Biometals (2007) 20:365–377
DOI 10.1007/s10534-006-9058-3

Iron acquisition in the dental pathogen *Actinobacillus actinomycetemcomitans*: What does it use as a source and how does it get this essential metal?

Eric R. Rhodes · Sharon Menke · Christopher Shoemaker · Andrew P. Tomaras · Glen McGillivary · Luis A. Actis

Received: 20 July 2006 / Accepted: 28 November 2006 / Published online: 6 January 2007

Abstract *Actinobacillus actinomycetemcomitans* requires iron to grow under limiting conditions imposed by synthetic and natural chelators. Although none of the strains tested used hemoglobin, lactoferrin or transferrin, all of them used $FeCl_3$ and hemin as iron sources under chelated conditions. Dot-blot binding assays showed that all strains bind lactoferrin, hemoglobin, and hemin but not transferrin. When compared with smooth strains, the rough isolates showed higher hemin binding activity, which was sensitive to proteinase K treatment. *A. actinomycetemcomitans* harbors the Fur-regulated *afeABCD* locus coding for iron acquisition in isogenic and non-isogenic cell backgrounds. The genome of this oral pathogen also harbors several other predicted iron uptake genes including the *hitABC* locus, which restored iron acquisition in the *E. coli* 1017 *ent* mutant. However, the disruption of this locus in the parental strain did not affect iron acquisition as drastically as the inactivation of AfeABCD, suggesting that the latter system could be more involved in iron transport than the HitABC system. The genome of this oral pathogen also harbors an active copy of the *exbBexbDtonB* operon, which could provide the energy needed for hemin acquisition. However, inactivation of each coding region of this operon did not affect the hemin and iron acquisition phenotypes of isogenic derivatives. This observation suggests that the function of these proteins could be replaced by those coded for by *tolQ*, *tolR* and *tolA* as it was described for other bacterial transport systems. Interruption of a *hasR* homolog, an actively transcribed gene that is predicted to code for an outer membrane hemophore receptor protein, did not affect the ability of an isogenic derivative to bind and use hemin under chelated conditions. This result also indicates that *A. actinomycetemcomitans* could produce more than one outer membrane hemin receptor as it was described in other human pathogens. All strains tested formed biofilms on plastic under iron-rich and iron-chelated conditions. However, smooth strains attached poorly and formed weaker biofilms when compared with rough isolates. The incubation of rough cells in the presence of $FeCl_3$ or hemin resulted in an increased number of smaller aggregates and microcolonies as compared to the fewer but larger aggregates formed when cells were grown in the presence of dipyridyl.

Keywords Iron acquisition · Hemin acquisition · Iron regulation · Biofilms

E. R. Rhodes · S. Menke · C. Shoemaker · A. P. Tomaras · G. McGillivary · L. A. Actis (✉)
Department of Microbiology, Miami University, 40 Pearson Hall, Oxford, OH 45056, USA
e-mail: actisla@muohio.edu

Introduction

Actinobacillus actinomycetemcomitans, recently renamed *Aggregatibacter actinomycetemcomitans* (Nørskov-Lauritsen and Kilian 2006), is a Gram-negative coccobacillus that colonizes the oral cavity of humans and Old World Primates. This oral pathogen has been strongly implicated in adult periodontitis (Zambon 1985), although the main disease that this bacterium causes is Localized Juvenile Periodontitis (LJP) also known as Localized Aggressive Periodontitis (LAP) (Armitage 1999; Araujo 2002). This is one of the most severe forms of periodontal disease that could lead to progressive destruction of dental tissue that eventually results in the damage of the supportive bone structure causing premature tooth loss (Meyer and Fives-Taylor 1997). *Actinobacillus actinomycetemcomitans* is a member of the HACEK bacterial group that has been implicated in the pathogenesis of extra-oral infections such as subacute endocarditis (Berbari et al. 1997). There is a lot of interest in periodontal infections being a risk factor for the development of cardiovascular diseases (Genco et al. 2002). In fact, *A. actinomycetemcomitans* has been detected in 18% of atherosclerotic plaque samples (Haraszthy et al. 2000).

There are a number of strains that are currently being studied, which have different colony morphology and behave differently under the same experimental conditions. Colony morphology and adhesion properties play a role in how these bacteria differ (Fine et al. 1999). Most fresh clinical isolates appear as rough colonies on agar plates and adhere to each other and to inanimate surfaces (Fine et al. 1999). Other strains have a smooth colony morphology and do not adhere to themselves or solid surfaces, typical behavior of strains that have been repeatedly cultured in rich broth (Fine et al. 1999). The transformation from the rough to smooth phenotype is associated with the loss of fimbriae (Kachlany et al. 2000). These phenotypic changes are also associated with the ability of this pathogen to cause disease in an experimental model that mimics the infection process in humans (Schreiner et al. 2003). The researchers used Sprague-Dawley rats that were inoculated orally by directly adding bacteria to their food for eight days. They monitored the infection by looking at the ability of the bacteria to colonize and persist in the oral cavity, to elicit a humoral immune response, and cause significant bone loss. The data obtained with this animal model demonstrated the necessity to study strains that are closer to the fresh clinical isolates obtained from LAP patients because strains with smooth colony morphology did not persist in the oral cavity, elicit a humoral immune response, or cause any bone loss in experimentally infected animals (Schreiner et al. 2003).

Binding and utilization of different iron sources

Like many other human pathogens, *A. actinomycetemcomitans* requires iron to grow under iron-limiting conditions similar to those encountered in the host. Such conditions are imposed by the production of iron-binding compounds such as ferritin, hemin, transferrin and particularly lactoferrin, which is present in secretions like saliva in the human oral cavity. Bacteria have responded to this nutrient limitation by expressing high-affinity iron acquisition systems that acquire iron by either interacting directly with host-binding proteins or scavenging iron from these proteins via the secretion and internalization of chelators known as siderophores (Crosa et al. 2004). Experimental evidence showed that *A. actinomycetemcomitans* does not produce high-affinity siderophores that could provide the needed iron under limiting conditions (Winston et al. 1993). This finding seems to be supported by the annotation of the genome sequence of the strain HK1651 (http://www.oralgen.lanl.gov/), which did not reveal the presence of genes required to code for the functions needed for the biosynthesis, secretion, internalization and utilization of a particular siderophore as it has been described for other pathogens (Crosa and Walsh 2002; Wandersman and Delepelaire 2004). On the other hand, these experimental and in silico observations are in accordance with the fact that this oral pathogen belongs to the *Pasteurellaecea* family in which some of its members, such as *Haemophilus influenzae*, are also known for their

inability to produce siderophore compounds (Gray-Owen and Schryvers 1996).

The direct interaction with host iron-holding products is an alternative pathway bacteria use to acquire iron. As it was the case for the production of siderophores, experimental evidence showed that *A. actinomycetemcomitans* does not bind and use human transferrin as an iron source (Winston et al. 1993; Hayashida et al. 2002). Such a finding is also in accordance with the gene annotation of this pathogen and the sequence analysis of some strains that revealed either the absence of active genes or the presence of transferrin-binding pseudogenes, a trait that seems to be common among different *A. actinomycetemcomitans* isolates (Winston et al. 1993; Hayashida et al. 2002). In contrast, the interaction of different *A. actinomycetemcomitans* isolates with lactoferrin seems to be more variable, with some strains unable to bind lactoferrin (Winston et al. 1993) and others capable of binding iodinated human and bovine lactoferrin (Alugupalli et al. 1995). The latter observation is in agreement with our experimental data obtained using strains representing different serotypes and colony morphologies. The mildly rough serotype b strain HK1651, a JP2 clone that overexpresses leukotoxin activity (Haubek et al. 1996) that was selected to sequence and annotate the genome of this oral pathogen (http://www.oralgen.lanl.gov/), was used as the reference strain to obtain the nucleotide sequence of genes of interest. The DF2200N and CU1000 strains, both of which are not JP2-like isolates (Kaplan et al. 2002), are aggregative isolates that express the phenotype of fresh clinical isolates and represent the serotypes groups a and f, respectively. The strain CU1060 is a smooth isogenic derivative of CU1000 with a mutation in the *flp-tadG* region that impairs the formation of long bundled fibrils (Kachlany et al. 2000). All four strains were able to bind human lactoferrin labeled with horseradish peroxidase, when tested using dot blot assays, independently of whether the cells were cultured under iron-rich or iron-chelated conditions. In spite of their ability to bind lactoferrin, none of the four strains were able to grow around filter disks containing human lactoferrin, either fully or partially saturated with iron when plated on AAGM agar supplemented with 250 μM dipyridyl.

Our experimental observations are in accordance with those made by Hayashida et al. (Hayashida et al. 2002) in which none of the 10 strains tested used lactoferrin as an iron source when streaked on chelated BHI agar. Taken together all these studies show that although some *A. actinomycetemcomitans* strains bind lactoferrin, none of the strains tested so far use this protein as an iron source under chelated conditions. The latter behavior is supported by the annotation of the HK1651 genome, which did not reveal the presence of genes coding for an active lactoferrin binding and utilization system as it was described in other bacterial pathogens that use this human protein as an iron source (Gray-Owen and Schryvers 1996). However, the cellular factors and/or proteins responsible for the binding of lactoferrin, which were implicated in a previous study (Alugupalli et al. 1995), remain poorly understood. Also poorly understood is the biological significance of the interaction of this oral pathogen with lactoferrin, which inhibits *A. actinomycetemcomitans* attachment to buccal epithelial cells when saturated with iron (Fine and Furgang 2002).

Hemoglobin is also an alternative source of iron that *A. actinomycetemcomitans* cells can use under chelated conditions (Grenier et al. 1997). More recent studies showed that such a capacity depends on the presence and expression of *hgpA*, a gene that codes for a homolog of the *H. influenzae* hemoglobin protein binding A (Hayashida et al. 2002). In our hands, none of the four strains we tested were able to use this compound as an iron source to grow under iron-chelated conditions, although all of them were able to bind biotin-labeled human hemoglobin when tested with dot blot assays. The failure to use this iron source could be explained by the presence of deleterious mutations such as those reported within the pseudogene found in the HK1651 and other strain genomes (Hayashida et al. 2002). However, the hemoglobin binding ability of HK1651 (as well as that of the other strains we tested) remains to be explained, considering that one report implicates the lipopolysaccharides produced by this bacterium in its ability to interact with hemoglobin (Grenier et al. 1997).

According to previous reports (Grenier et al. 1997; Graber et al. 1998; Hayashida et al. 2002), hemin is another compound that this oral pathogen could bind and use as an iron source to grow under iron-limiting conditions. These early observations are in agreement with our experimental data showing that the strains HK1651, CU1000, CU1060, and DF2200N grow around filter disks saturated with hemin deposited on chelated AAGM agar plates. Furthermore, the incubation of these strains in AAGM broth containing hemin resulted in cell pellets that were stained after washing with saline solution (Fig. 1A, top panel), indicating the ability of the cells to bind this iron-containing compound. This ability was confirmed with dot blot assays that were developed with 3,3′-dimethoxybenzidine dihydrochloride (DMB) (Fig. 1A, bottom panel), which detects the hemin-associated peroxidase activity (Fouz et al. 1996). Interestingly, the hemin-binding activity was significantly diminished when the cells were pretreated with proteinase K, while no significant changes were observed when the samples were incubated with sodium metaperiodate. Visual inspection of the pellets of cells incubated in the presence of hemin showed that the cell pellet of the smooth strain CU1060 was less stained than that of rough cells (compare samples 2 and 3 with sample 4 in top panel of Fig. 1A). This observation indicates that rough strains bind more hemin than smooth isolates, a possibility that was examined by testing their ability to bind Congo red, which could reflect hemin binding as it was described for other pathogens such as *Shigella flexneri* (Stugard et al. 1989) and *Y. pestis* (Perry et al. 1990). Colorimetric assays, which measured the amount of Congo red eluted with DMSO from cells cultured in AAGM broth containing 30 μg/ml of this stain (Fig. 1B), showed that the binding of Congo red is proportional to the roughness and aggregative nature of the *A. actinomycetemcomitans* strains. Although not shown, the DF2200N cells displayed a similar behavior when tested under the same experimental conditions.

Finally, the iron-utilization assays also showed that all strains we tested are able to use inorganic iron ($FeCl_3$ solubilized in 0.1 N HCl) to grow on chelated AAGM agar plates. Altogether the

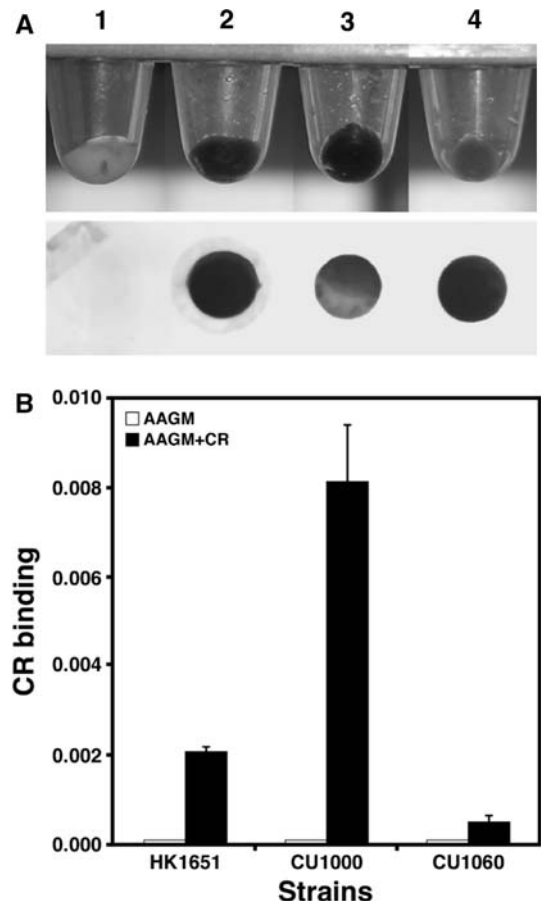

Fig. 1 Binding of hemin and Congo red by bacterial cells. (**A**) The binding of hemin was determined by visual inspection after the *Escherichia coli* DH5α (1) and *A. actinomycetemcomitans* HK1651 (2), CU1000 (3) and CU1060 (4) cells were washed with saline solution or tested with dot-blot assays using DMB to detect the peroxidase activity associated with hemin. *E. coli* DH5α was used as a negative control. (**B**) The binding of Congo red by cells cultured in AAGM agar containing 30 μg/ml of this stain, which was eluted from the cells with dimethyl sulfoxide, was quantified photocolorimetrically. The amount of stain eluted from the cells was normalized to the protein content of each sample. Error bars represent 1 SD

results of our studies and those published before demonstrate that hemin and inorganic iron are sources that are used by all tested strains, while a significant variability to bind and use iron sources is apparent among different clinical isolates of this oral pathogen. Furthermore, the observation that the hemin binding activity is susceptible to treatment with proteinase K indicates that this

iron-containing compound interacts with an unidentified protein that appears to be located on the outer membrane facing the extracellular environment.

The AfeABCD and HitABC PBT systems

The afeABCD *operon*

The utilization of inorganic iron to grow under chelated conditions indicates that *A. actinomycetemcomitans* strains could acquire this metal through the expression of periplasmic-binding protein-dependent transport (PBT) systems that require neither an outer membrane receptor nor a specific ligand and function independently of the TonB-ExbB-ExbD system. The first description of a PBT system was that of the *S. marcescens* SfuABC system (Angerer et al. 1990; Angerer et al. 1992), which was followed by reports describing similar transport systems in pathogens such as *H. influenzae* (Sanders et al. 1994), *Neisseria* (Chen et al. 1993), *Y. enterocolitica* (Saken et al. 2000; Gong et al. 2001), and *Y. pestis* (Bearden et al. 1998; Bearden and Perry 1999).

The annotation of the HK1651 genome (http://www.oralgen.lanl.gov/) and our in silico analyses showed the presence of at least four gene clusters coding for proteins related to components of PBT systems described in other bacterial pathogens (Fig. 2). We initiated the analysis of these systems by testing the expression of the *Y. pestis yfeABCD* homolog, which we named *afeABCD* (Rhodes et al. 2005). PCR cloning and nucleotide sequence analyses showed that the genome of the strains CU1000 and DF2200N also harbor this locus, which proved to be expressed as a polycistronic message in all three strains. The transcription of this locus is controlled by a promoter element located upstream of *afeA*, which contains a Fur box that mediates the binding of the Fur repressor proteins and controls the differential transcription of this operon in response to the iron content of the medium. Computer analysis of these genes showed that they code for all the components of a PBT iron transport system, in which AfeA is the putative periplasmic iron-binding protein and the AfeBCD proteins form the permease and ATPase components of this ABC metal transporter. The functionality of this PBT system was confirmed by its ability to restore the growth of *E. coli* 1017 in the presence of dipyridyl, when cloned and transformed into this enterobactin deficient strain. This complementing activity was abolished when any of the coding regions of this operon was inactivated by in vitro transposition with the EZ-Tn5 ⟨KAN-2⟩ element. The results obtained with this heterologous cell background were confirmed by testing the iron acquisition capacity of isogenic insertion derivatives obtained by transforming artificially induced DF2200N competent cells with a suicide-cloning vector that harbors DNA uptake sequences, in which the insertion derivatives of the *afe* genes were cloned. Such an approach showed that the expression of all AfeABCD components is needed for the cells to grow under iron-chelated conditions (Fig. 3). Furthermore, the immunoblot analysis of *E. coli* 1017 and DF2200N harboring either the parental *afeA* allele or the *afeA*:: EZ-Tn5 ⟨KAN-2⟩ insertion derivative linked the expression of this coding region to the production of a 32-kDa protein related to the *H. influenzae* YfeA periplasmic protein detected in the *A. actinomycetemcomitans* Y4 strain (Graber

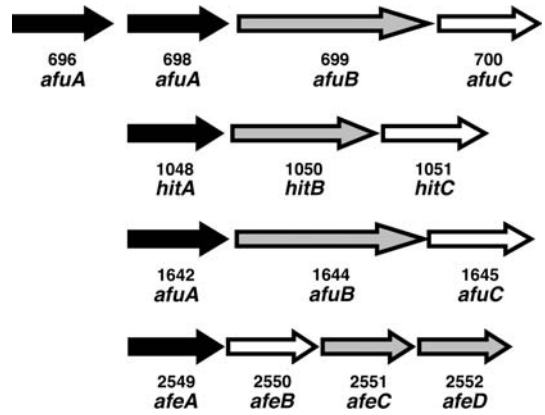

Fig. 2 Genetic organization of *A. actinomycetemcomitans* HK1651 open reading frames predicted to code for PBT systems involved in iron acquisition. The numbers represent the AA coding regions according to the genome annotation of this strain. The arrows represent the location and direction of transcription of each coding region. Open reading frames coding for equivalent functions in each system are identified with arrows of the same design

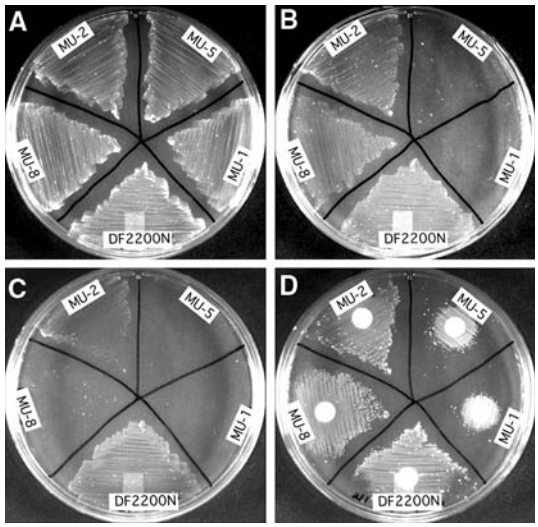

Fig. 3 Analysis of the iron uptake proficiency of the *A. actinomycetemcomitans* isogenic mutants in *afeA* (MU-1), *afeB* (MU-5), *hitA* (MU-8), *hitB* (MU-2) and the parental strain DF2200N. Growth of the strains was compared after plating on AAGM agar (**A**), AAGM agar supplemented with 235 μM dipyridyl (**B**), or 265 μM dipyridyl (**C**). (**D**) Growth around $FeCl_3$-saturated paper filter discs on AAGM agar plates supplemented with 325 μM dipyridyl of the DF2200N parental strain as compared to the MU-1, MU-5, MU-8, and MU-2 isogenic insertion derivatives

et al. 1998). It is worthy to note that the growth of cells harboring interrupted *afe* genes was rescued by adding $FeCl_3$ (Fig. 3) but not $ZnCl_2$ or $MnCl_2$ to iron-chelated media, an observation that indicates the iron specificity of this PBT system. It is also important to note that the DF2200N isogenic derivative harboring the *afeA*::EZ-Tn5 ⟨KAN-2⟩ allele was able to grow around filter disks saturated with hemin when plated on chelated AAGM agar plates. This result indicates that the AfeABCD system is involved in the acquisition of inorganic iron but not in the utilization of hemin as an iron source.

The hitABC *operon*

An approach similar to that used for the analysis of *afeABCD* showed that the *hitABC* locus identified during the annotation of the HK1651 genome (Fig. 2) is also present in the genome of the strains CU1000 and DF2200N. Furthermore, all three strains express this locus at the transcriptional level when cultured in AAGM under iron-chelated conditions. According to the gene annotation data, the product of *hitA* is a periplasmic iron binding protein while the HitB and HitC are proteins with predicted permease and ATPase activities, respectively. All these proteins are highly related to other bacterial proteins, particularly those of *H. influenzae*, which are part of predicted ABC metal transporters. Interestingly, this operon has only one gene coding for a permease (*hitB*) that is located between the *hitA* periplasmic iron binding protein gene and the *hitC* ATPase gene (Fig. 2). In contrast, the *afeABCD* locus includes two genes, *afeC* and *afeD*, coding for permeases, both of which are located at the end of the operon after *afeB*, which codes for the ATPase component of this ABC transporter. These observations and predictions are in accordance with the ability of this operon to complement the iron uptake deficiency of *E. coli* 1017, when cloned in the vector pACYC184 and transformed into this *ent* mutant strain. Insertion of EZ::TN ⟨*ori*V/KAN-2⟩ into each of the coding regions of this operon abolished the complementing activity, a result that proves that this locus codes for iron acquisition functions that are expressed in a heterologous cell background.

The role of the HitABC system in iron acquisition was further confirmed by creating isogenic derivatives in which each parental allele was replaced by homologous recombination with the cognate EZ::TN ⟨*ori*V/KAN-2⟩ insertion modification. Plating the *hitA* and *hitB* insertion derivatives, named MU-8 and MU-2, respectively, on AAGM agar with increasing dipyridyl concentrations showed that the inactivation of these components of the *hitABC* locus affects the ability of *A. actinomycetemcomitans* to grow under conditions of increasing iron chelation (Fig. 3). However, these isogenic derivatives were not as affected in their ability to grow under iron-chelated conditions as the MU-1 and MU-5 derivatives, which harbor a transposon insertion in *afeA* and *afeB*, respectively. Although all four mutant grew as well as the parental strain when plated on AAGM agar (panel A), the addition of 235 μM dipyridyl diminished but not abolished the growth of the MU-2 and MU-8 insertion derivatives with almost no effect on the DF2200N

parental strain (panel B). In contrast, this concentration of iron chelator was enough to reduce the growth of the MU-1 and MU-5 mutants drastically. The increase of the iron chelator concentration to 265 μM affected the growth of the parental strain slightly and reduced the growth of the MU-2 and MU-8 mutants further, while inhibiting the growth of the MU-1 and MU-5 mutants completely (panel C). There was also a significant difference in the response of these mutants when fed with inorganic iron. The HitA (MU-8) and HitB (MU-2) mutants formed similar growth halos on AAGM agar containing 325 μM dipyridyl around filter disks saturated with $FeCl_3$ (Fig. 3, panel D). These growth halos were just slightly smaller in size to that produced by the parental strain. In contrast, the growth halos formed by the AfeA (MU-1) and AfeB (MU-5) mutants were much smaller. The same results were obtained when the disks were saturated with ferric citrate.

Taken together, these results show that the *hitABC* operon codes for an iron acquisition system that allows *A. actinomycetemcomitans* to grow under iron-chelated conditions as it was observed with the AfeABCD system (Rhodes et al. 2005). However, isogenic mutants affected in the expression of the latter system are more sensitive to iron-chelated conditions and cannot use inorganic iron as a sole source of iron as well as the derivatives affected in the expression of the HitABC system. This suggests that under the conditions we tested the AfeABCD system plays a more significant role in iron acquisition than the HitABC system. Whether this is also the case during the pathogenesis of oral infections is a possibility that remains to be investigated using these mutants in the experimental infection model that mimics the outcome of the disease this pathogen causes in the human oral cavity (Schreiner et al. 2003). We are testing the role of the HK1651 *afuAABC* and *afuABC* loci (Fig. 2), which also proved to be present in the genome of the strains CU1000 and DF2200N and been expressed in all three strains when cultured under iron-chelated conditions. Interestingly, RT-PCR showed that the first coding region of the *afuAABC* locus is the only one that is not part of a polycistronic locus, a result that is compatible with the presence of a predicted rho-independent transcriptional termination signal between the coding regions AA00696 and AA00698 (Fig. 2).

Hemin utilization functions

Hemin uptake functions

Dot-blot and iron utilization assays showed that *A. actinomycetemcomitans* binds and uses hemin as an iron source when cultured under iron-chelated conditions. Bacteria acquire iron from hemin either through hemophore-dependent or -independent mechanisms, all of which require energy provided by the TonB-ExbB-ExbD transducing system (Wandersman and Delepelaire 2004). Currently, the cellular products and molecular mechanisms by which *A. actinomycetemcomitans* interacts with hemin have not been identified and characterized. Furthermore, the annotation of the HK1651 showed that, with the exception of the predicted coding region AA02782, there are no genes coding for proteins significantly related to hemin acquisition systems. Based on the insights gained with these in silico analyses, we initiated our analysis of hemin utilization functions in this oral pathogen by using a directed approach focused on the *hasR* (AA02782) locus. The product of this gene has been annotated as an outer membrane hemophore receptor protein with the highest similarity to the *P. multocida* HasR predicted protein. In *A. actinomycetemcomitans*, the *hasR* homolog is predicted to code for an 875-amino acid protein with a molecular mass of 99.7 kDa. This predicted protein has a domain that is similar to the TonB dependent/Ligand-Gated channel domain of FepA, an outer membrane iron siderophore transporter from *E. coli*. The domain consists of a monomeric 22 strand anti-parallel beta-barrel, and N-terminal residues that form a plug on the periplasmic end of the barrel (Klebba 2004). PCR and RT-PCR showed that this HK1651 locus is also present in the chromosome of the strain CU1000 and DF2200N, with all of them being transcribed in cells cultured under iron-chelated conditions.

The role of the HasR protein in hemin binding and utilization was tested by creating an isogenic derivative by in vitro transposition mutagenesis and allelic exchange as we did for the analysis of the *afeABCD* operon (Rhodes et al. 2005). The derivative with an insertion in the *hasR* gene (MU-4) grew just as well as DF2200N parental strain when plated on AAGM agar without any supplementation while both of them were not able to grow on this medium containing 325 μM dipyridyl. Iron utilization experiments showed that both the parental strain DF2200N and the MU-4 isogenic derivative harboring a *hasR*::EZ::TN ⟨*ori*V/KAN-2⟩ allele grew around hemin-saturated filter disks deposited on chelated AAGM agar (Fig. 4A). Incubation of MU-4 and DF2200N cells in AAGM broth containing hemin resulted in cell pellets that were similarly stained, an indication that the insertion inactivation of *hasR* does not affect the binding of this iron-containing compound.

Taken together, these results indicate that *hasR* is not essential for the ability of *A. actinomycetemcomitans* to bind and use hemin as an iron source. At the present time it is not possible to establish whether HasR plays any role in this process because this bacterium may be able to produce other hemin receptors that complement the function of the interrupted HasR. In fact, many pathogens have multiple hemin receptors. *Vibrio cholerae* has three different hemin receptors in which there must be a mutation in all of them for the bacterium to be unable to use hemin as a sole source of iron (Mey and Payne 2001). The possibility that this is also the situation with *A. actinomycetemcomitans* is supported by the observation that the HK1651 genome has four additional open reading frames that could function in hemin acquisition. They are a predicted hemin receptor (AA00490), a putative hemin storage protein (AA00491) or hypothetical proteins (AA00492 and AA00493) that could be part of an operon with the previous gene, which could function in hemin utilization. The role of these genes and their products is being analyzed with the same approach used for the study of *hasR*.

Energy transducing functions

As mentioned earlier, bacterial hemin utilization is an active process that requires the energy provided by the TonB-ExbB-ExbD transducing system (Wandersman and Delepelaire 2004). In accordance with the ability of *A. actinomycetemcomitans* to use this compound as a source to grow under iron-chelated conditions, the annotation of the HK1651 genome showed the presence of the coding regions AA01124, AA01124.1 and

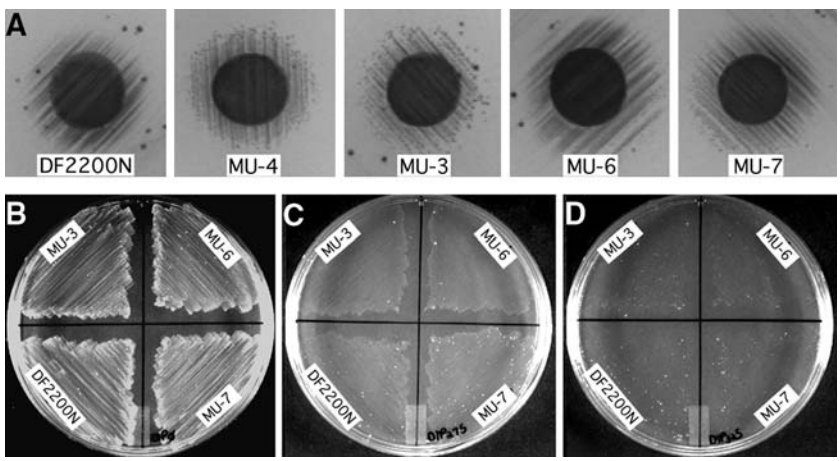

Fig. 4 Analysis of the hemin utilization and iron acquisition proficiency of the *A. actinomycetemcomitans* isogenic mutants in *exbB* (MU-3), *exbD* (MU-6), *tonB* (MU-7), *hasR* (MU-4) and the parental strain DF2200N. (**A**) Growth around filter disks saturated with hemin was compared after the strains were streaked on AAGM agar supplemented with 325 μM dipyridyl. Growth of the parental strain DF2200N as compared to MU-3, MU-6 and MU-7 on AAGM agar (**B**), or AAGM agar supplemented with 275 μM (**C**) or 325 μM (**D**) dipyridyl

AA01127. These genes are predicted to code for the *A. actinomycetemcomitans* ExbB, ExbD and TonB homologs, respectively. PCR and RT-PCR showed that strains HK1651, CU1000 and DF2200N all contain and express these three genes, which are part of a polycistronic transcript.

The predicted ExbB protein contains 156 amino acids, which is 17.6 kDa in size. Amino acids 73–89 and 105–121 are predicted to each form a transmembrane domain. ExbB has a conserved domain similar to the *E. coli* MotA protein (Marchler-Bauer et al. 2005), which is an essential component of the flagellar motor that uses a proton gradient to generate rotational motion in the flagella. This conserved domain belongs to the MotA/TolQ/ExbB proton channel family, which is made of integral membrane proteins. ExbD is a predicted protein of 148 amino acids and is 16.2 kDa in size. A conserved-domain search showed that ExbD is similar to the biopolymer transport proteins ExbD/TolR. ExbD and TolR are involved in TonB-dependent transport of various receptor bound substrates (Braun 1989; Bradbeer 1993; Cascales et al. 2001). TonB is a predicted protein consisting of 252 amino acids and has a hypothetical molecular weight of 27.1 kDa, which is the typical size of TonB proteins from other bacteria. TonB is a periplasmic protein that links the inner and outer membranes and provides the energy needed by outer membrane receptors to transport their respective substrates (Postle and Kadner 2003).

The role of the *A. actinomycetemcomitans* TonB-ExbB-ExbD system in hemin utilization was investigated by creating isogenic derivatives in which either *exbB, exbD* or *tonB* was interrupted with the EZ::TN ⟨*ori*V/KAN-2⟩ transposon. The *A. actinomycetemcomitans* isogenic derivatives with insertions in *exbB, exbD* and *tonB* were named MU-3, MU-6 and MU-7, respectively. The growth of the parental strain and all three mutants was comparable when they were plated either on plain AAGM agar or AAGM agar containing increasing concentrations of dipyridyl (Fig. 4, panels B-D). All strains also showed similar growth around filter disks saturated with hemin that were deposited on AAGM agar containing 325 μM dipyridyl (Fig. 4, panel A).

Altogether these results indicate that the *A. actinomycetemcomitans* ExbB-ExbD-TonB homologs are not essential for hemin acquisition and another system must be providing the energy needed for the functioning of the hemin uptake system. In fact, the annotation of the HK1651 genome shows that AA02836 and AA02834 are paralogs of *exbB* and *exbD*, respectively. The genes AA02836 and AA02834 were annotated as *tolQ* and *tolR*, respectively. There is another gene down stream from *tolR*, AA02832, which was annotated as *tolA*. PCR and RT-PCR showed that all three of these genes are present and expressed in strain DF2200N, HK1651 and CU1000. The significance of this observation is that in *E. coli* the TolQ, TolR, and TolA proteins are required for group A colicins to be transferred from their receptors in the outer membrane to the inner membrane (Derouiche et al. 1995). The TolQ and TolR proteins interact with and energize TolA in a similar mechanism as ExbB, ExbD and TonB (Journet et al. 1999; Cascales et al. 2001; Germon et al. 2001). The topologies of TonB, ExbB, and ExbD are very similar to those of TolA, TolQ, and TolR, respectively. In fact, TolQ and TolR are orthologous to ExbB and ExbD, respectively, whereas TolA and TonB are homologous (Cascales et al. 2001). In direct experiments investigating the transport of cobalamin (Bradbeer 1993) and colicin sensitivity (Braun 1989) in *E. coli*, the researchers showed that the *tolQ* gene product can partially replace the function of the ExbB protein. In addition, it was shown that TolQ-TolR interacts through TonB, and ExbB-ExbD interacts through TolA with their outer membrane receptors (Braun and Herrmann 1993). Therefore, the *tolQ* and *tolR* genes located in the genome of *A. actinomycetemcomitans* could be complementing the mutations in *exbB* and *exbD*. There are no reports that show a complementation effect by the *tolA* gene product in any *tonB* mutants. However, when the anchor of TonB was replaced with that of TolA it still could function in activating transport (Witty et al. 2002) suggesting that the TolA and TonB C-terminal domains have a common evolutionary origin and are related by means of domain swapping. Thus, TolQ, TolR and TolA could be complementing all the functions of ExbB, ExbD and TonB respectively, not just those

involved in hemin or iron acquisition. We are interrupting *tolA* in MU-7, *tolQ* in MU-3, and *tolR* in MU-6 to determine if this is the case in *A. actinomycetemcomitans*.

Effects of iron on biofilm formation

Iron is an important environmental signal that controls the differential expression of a large number of genes, some of which code for important bacterial virulence factors including the formation of biofilms. It has been shown that biofilm formation by human pathogens such as *Acinetobacter baumannii* (Tomaras et al. 2003), *Pseudomonas aeruginosa* (Singh 2004; Banin et al. 2005), *Staphylococcus aureus* (Johnson et al. 2005) and *Streptococcus mutans* (Berlutti et al. 2004) is controlled by iron. This regulation could be mediated via Fur-dependent as well as Fur-independent regulatory systems. Although there are some publications describing the production of Fur and the effect of iron on gene expression in *A. actinomycetemcomitans* (Winston et al. 1993; Spitznagel et al. 1995; Willemsen et al. 1997; Graber et al. 1998; Haraszthy et al. 2002; Fong et al. 2003; Rhodes et al. 2005), most of the components of the iron modulon and Fur regulon in this oral pathogen have remained unidentified. In addition, very little is known about the mechanisms and environmental signals that could control the formation of biofilms by *A. actinomycetemcomitans.* Based on these considerations, we decided to examine the effects of iron-rich and iron-chelated conditions on the formation of *A. actinomycetemcomitans* biofilms, which in the case of those formed in the human oral cavity are known as dental plaques (Kolenbrander et al. 2005).

Biofilm assays, in which cells adhered to polystyrene tubes are stained with crystal violet after static incubation, showed that CU1000 cells attach to and form robust biofilms on the wall and at the bottom of polystyrene tubes when grown in AAGM either under iron-rich or iron-chelated conditions. However, cells cultured in the presence of hemin formed aggregates that appeared denser and more localized at the bottom of the tubes than those formed under the other experimental conditions. The DF2200N strain also formed strong biofilms of similar densities under the same experimental conditions, although these biofilms were located mostly at the bottom of the tubes. In contrast, the CU1060 smooth derivative of the CU1000 rough strain, attached poorly to and formed weak biofilms, particularly when grown in AAGM supplemented with $FeCl_3$. The presence of hemin in the culture medium seems to enhance cell attachment and formation of biofilms, which were distributed uniformly on the tube and were less dense than those formed by the CU1000 and DF2200N rough strains. Photocolorimetric quantification of the stain retained by the attached cells supported these macroscopic observations.

Scanning electron microscopy (SEM) of biofilms formed on the surface of plastic coverslips also showed that the iron content of the medium affects cell adherence and biofilm formation. Incubation of DF2200N in AAGM broth supplemented with $FeCl_3$ resulted in the formation of a larger number of small aggregates and microcolonies when compared with those formed by cells incubated in AAGM without any supplementation (compare panels A and B of Fig. 5). Interestingly, the aggregates formed in the presence of inorganic iron were connected by or attached to

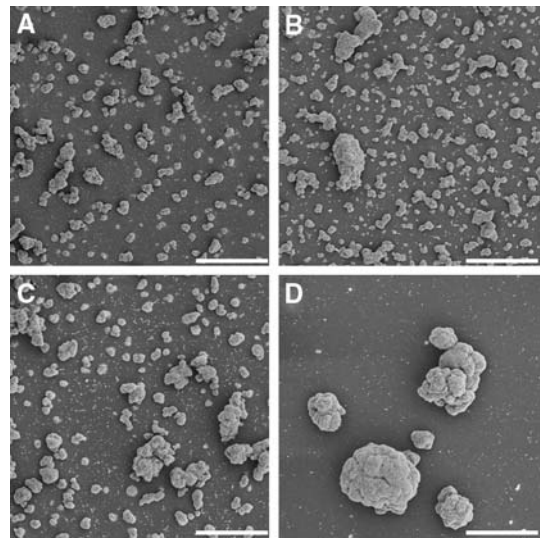

Fig. 5 SEM of DF2200N cells attached to plastic coverslips. Cells were cultured in AAGM (**A**) or AAGM supplemented with $FeCl_3$ (**B**), hemin (**C**), or dipyridyl (**D**). Bars in all panels represent 100 μm

amorphous filaments, which were either barely seen or absent in the structures formed when the cells were incubated in non-supplemented AAGM or AAGM broth containing hemin or dipyridyl. The addition of hemin to the culture medium promoted the formation of very dense and tightly packed aggregates of various sizes while the incubation of the cells in chelated medium resulted in the formation of mostly large conglomerates with few small aggregates and microcolonies (compare panels B-D of Fig. 5). Similar observations were made with the rough strain CU1000.

A similar SEM analysis showed that the CU1060 smooth derivative also form biofilms on the coverslips (Fig. 6), although the cell aggregates were simpler and less dense when compared with those formed by the rough strains (Fig. 5). It is apparent that the presence of $FeCl_3$ in the medium reduces significantly the number of aggregates formed on the coverslip surface and alters the size and shape of the cells, which are smaller and more rounded than those seen under the other experimental conditions used in this study (Fig. 6B). In contrast, the addition of hemin to the culture medium enhances the number of attached cells and aggregates formed on the plastic surface (panel C). The biofilms formed in the presence of dipyridyl showed a density and structure similar to those formed in AAGM broth, although the presence of the iron chelator seemed to induce the formation of cell chains, some of which remain attached to each other (panel D).

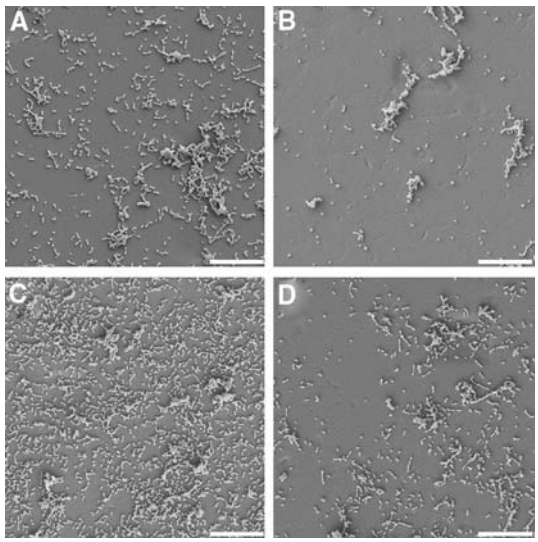

Fig. 6 SEM of CU1060 cells attached to plastic coverslips. Cells were cultured in AAGM (**A**) or AAGM supplemented with $FeCl_3$ (**B**), hemin (**C**), or dipyridyl (**D**). Bars in all panels represent 10 μm

Altogether these results indicate that the type of structures *A. actinomycetemcomitans* rough and smooth strains form on abiotic surfaces depends on the iron content of the medium. These findings are in agreement with those reported for other important human pathogens such as *P. aeruginosa* (Singh et al. 2002; Singh 2004; Banin et al. 2005), *S. mutants* (Berlutti et al. 2004) and *S. aureus* (Johnson et al. 2005). These studies together with our data indicate that iron and iron-chelating compounds play a role in the type of cell aggregates and biofilm structures bacteria, including oral pathogens, form under iron-rich and iron-chelated conditions. It is possible that some of these factors, such as the expression of active iron acquisition systems and the production of Fur, influence different stages of the *A. actinomycetemcomitans* biofilm formation process. It is also possible that this process is affected by the presence of human lactoferrin, an iron-binding protein that plays a significant role in oral biology. Such a potential effect is supported by the observation that iron-saturated lactoferrin reduces the attachment of this oral pathogen to buccal epithelial cells (Fine and Furgang 2002). Some of these possibilities are being tested with different isogenic derivatives cultured under different experimental conditions, some of which resemble those found in the human oral cavity.

Acknowledgments Funds from Miami University and Public Health DE13657-02 and NSF 0420479 Grants supported the research work presented in this manuscript. We thank Richard Edelmann, Matt Duley and the Miami University Electron Microscopy Facility for their help with electron microscopy. We also thank C. Wood, coordinator of the Miami University Center of Bioinformatics and Functional Genomics for his support and assistance with automated DNA sequencing and nucleotide sequence analysis. We are grateful to Dr. D. Dyer (University of Oklahoma Health Science Center) for making available the nucleotide sequence of the *A. actinomycetemcomitans* HK1651 genome. We thank D. Figurski (College of Physicians and Surgeons of Columbia University) and D. Fine and S. Kachlany (New Jersey Dental School) for

giving us the *A. actinomycetemcomitans* strains used in our studies as well as Dr. R. Perry (University of Kentucky) for providing the *E. coli* 1017 strain.

References

Alugupalli KR, Kalfs S, Ewardsson S, Naidu AS (1995) Lactoferrin interaction with *Actinobacillus actinomycetemcomitans*. Oral Microbiol Immunol 10:35–41

Angerer A, Gaisser S, Braun V (1990) Nucleotide sequence of *sfuA*, *sfuB*, and *sfuC* genes of *Serratia marcescens*. J Bacteriol 172:572–578

Angerer A, Klupp B, Braun V (1992) Iron transport systems of *Serratia marcescens*. J Bacteriol 174:1378–1387

Araujo M (2002) Localized juvenile periodontitis or localized aggressive periodontitis. J Mass Dent Soc 51:14–18

Armitage GC (1999) Development of a classification system for periodontal diseases and conditions. Ann Periodontol 4:1–6

Banin E, Vasil ML, Greenberg EP (2005) Iron and *Pseudomonas aeruginosa* biofilm formation. Proc Natl Acad Sci USA 102:11076–11081

Bearden SW, Perry RD (1999) The YFE system of *Yersinia pestis* transports iron and manganese and is required for full virulence of plague. Mol Microbiol 32:403–414

Bearden SW, Staggs TM, Perry RD (1998) An ABC transport system of *Yersinia pestis* allows utilization of chelated iron by *Escherichia coli* SAB11. J Bacteriol 180:1135–1147

Berbari EF, Cockerill FR III, Steckelberg JM (1997) Infective endocarditis due to unusual or fastidious microorganisms. Mayo Clin Proc 72:532–542

Berlutti F, Ajello M, Bosso P et al (2004) Both lactoferrin and iron influence aggregation and biofilm formation in *Streptococcus mutans*. Biometals 17:271–278

Bradbeer C (1993) The proton motive force drives the outer membrane transport of cobalamin in *Escherichia coli*. J Bacteriol 175:3146–3150

Braun V (1989) The structurally related *exbB* and *tolQ* genes are interchangeable in conferring *tonB*-dependent colicin, bacteriophage, and albomycin sensitivity. J Bacteriol 171:6387–6390

Braun V, Herrmann C (1993) Evolutionary relationship of uptake systems for biopolymers in *Escherichia coli*: cross-complementation between the TonB-ExbB-ExbD and the TolA-TolQ-TolR proteins. Mol Microbiol 8:261–268

Cascales E, Lloubes R, Sturgis JN (2001) The TolQ-TolR proteins energize TolA and share homologies with the flagellar motor proteins MotA-MotB. Mol Microbiol 42:795–807

Chen C-Y, Berish SA, Morse SA, Mietzner TA (1993) The ferric-iron binding protein of pathogenic *Neisseria* spp. functions as a periplasmic transport protein in iron acquisition from human transferrin. Mol Microbiol 10:311–318

Crosa JH, Mey AR, Payne SM (2004) Iron transport in bacteria. ASM Press, Washington, DC

Crosa JH, Walsh CT (2002) Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. Microbiol Mol Biol Rev 66:223–249

Derouiche R, Benedetti H, Lazzaroni JC, Lazdunski C, Lloubes R (1995) Protein complex within *Escherichia coli* inner membrane. TolA N-terminal domain interacts with TolQ and TolR proteins. J Biol Chem 270:11078–11084

Fine DH, Furgang D (2002) Lactoferrin iron levels affect attachment of *Actinobacillus actinomycetemcomitans* to buccal epithelial cells. J Periodontol 73:616–623

Fine DH, Furgang D, Schreiner HC et al (1999) Phenotypic variation in Actinobacillus actinomycetemcomitans during laboratory growth: implications for virulence. Microbiology 145:1335–1347

Fong KP, Gao L, Demuth DR (2003) *luxS* and *arcB* control aerobic growth of *Actinobacillus actinomycetemcomitans* under iron limitation. Infect Immun 71:298–308

Fouz B, Mazoy R, Lemos ML, del Olmo MJ, Amaro C (1996) Utilization of hemin and hemoglobin by *Vibrio vulnificus biotype* 2. Appl Environ Microbiol 62:2806–2810

Genco R, Offenbacher S, Beck J (2002) Periodontal disease and cardiovascular disease: epidemiology and possible mechanisms. J Am Dent Assoc 133:14S-22S

Germon P, Ray MC, Vianney A, Lazzaroni JC (2001) Energy-dependent conformational change in the TolA protein of *Escherichia coli* involves its N-terminal domain, TolQ, and TolR. J Bacteriol 183:4110–4114

Gong S, Bearden SW, Geoffroy VA, Fetherston JD, Perry RD (2001) Characterization of the *Yersinia pestis* Yfu ABC inorganic iron transport system. Infect Immun 69:2829–2837

Graber KR, Smoot LM, Actis LA (1998) Expression of iron binding proteins and hemin binding activity in the dental pathogen *Actinobacillus actinomycetemcomitans*. FEMS Microbiol Lett 163:135–142

Gray-Owen SD, Schryvers AB (1996) Bacterial transferrin and lactoferrin receptors. Trends Microbiol 4:185–191

Grenier D, Leduc A, Myrand D (1997) Interaction between *Actinobacillus actinomycetemcomitans* lipopolysaccharides and human hemoglobin. FEMS Microbiol Lett 151:77–81

Haraszthy VI, Lally ET, Haraszthy GG, Zambon JJ (2002) Molecular cloning of the *fur* gene from *Actinobacillus actinomycetemcomitans*. Infect Immun 70:3170–3179

Haraszthy VI, Zambon JJ, Trevisan M, Zeid M, Genco RJ (2000) Identification of periodontal pathogens in atheromatous plaques. J Periodontol 71:1554–1560

Haubek D, Poulsen K, Westergaard J, Dahlen G, Kilian M (1996) Highly toxic clone of *Actinobacillus actinomycetemcomitans* in geographically widespread cases of juvenile periodontitis in adolescents of African origin. J Clin Microbiol 34:1576–1578

Hayashida H, Poulsen K, Kilian M (2002) Differences in iron acquisition from human haemoglobin among

strains of *Actinobacillus actinomycetemcomitans*. Microbiology 148:3993–4001

Johnson M, Cockayne A, Williams PH, Morrissey JA (2005) Iron-responsive regulation of biofilm formation in *Staphylococcus aureus* involves *fur*-dependent and *fur*-independent mechanisms. J Bacteriol 187:8211–8215

Journet L, Rigal A, Lazdunski C, Benedetti H (1999) Role of TolR N-terminal, central, and C-terminal domains in dimerization and interaction with TolA and TolQ. J Bacteriol 181:4476–4484

Kachlany SC, Planet PJ, Bhattacharjee MK et al (2000) Nonspecific adherence by Actinobacillus actinomycetemcomitans requires genes widespread in bacteria and archaea. J Bacteriol 182:6169–6176

Kaplan JB, Schreiner HC, Furgang D, Fine DH (2002) Population structure and genetic diversity of *Actinobacillus actinomycetemcomitans* strains isolated from localized juvenile periodontitis patients. J Clin Microbiol 40:1181–1187

Klebba PE (2004) Transport biochemistry of FepA. In: Crosa JH, Mey AR, Payne SM (eds) Iron transport in bacteria. ASM Press, Washington, DC, pp. 147–157

Kolenbrander PE, Egland PG, Diaz PI, Palmer RJ Jr (2005) Genome-genome interactions: bacterial communities in initial dental plaque. Trends Microbiol 13:11–15

Marchler-Bauer A, Anderson JB, Cherukuri PF et al (2005) CDD: a conserved domain database for protein classification. Nucleic Acids Res 33:D192–D196

Mey AR, Payne SM (2001) Haem utilization in *Vibrio cholerae* involves multiple TonB-dependent haem receptors. Mol Microbiol 42:835–849

Meyer DD, Fives-Taylor PM (1997) The role of *Actinobacillus actinomycetemcomitans* in the pathogenesis of periodontal disease. Trends Microbiol 6:224–228

Nørskov-Lauritsen N, Kilian M (2006) Reclassification of *Actinobacillus actinomycetemcomitans*, *Haemophilus aphrophilus*, *Haemophilus paraphrophilus* and *Haemophilus segnis* as *Aggregatibacter actinomycetemcomitans* gen. nov., comb. nov., *Aggregatibacter aphrophilus* comb. nov. and *Aggregatibacter segnis* comb. nov., and emended description of *Aggregatibacter aphrophilus* to include V factor-dependent and V factor-independent isolates. Int J Syst Evol Microbiol (in press)

Perry RD, Pendrak ML, Schuetze P (1990) Identification and cloning of a hemin storage locus involved in the pigmentation phenotype of *Yersinia pestis*. J Bacteriol 172:5929–5937

Postle K, Kadner RJ (2003) Touch and go: tying TonB to transport. Mol Microbiol 49:869–882

Rhodes ER, Tomaras AP, McGillivary G, Connerly PL, Actis LA (2005) Genetic and functional analyses of the *Actinobacillus actinomycetemcomitans* AfeABCD siderophore-independent iron acquisition system. Infect Immun 73:3758–3763

Saken E, Rakin A, Heesemann J (2000) Molecular characterization of a novel siderophore-independent iron transport system in *Yersinia*. Int J Med Microbiol 290:51–60

Sanders JD, Cole LD, Hansen EJ (1994) Identification of a locus involved in the utilization of iron by *Haemophilus influenzae*. Infect Immun 62:4515–4525

Schreiner HC, Sinatra K, Kaplan JB et al (2003) Tight-adherence genes of Actinobacillus actinomycetemcomitans are required for virulence in a rat model. Proc Natl Acad Sci USA 100:7295–7300

Singh PK (2004) Iron sequestration by human lactoferrin stimulates *P. aeruginosa* surface motility and blocks biofilm formation. Biometals 17:267–270

Singh PK, Parsek MR, Greenberg EP, Welsh MJ (2002) A component of innate immunity prevents bacterial biofilm development. Nature 417:552–555

Spitznagel J Jr, Kraig E, Kolodrubetz D (1995) The regulation of leukotoxin production in *Actinobacillus actinomycetemcomitans* strain JP2. Adv Dent Res 9:48–54

Stugard CE, Daskaleros PA, Payne SM (1989) A 101-kilodalton heme-binding protein associated with Congo red binding and virulence of *Shigella flexneri* and enteroinvasive *Escherichia coli* strains. Infect Immun 57:3534–3539

Tomaras AP, Dorsey CW, Edelmann RE, Actis LA (2003) Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system. Microbiology 149:3473–3484

Wandersman C, Delepelaire P (2004) Bacterial iron sources: from siderophores to hemophores. Annu Rev Microbiol 58:611–647

Willemsen PTJ, Vulto I, Boxem M, De Graaf J (1997) Characterization of a periplasmic protein involved in iron utilization of *Actinobacillus actinomycetemcomitans*. J Bacteriol 179:4949–4952

Winston JL, Chen C-K, Neiders ME, Dyer DW (1993) Membrane protein expression by *Actinobacillus actinomycetemcomitans* in response to iron availability. J Dent Res 72:1366–1373

Witty M, Sanz C, Shah A et al (2002) Structure of the periplasmic domain of Pseudomonas aeruginosa TolA: evidence for an evolutionary relationship with the TonB transporter protein. EMBO J 21:4207–4218

Zambon JJ (1985) *Actinobacillus actinomycetemcomitans* in human periodontal disease. J Clin Periodontol 12:1–20