Identification of a conserved Moraxella catarrhalis haemoglobin-utilization protein, MhuA

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Moraxella catarrhalis is a leading cause of acute otitis media in children and is a cause of respiratory disease in adults with underlying lung disease. This organism is a strict human pathogen that has an absolute requirement for iron in order to grow and cause disease. Previous studies identified transferrin and lactoferrin receptors used by M. catarrhalis to obtain iron from the human host, yet other iron-acquisition systems remain undefined. In this study, it is demonstrated that this strict mucosal pathogen can utilize haemoglobin (Hb) as a sole source of iron for growth. A novel 107 kDa outer-membrane protein involved in Hb utilization by this pathogen was also identified. An isogenic mutant defective in this Moraxella Hb-utilization protein (MhuA), 7169::mhuA, showed a significant lag during growth in the presence of Hb as the sole iron source. This protein appears to be expressed constitutively, regardless of growth conditions, and a mAb directed to MhuA demonstrated that this protein contains highly conserved, surface-exposed epitopes. Data demonstrating that expression of MhuA may be highly specific to isolates of M. catarrhalis are also presented, suggesting a potential role as a diagnostic marker. To our knowledge, this is the first report demonstrating that M. catarrhalis expresses an Hb-binding protein and that this bacterium can utilize Hb as a sole iron source for growth.

INTRODUCTION

Moraxella catarrhalis is a Gram-negative diplococcus that primarily infects young children and also infects older adults with underlying lung disease, such as chronic obstructive pulmonary disease (COPD) (Bakri et al., 2002; Karalus & Campagnari, 2000; McMichael, 2000; Murphy, 1996; Verduin et al., 2002). This organism is a major cause of bacteria-induced otitis media and sinusitis, and has also been implicated as a potential cause of nosocomial pneumonia in some hospital settings (Aebi et al., 1998). There is a substantial burden on the health-care industry due to direct and indirect costs that are involved in the treatment of the estimated 24 million cases of childhood acute otitis media (AOM) that occur each year in the USA (Murphy, 1996; Stool & Field, 1989; Teele et al., 2001). In addition, AOM is the single most prominent reason for prescribing antibiotics to children (Teele et al., 2001). It is a contributing factor to the rapid emergence of β-lactamase-positive clinical isolates of M. catarrhalis and, thus, antibiotic therapy to treat AOM and exacerbations of COPD is becoming a serious challenge (Bakri et al., 2002; Dagan, 2000). Therefore, many of the research efforts involving M. catarrhalis have focused on the identification of potential vaccine antigens to prevent colonization and disease (Cripps & Kyd, 2003; Hu et al., 2000; Jiao et al., 2002).

M. catarrhalis is a strict human pathogen that has been reported to colonize a significant number of young, healthy children (Faden, 2001). It also appears that this organism can maintain a presence in the human lung, primarily in individuals with COPD (Bakri et al., 2002). Despite these data, there is very little information regarding the basic biological mechanisms that M. catarrhalis uses to survive in the human host. One major hurdle that all successful pathogens must overcome is the lack of free iron in vivo. It is well-established that, like most other organisms, M. catarrhalis has a strict requirement for iron (Campagnari et al., 1994, 1996; Furano & Campagnari, 2003; Luke & Campagnari, 1999). M. catarrhalis can obtain iron for growth and metabolism through the binding of either transferrin or lactoferrin, two of the human iron-binding proteins (Campagnari et al., 1994; Luke & Campagnari, 1999; Luke et al., 1999; Schryvers et al., 1998; Stojićković et al., 1996). These two specific receptor

Abbreviations: AOM, acute otitis media; COPD, chronic obstructive pulmonary disease; Hb, haemoglobin; Hm, haemin; OMP, outer-membrane protein.

The GenBank/EMBL/DDBJ accession number for the M. catarrhalis 7169 mhuA nucleic acid sequence and MhuA amino acid sequence reported in this paper is AY574198.
systems are similar in composition, with an integral outer-membrane protein (OMP) component and an outer-membrane lipoprotein component that work together to bind the iron-carrier protein and internalize the iron moiety. These well-known transferrin- and lactoferrin-binding systems have been described for many other pathogens, including pathogenic species of the genera Neisseria and Haemophilus (Cornellis & Spirling, 1994; Gray-Owen & Schryvers, 1996; Schryvers, 1989). Investigators have provided evidence that these surface receptors have vaccine potential, although significant heterogeneity has been reported (Ala’Aldeen & Borriello, 1996; Ala’Aldeen et al., 1994; Johnson et al., 1999; Masri & Cornellis, 2002; Rokbi et al., 2000; West et al., 2001).

We now present data demonstrating that M. catarrhalis can utilize haemoglobin (Hb) for in vitro growth. We have identified and characterized an OMP, MhuA, that we hypothesize to be an integral component of a newly identified haemoprotein-utilization system for this bacterium. A mAb specific to MhuA, 3F5-5E5, demonstrates that expression of this surface-exposed protein is conserved among a wide panel of M. catarrhalis clinical isolates and that the epitope recognized by this mAb appears to be species-specific, as we have not detected this determinant on any other bacterium evaluated. These data provide new information regarding another mechanism utilized by M. catarrhalis for survival in the human host and also provide new insight into the biological systems available to this important mucosal pathogen.

**METHODS**

**Bacterial strains and culture conditions.** M. catarrhalis 7169 was isolated from the middle ear of a child with otitis media (Dr Howard Faden, Buffalo, NY, USA) and this wild-type strain was used to construct the MhuA-deficient mutant 7169:: mhuA. Clinical isolates of M. catarrhalis were obtained from Dr Mark Achtmann (Max-Planck-Institute, Berlin, Germany) or from laboratory stocks. Routine culture of M. catarrhalis was performed at 35 °C in 5% CO₂ on brain heart infusion (BHI) or GC agar plates or at 37 °C with rotary shaking at 225 r.p.m. in the appropriate liquid medium. The mutant strain 7169:: mhuA was cultured in the presence of 20 μg kanamycin ml⁻¹. *Escherichia coli* XL-1 Blue cells were cultured on Luria-Bertani (LB) agar plates or in liquid LB medium in the presence of 50 μg ampicillin ml⁻¹. *E. coli* (HB101) was cultured in the presence of 2 M NaCl or 2 M guanidine/HCl (50 M H2O. Hb, Hm and Fe(NO3)3 were sterilized before addition to culture media. Haemoglobin (Hb) was prepared by using a previously described method (Russo et al., 1993). All standard molecular-biology reagents, including T4 ligase and restriction endonucleases, were purchased from either Promega or New England Biolabs and were utilized according to standard protocols. PCR-amplification analyses were performed by using genomic M. catarrhalis 7169 DNA with Platinum Taq High Fidelity Polymerase (Invitrogen Life Technologies). All PCR products and plasmid constructs were purified by using MiniElute kits or QIAprep spin kits, respectively (Qiagen). DNA sequencing was performed by the RPCI Biopolymer Facility, Roswell Park Cancer Institute, Buffalo, NY, USA, and analysed with MacVector software (version 7.2; Genetics Computer Group).

**Cloning and mutagenesis of M. catarrhalis 7169 mhuA.** mhuA was identified through BLAST searches based on homology to other TonB-dependent proteins in GenBank. PCR primers were designed for cloning mhuA based on the nucleotide sequence submitted under Incyte Genomics sequence 6, patent WO0078968, GenBank accession no. AX067431. PCR amplification between 75 bp upstream of the predicted 5’ transcription start site and 197 bp upstream of the 3’ predicted stop site was performed by using primers 605 (5’-TGATTGTTGATACAGTAGG-3’) (sense) and 606 (5’-TGTGGCATTCAAAGGGCTC-3’) (antisense). Primers 605 and 606 resulted in a 2782 bp product that was ligated into pGEM-T Easy (Promega), resulting in pTB6-AH. E. coli XL-1 Blue cells were transformed with pTB6-AH by using electroporation. PCR analysis, restriction digestion and sequence analysis were performed on this plasmid to confirm the nucleotide organization of the 7169 mhuA.

A deletion–insertion isogenic mutant of 7169 mhuA was constructed by using PCR, using pTB6-AH as a template. Primers 653 (5’-ATCTCGAGCAAGGCTAGGCTAAGCAAATG-3’) (sense) and 654 (5’-GGGAATCTTCAATCATCTTCTGCACGCTG-3’) (antisense), with engineered Xhol and BglII sites, respectively (underlined), were designed to create a 2081 bp deletion internal to the ORF of mhuA. Amplification of aphA-3, the non-polar kanamycin-resistance cassette from pUC18K, was performed by using primers 417 (5’-TATAAGATCTGGGTGACTAACTAGGAGGAATAAATGGCTA-3’) (sense), with engineered Xhol and BglII sites, respectively (underlined, were designed to create a 2081 bp deletion internal to the ORF of mhuA.

**Hb-agarose binding.** Hb-agarose binding was performed essentially as described by Dashper et al. (2000) with the following modifications. Briefly, 200 μl Hb–agarose (Sigma) was washed with 100 mM NaCl, 25 mM Tris/HCl (pH 7.4). Washes were performed three times by resuspending the agarose in 500 μl buffer and centrifuging (7000 r.p.m. for 5 min). Zwittergent-extracted OMP preparations (50 μl) from each of the CDM/Hb, CDM/Hm and CDM 100 cultures (10 ml) containing 10 μM desferal with or without 5 μM bovine haemin (CDM/Hm; Sigma) were subjected to SDS-PAGE and Western blot (mAb 3F5-5E5) analyses.

**Growth analyses.** Chelex-treated CDM was used to culture M. catarrhalis in the presence of 10 μM desferal with or without 5 μM human Hb (CDM/Hb; Sigma), 8 μM bovine haemin (CDM/Hm; Sigma) or 100 μM Fe(NO3)3 (CDM 100; Sigma) (Campagnari et al., 1994). Hb was solubilized by using 10 mM HEPES (pH 7-4), Hm was solubilized with 0-1 M NaOH and Fe(NO3)3 was solubilized in H2O. Hb, Hm and Fe(NO3)3 were sterilized before addition to culture media by using a 0.2 μm pore-size filter (Pall). CDM 0, CDM 100 and CDM/Hb cultures (10 ml) containing 10 μM desferal were

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inoculated to a starting OD₆₀₀ (BioPhotometer; Eppendorf) of 0.1 from overnight GC agar plate-grown *M. catarrhalis* 7169 or 7169::*mhuA*. For studies with Hm, CDM 0 and CDM/Hm cultures (10 ml) were also inoculated to a starting OD₆₀₀ of 0.1 from overnight GC agar plate-grown *M. catarrhalis* 7169 or 7169::*mhuA*, but these strains were cultured at 37 °C with rotary shaking at 225 r.p.m. overnight (16 h) in either CDM 0 or CDM/Hm, centrifuged at 3000 r.p.m. for 10 min and fresh 10 ml cultures (CDM 0 or CDM/Hm plus desferal) were inoculated to a starting OD₆₀₀ of 0.06 for these studies. All strains were cultured at 37 °C with rotary shaking at 225 r.p.m. over the time course indicated. Representative curves are shown for CDM 100 and CDM/Hm growth analyses that have been repeated at least three times, and the CDM/Hb curve shown is the mean ± SD of three independent experiments.

**RT-PCR analysis of *mhuA***. Transcriptional analysis of *mhuA* was performed by using wild-type *M. catarrhalis* 7169. RNA was isolated from this strain after culture in CDM 0, CDM 100, CDM/Hm or CDM/Hb (as described above) by using an RNeasy Mini kit (Qiagen). The resulting concentration of RNA was standardized for all four conditions to 15 ng µl⁻¹ for use in RT-PCR analysis with primers internal to *mhuA*: primer 5′-TCATTCAAT-TGAAGGCGAGGC-3′ (sense) and primer 544, 5′-GTATACCCGCTAG-TTTAGCCG-3′ (antisense), resulting in a product of 1217 bp.

**OMP preparation, SDS-PAGE and Western blot.** *M. catarrhalis* strains 7169 and 7169::*mhuA* were cultured in 250 ml CDM broth containing 10 µM desferal plus 5 µM Hb, 8 µM Hm, 100 µM Fe(NO₃)₃ or no exogenous iron (conditions described above). After 16 h, cultures were harvested and OMPs were isolated by Zwittergent extraction (Campagnari et al., 1994, 1996). OMPs were analysed by SDS-PAGE (7% gel). Western blot, colony-lift assays and flow-cytometry analyses with mAb 3F5-5E5 were performed by using our standard methods (Campagnari et al., 1994, 1996; Luke et al., 1999).

mAb 3F5-5E5. mAb 3F5-5E5 (anti-MhuA) was developed against iron-stressed *M. catarrhalis* 25240 by injecting BALB/c mice using a previously described protocol (Campagnari et al., 1996).

**RESULTS**

**Identification and characterization of *M. catarrhalis* 7169 *mhuA***

Preliminary data in our laboratory demonstrated that *M. catarrhalis* 7169 could utilize various haemoproteins, including Hm, human Hb and Hb from various other species, as the sole iron source for *in vitro* growth. By using known TonB-dependent haemoprotein-receptor sequences, we performed a BLAST search against the unannotated, patented *M. catarrhalis* sequence in GenBank to find potential homologues of haemoprotein receptors. One *M. catarrhalis* ORF of 2886 bp (961 aa), with a predicted molecular mass of 107 kDa, was cloned and sequenced. Based on the BLAST homology results and the data presented below, we designated this protein MhuA (*Moraxella* Hb utilization).

In order to determine whether MhuA was involved in the ability of *M. catarrhalis* 7169 to utilize haemoproteins for growth, an isogenic *mhuA* mutant was constructed and termed 7169::*mhuA*, as described above.

**MhuA binding of Hb–agarose**

To determine the ability of MhuA to bind Hb directly, OMPs from *M. catarrhalis* 7169 were tested for their ability to bind Hb–agarose. The eluted protein fractions were analysed by SDS-PAGE, revealing the presence of multiple bands (Fig. 1a). However, a Western blot (Fig. 1b) probed with mAb 3F5-5E5 demonstrated that MhuA was present both in the Hb–agarose-bound fraction (Fig. 1b, lane 3) and in the 2 M guanidine/HCl-eluted fraction (Fig. 1b, lane 6). Furthermore, it is important to note that MhuA was not detected in the concentrated wash samples (Fig. 1b, lane 4), nor was there any evidence of this protein in the 3 M NaCl-elution fraction (Fig. 1b, lane 5). These studies confirm that MhuA can bind directly to human Hb and these data also suggest that there is a strong interaction between these two components.

**Growth analyses of *M. catarrhalis* 7169 and 7169::*mhuA* with Hb as the sole iron source**

To determine whether MhuA was specific for Hb, growth analyses were performed by comparing the ability of *M. catarrhalis* 7169 versus 7169::*mhuA* to grow with iron [Fe(NO₃)₃], Hm or Hb as sole iron sources. The ability of *M. catarrhalis* 7169::*mhuA* to utilize Fe(NO₃)₃ (Fig. 2a) or Hm (Fig. 2b) as the sole iron source was unchanged, compared with that of *M. catarrhalis* 7169. In the presence of Hb, wild-type *M. catarrhalis* 7169 exhibited growth rates consistent with those seen when the organism was cultured with Fe(NO₃)₃ (Fig. 2c). In contrast, *M. catarrhalis* 7169::*mhuA* showed a significantly (*P* < 0.04) decreased ability to utilize Hb as a sole source of iron, and this restricted growth was consistent throughout the entire time course of the experiment (Fig. 2c). When cultures were allowed to grow for 24 h, *M. catarrhalis* 7169::*mhuA* was unable to obtain wild-type levels of growth (data not shown).

![Fig. 1. Hb–agarose binding of MhuA. (a) SDS-PAGE (7%); (b) Western blot (mAb 3F5-5E5). Lanes: 1, CDM/Hb 7169 OMPs; 2, CDM/Hb 7169::mhuA OMPs; 3, Hb–agarose beads after incubation with CDM/Hb 7169 OMPs; 4, concentrated washes; 5, 3 M NaCl elution; 6, 2 M guanidine/HCl elution. Asterisks indicate the presence of MhuA.](http://mic.sgmjournals.org)
Expression of MhuA under various growth conditions

To determine the effects of various iron sources on the expression of MhuA, RT-PCR analysis of mhuA transcription and OMP analysis of wild-type M. catarrhalis 7169 were performed following growth in either CDM 0, CDM 100, CDM/Hm or CDM/Hb. RNA was isolated from each culture and the concentration was standardized for use in RT-PCR. Equal amounts of product were loaded onto an agarose gel, which revealed that mhuA transcript was detected under all conditions evaluated (Fig. 3).

Analysis of OMP profiles, depicted by SDS-PAGE in Fig. 4(a), demonstrated the loss of a 107 kDa band seen in lanes 2, 4 and 6, as expected for strain 7169::mhuA. MhuA was expressed in M. catarrhalis 7169 under all conditions tested (Fig. 4a, lanes 1, 3 and 5). TonB-dependent OMP expression is often iron-regulated and can be haem-regulated (Genco & Dixon, 2001; Wandersman & Stojiljkovic, 2000). It is important to note that the expression of MhuA appears unchanged under conditions of iron stress and haem stress.

Specificity and epitope conservation of mAb 3F5-5E5 to M. catarrhalis MhuA

mAb 3F5-5E5 was developed to iron-stressed OMPs of M. catarrhalis strain 25240 and previous Western blot analysis demonstrated that this antibody reacted to a 107 kDa protein expressed in M. catarrhalis 7169 (data not shown). To confirm the expression of MhuA, Western blot analysis was performed on the SDS-PAGE gel in Fig. 4(a), comparing reactivity of mAb 3F5-5E5 to MhuA expressed under the various growth conditions. Fig. 4(b) demonstrates that mAb 3F5-5E5 was specific for MhuA under all conditions. These data, together with the RT-PCR data,
suggest strongly that MhuA is expressed constitutively by *M. catarrhalis* 7169.

In order to determine the level of conservation exhibited by MhuA, whole bacterial samples were prepared from a series of *M. catarrhalis* clinical isolates from various geographical locations. Fig. 5 is a representative Western blot analysis probed with mAb 3F5-5E5, demonstrating that all of these isolates expressed MhuA. In addition, all of these strains were reactive by colony-lift analyses and flow cytometry (data not shown). These studies confirm that the Hb-utilization protein MhuA is surface-exposed and conserved among strains associated with human disease. These analyses were also performed on the Gram-negative organisms *Moraxella bovis*, *Moraxella lacunata*, *Haemophilus ducreyi*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Neisseria cinnerea*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. MhuA expression was not detected in any of these other species, suggesting that the epitope recognized by mAb 3F5-5E5 may be specific to *M. catarrhalis* (data not shown).

**DISCUSSION**

One of the overlooked areas in *Moraxella* pathogenesis is nutrient acquisition. Whilst it is assumed that this pathogen has many mechanisms that are similar to those of other human mucosal pathogens, the only systems that have been reported involve iron acquisition through the use of transferrin, lactoferrin and haem (Campagnari *et al.*, 1994; Furano & Campagnari, 2004; Luke & Campagnari, 1999; Luke *et al.*, 1999; Schryvers *et al.*, 1998; Stojiljkovic *et al.*, 1996). As stated previously, iron is an essential element that is required for growth and metabolism of most bacteria and understanding the strategies of iron uptake for *M. catarrhalis* will provide important information relating to the ability of this pathogen to colonize and survive on host tissues.

Whilst it has previously been shown that this *M. catarrhalis* strain expresses transferrin- and lactoferrin-receptor systems and can utilize these iron-carrier proteins for growth, the actual mechanism as to how these systems function has not been clearly defined (Campagnari *et al.*, 1994; Luke & Campagnari, 1999; Luke *et al.*, 1999; Schryvers *et al.*, 1998). It was initially thought that these proteins probably function in the same manner as the other, well-studied transferrin and lactoferrin receptors. However, in the case of the transferrin receptors in *Moraxella*, these genes are arranged in the opposite orientation and there is a third, undefined ORF present; thus, there may be functional differences. Until now, the lactoferrin and transferrin receptors are the only known iron-acquisition systems that have been reported for *M. catarrhalis*. In addition, this bacterium does not secrete siderophores and no haemolysins have been identified (Campagnari *et al.*, 1994).

Haem is the most abundant source of iron in the human body (Otto *et al.*, 1992). Previous studies have demonstrated

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**Fig. 4.** OMP-profile analyses of wild-type *M. catarrhalis* 7169 and 7169::mhuA by SDS-PAGE (7% gel) (a) and Western blot with mAb 3F5-5E5 (b). Lanes: 1, strain 7169 grown in CDM 100; 2, mutant 7169::mhuA grown in CDM 100; 3, strain 7169 grown in CDM/Hm; 4, mutant 7169::mhuA grown in CDM/Hm; 5, strain 7169 grown in CDM/Hb; 6, mutant 7169::mhuA grown in CDM/Hb. Molecular masses (kDa) are shown. *M. catarrhalis* 7169 MhuA appears to be expressed at similar levels under all growth conditions tested.

**Fig. 5.** Conservation of MhuA among a panel of *M. catarrhalis* geographically diverse clinical isolates. (a) SDS-PAGE (7% gel) analysis of whole-bacterial preparations from *M. catarrhalis* 7169 (1), CCUG 3292 (2), O35E (3), 7544 (4), RS10 (5), 43617 (6), 7477 (7), 7680 (8), 7530 (9), 7608 (10), 25240 (11), 340535 (12) and 7169::mhuA (13). (b) Western blot analysis with mAb 3F5-5E5 reveals that MhuA is expressed in all strains (7169::mhuA was used as a negative control).
that numerous bacteria can utilize haem, Hb and other haemoprotein complexes as both haem and iron sources (Chen et al., 1996; Genco & Dixon, 2001; Lewis et al., 1997, 1998, 1999; Stojiljkovic et al., 1996; Wandersman & Stojiljkovic, 2000). Multiple different haemoprotein-utilization systems, some of which are redundant, have been characterized for pathogenic species of the genera Neisseria, Haemophilus, Escherichia, Shigella, Yersinia, Pseudomonas, Vibrio and Serratia (Morton et al., 1999; Ochsner et al., 2000; Wandersman & Stojiljkovic, 2000). Many of these systems are related by their reliance on the energy-transducing TonB/ExbB/ExbD protein complex that is present in the inner membrane and periplasmic space. It is thought that, during inflammation, both Hb and free haem are present on mucosal surfaces, making them viable options as iron sources for these pathogens (Schryvers & Stojiljkovic, 1999).

As M. catarrhalis is a mucosal pathogen, the availability of haem and Hb would provide an obvious advantage, particularly in the middle ear during AOM. To date, there has not been any such system described for M. catarrhalis. In fact, there are conflicting reports involving this bacterium that, during inflammation, both Hb and free haem are present as iron sources for these pathogens (Schryvers & Stojiljkovic, 1999).

Another important observation presented in our studies is the identification of the MhuA protein, which appears to be a highly conserved OMP that binds Hb and is probably involved in the subsequent utilization of this iron source. Whilst our studies have not as yet defined the specific role of MhuA, the significant growth difference between the mutant and the wild-type, as we show in the growth studies, relates directly to the disruption of a single gene. These data provide further support for the involvement of MhuA in Hb utilization. The binding of other unidentified proteins to the Hb-agarose column is consistent with previous data obtained by other investigators studying the Hb-binding activity of proteins from various organisms (Archambault et al., 2003; Bracken et al., 1999; Dashper et al., 2000; Lee & Levesque, 1997; Sengupta et al., 1999). Due to the varied haem and iron sources that are present in the human host and the varied degree of receptor specificities for haemoproteins, it is not surprising that multiple receptor systems exist for haem uptake in many bacterial species (Cope et al., 1995, 1998; Genco & Dixon, 2001; Lewis et al., 1999; Wandersman & Stojiljkovic, 2000). Like these related organisms, other Hb-binding and -utilization proteins are likely to exist for M. catarrhalis, suggesting the possibility of redundant systems.

This study is the first to demonstrate the ability of M. catarrhalis to grow with Hb as the sole iron source and to define a putative Hb receptor. Future studies will be designed to determine the actual function of MhuA in this Hb system and also to determine whether in vivo expression elicits human antibodies. However, far more studies are needed to define the steps involved in colonization and survival on human mucosal surfaces. It will be important to continue to characterize the mechanism(s) of iron uptake via haemoproteins by M. catarrhalis, in order to increase our knowledge of the biological systems utilized by this insufficiently studied bacterium and the role that these systems play in colonization and pathogenesis.

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M. catarrhalis and haemoglobin utilization


