restricted condition) were harvested by
centrifugation, washed in PBS and suspended in the same buffer containing 20 mM EDTA and 0.5% Triton X-100 (2.5 ml). An equal volume of glass beads (150–200 μm) was added and the suspension was shaken (450 rpm) for 20 h at 4°C. After centrifugation (8000 g for 20 min) to remove cells, the supernatant containing the extracted cell surface proteins was filtered (0.2 μm). Cell surface proteins (20 μg protein) were mixed with an equal volume of solubilizing buffer (125 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 2% mercaptoethanol and 0.01% Bromophenol blue) and boiled for 10 min. Samples were analysed by SDS-12.5% PAGE according to the procedure of Laemmli [11], and proteins were stained with Coomassie brilliant blue.

2.4. Detection of siderophore activity

The universal siderophore assay of Schwyn and Neilands [12] was used to evaluate production of siderophores by S. intermedius ATCC 27335. Culture supernatants (adjusted at pH 7.0) from bacteria grown (48 h) in MBB-glucose treated with Chelex 100 were mixed with plain Chrome azurol S solution and the absorbance at 630 nm was measured. The culture supernatant obtained from a strain of Ustilago maydis cultivated under an iron restricted condition was used as a positive control [13].

2.5. Determination of transferrin-binding activity

The binding of human transferrin by S. intermedius ATCC 27335 was evaluated by a solid-phase dot-blot enzyme assay. A nitrocellulose membrane was spotted with 2 μl of a cell suspension (optical density at 660 nm = 0.1 in 50 mM phosphate-buffered saline (pH 7.2; PBS)) of bacteria grown (48 h) in MBB-glucose treated with Chelex-100. The membrane was incubated in 20 mM Tris buffer (pH 7.5)-0.5 M NaCl (TBS) containing 3% gelatin for 1 h at room temperature with shaking. The membrane was then transferred into TBS containing 1.5% gelatin and 1 μg/ml of horseradish peroxidase (HRP)-conjugated human transferrin (Bio/Can Scientific, Mississauga, Ont.), and incubated for 4 h at room temperature with shaking. The membrane was washed (4×15 min) in TBS containing 0.05% Tween-20, and then stained with the Bio-Rad colour development kit (Bio-Rad) according to the manufacturer’s instructions. A strain of Moraxella catarrhalis (PD) was used as a positive control [14].

2.6. Determination of ferric reductase activity

The procedure of Morrissey et al. [15] was used to detect ferric reductase activity in supernatant and whole cells of S. intermedius ATCC 27335 from cultures (48 h) in MBB-glucose treated with Chelex 100. An aliquot of 500 μl of the culture was centrifuged and cells were suspended in 1 ml of the assay buffer (50 mM sodium citrate, pH 6.5, 5% glucose) containing 1 mM ferric chloride and 1 mM bathophenanthroline disulfonate. The supernatant (pH adjusted at 7.0) of the culture was mixed with 500 μl of the assay buffer (2-fold concentrated). An uninoculated culture medium served as a negative control. Samples were incubated at 30°C for 10 min, after which the cells were removed by centrifugation and the absorbance at 520 nm of the assay mixture supernatant was measured. The level of ferrous ions produced was estimated from a calibration curve constructed from a solution of known ion concentrations.

2.7. Effect of pH decrease during bacterial growth on the release of iron from transferrin

S. intermedius ATCC 27335 was cultivated in MBB-glucose media containing 2,2'-dipyridyl and holotransferrin as the source of iron, and samples were obtained at various times during the incubation. For each sample, the optical density at 660 nm, the pH of the culture and the level of iron saturation of transferrin, evaluated by the electrophoretic procedure [10], were determined. The effect of adding 200 mM Tris buffer pH 7.2 to MBB-glucose media containing 2,2'-dipyridyl and either holotransferrin or ferrous sulfate on the ability of S. intermedius ATCC 27335 to grow was also evaluated.

3. Results

Results of the growth studies for S. intermedius ATCC 27335 cultivated under the various iron con-
ditions are summarised in Fig. 1. When the MBB-glucose medium was rendered iron-restricted by adding 2,2′-dipyridyl or by treatment with the chelating resin, no growth of *S. intermedius* ATCC 27335 was observed after 36 h of incubation. Adding either ferrous sulfate or holotransferrin allowed a significant growth that was comparable to the one obtained in a non-iron chelated medium. Growth rates were not significantly different for culture in the presence of ferrous sulfate or holotransferrin (data not shown).

Using apotransferrin, the iron-free form of transferrin, instead of apotransferrin did not allow growth of *S. intermedius*. To test whether *S. intermedius* could acquire iron from transferrin under a condition more closely representing the in vivo situation, its capacity to grow in serum was evaluated. Active human serum allowed growth of *S. intermedius* up to a final optical density of 0.25 (660 nm). All the above growth assays were carried out in triplicate and showed reproducibility.

The transferrin added to MBB-glucose medium containing 2,2′-dipyridyl was analysed following growth of *S. intermedius* ATCC 27335. No breakdown products of transferrin could be seen by SDS-PAGE analysis of the culture supernatant indicating that proteolytic enzymes active on transferrin are not produced by *S. intermedius* (data not shown). Performing an urea/borate/EDTA-PAGE analysis of the culture supernatant revealed removal of the iron from holotransferrin (Fig. 2). A similar result was obtained with *S. intermedius* strain 41.

It was observed that when the incubation was extended to 48 h, some growth (OD$_{660}$ nm = 0.55) of *S. intermedius* was obtained in the iron-depleted medium containing no iron supplement. This condition of growth limitation was used for the following experiments. The effect of an iron-restricted condition on the expression of cell surface proteins by *S. intermedius* ATCC 27335 was investigated by comparing growth (48 h) in MBB-glucose and MBB-glucose containing 2,2′-dipyridyl. By SDS-PAGE analysis,
the glass bead extract obtained from whole cells was found to contain a large variety of cell surface-associated proteins (Fig. 3, lane 2). Few differences were noted except for a protein of 58 kDa that was overexpressed following growth under the iron poor condition (Fig. 3, lane 3).

Further experiments with *S. intermedius* ATCC 27335 were carried out to investigate the mechanism of iron acquisition from human transferrin. Siderophore activity in the supernatant of *S. intermedius* grown in the MBB-glucose medium treated with Chelex 100 could not be detected by the universal procedure described by Schwyn and Neilands [12]. The culture supernatant of *U. maidis* gave a strong positive reaction. *S. intermedius* grown under iron restriction was analysed to determine whether transferrin could bind to the cell surface. A solid-phase dot-blot enzyme assay was used in which whole cells were immobilised on a nitrocellulose membrane which was then probed with HRP-conjugated human transferrin. Using this binding assay, no reactivity was found with *S. intermedius*, whereas the control *M. catarrhalis* gave a strong positive reaction (data not shown). Lastly, reductase activity could not be detected in the supernatant of *S. intermedius* grown in the iron-restricted culture medium. Bacterial cells were also devoid of reductase activity.

Incubation of holotransferrin with cells of *S. intermedius* grown under iron restriction did not result in removal of iron from the molecule, as determined by urea/borate/EDTA-PAGE analysis. A similar experiment performed with the supernatant resulted in the generation of apotransferrin, the iron-free form. This transformation did not occur when the supernatant was readjusted to neutrality (pH 7.0) prior to carrying out the assay. This result suggested that the abil-

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**Fig. 3.** SDS-polyacrylamide gel electrophoretic analysis of cell surface proteins obtained from *S. intermedius* ATCC 27335 grown in either MBB-glucose (lane 2) or MBB-glucose medium containing 2,2'-dipyridyl (lane 3). Molecular weight markers (lane 1) were: β-galactosidase (121 kDa), bovine serum albumin (82 kDa), ovalbumin (50.2 kDa), carbonic anhydrase (34.2 kDa), trypsin inhibitor (28.1 kDa) and lysozyme (19.4 kDa). The arrowhead indicates position of the 58 kDa protein.

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**Fig. 4.** Urea/borate/EDTA polyacrylamide gel electrophoretic analysis and pH of culture supernatants collected during growth of *S. intermedius* ATCC 27335 in MBB-glucose medium containing 2,2'-dipyridyl and supplemented with holotransferrin. Lane 1, control apotransferrin (iron-free form); lane 2, culture at time 0 (pH 7.1); lane 3, culture at OD660 nm = 0.27 (pH 6.6); lane 4, culture at OD660 nm = 0.35 (pH 6.4); lane 5, culture at OD660 nm = 0.66 (pH 5.8); lane 6, culture at OD660 nm = 0.84 (pH 5.1).
ity of *S. intermedius* to lower the pH during growth may be associated to its capacity to use iron from transferrin. The pH at which the iron is removed from holotransferrin during growth of *S. intermedius* was determined by analyzing samples obtained at various times during the incubation (Fig. 4). Iron removal from holotransferrin with production of the monomeric form in which the iron is associated with either the N- or C-domain binding site started to occur when the pH of the culture reached 6.6 (OD$_{660}$ nm = 0.27). The iron-free form of transferrin began to appear in the supernatant when the culture reached a pH of 5.8 (OD$_{660}$ nm = 0.66). At the stationary growth phase (final OD$_{660}$ nm = 0.84; final pH 5.1), the apotransferrin had increased in proportion while the holotransferrin was undetectable. To support the importance of the pH decrease for growth of *S. intermedius*, the MBB-glucose media containing 2,2$^{3}$P-dipyridyl and either holotransferrin or ferrous sulfate was buffered by adding 200 mM Tris at pH 7.2. It was found that under this condition, no bacterial growth occurred when holotransferrin was the source of iron. On the other hand, the high buffering capacity had no effect when ferrous sulfate was used as source of iron.

4. Discussion

*S. intermedius* was found to be able to grow in the presence of holotransferrin as the sole source of iron, whereas the iron-free form of transferrin did not allow growth under the same assay conditions. Experiments were carried out to determine whether *S. intermedius* utilises the iron bound to transferrin via the classical mechanisms reported for other pathogens [7–9]. Neither siderophore activity nor reductase activity could be detected. Moreover, cells of *S. intermedius* did not show proteolytic activity toward transferrin and transferrin-binding activity. This latter observation is in agreement with the study of Beighton et al. [16] who reported that among oral streptococci, *Streptococcus oralis* and *Streptococcus mitis* exhibited high affinity for transferrin whereas members of the SMG (former nomenclature) had only a weak capacity to bind transferrin.

Since the pH may have an effect on the affinity of iron for transferrin, we investigated the possibility that modification of this environmental parameter may represent the mechanism of iron acquisition for *S. intermedius*. Our results indicated that *S. intermedius* could rapidly decrease the pH of the medium resulting in a release of iron from transferrin. A mechanism of iron acquisition from transferrin based on a modification of the environment has been previously suggested for *Clostridium perfringens* [17]. The growth of this bacterial species in vivo leads to a significant fall in the oxidation-reduction potential ($E_h$) and pH of the infected tissue ($E_h$ = −400 mV and pH 6.5). These highly reduced conditions and acidic pH, allowed the release of iron from transferrin and permitted growth of *C. perfringens*.

Few studies have been done concerning the iron requirements and mechanisms of iron acquisition in streptococci. von Hunolstein et al. [18] reported that oral and non-oral strains of *Streptococcus* spp. could grow in media which have a very low iron content (<0.1 μM). They also suggested that these low iron requirements could account for the lack of antibacterial activity of human transferrin on streptococci. Iron acquisition in *Streptococcus mutans* was studied by Evans et al. [19]. It was found that the bacterium did not produce siderophores and transports only ferrous iron utilizing reductants furnished by the glucose metabolism to reduce iron prior to its uptake.

Besides being important for multiplication of microorganisms, iron also regulates the synthesis of bacterial proteins that may be important in virulence. We observed that cultivation of *S. intermedius* under an iron-poor condition was associated with the over expression of a 58 kDa cell surface protein. Further studies should indicate whether this protein has a role to play in iron acquisition or pathogenicity.

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References