Evaluation of different iron sources and their influence in biofilm formation by the dental pathogen *Actinobacillus actinomycetemcomitans*

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Actinobacillus actinomycetemcomitans, a pathogen associated with oral and extra-oral infections, requires iron to grow under limiting conditions. Although incapable of producing siderophores, this pathogen could acquire iron by direct interaction with compounds such as haemin, haemoglobin, lactoferrin and transferrin. In this work the ability of different *A. actinomycetemcomitans* strains to bind and use different iron sources was tested. None of the strains tested used haemoglobin, lactoferrin or transferrin as sole sources of iron. However, all of them used FeCl₃ and haemin as iron sources under chelated conditions. Dot-blot binding assays showed that all strains bind lactoferrin, haemoglobin and haemin, but not transferrin. Insertion inactivation of *hmsF*, which encodes a predicted cell-envelope protein related to haemin-storage proteins produced by other pathogens, reduced haemin and Congo red binding drastically without affecting haemin utilization as an iron source under chelated conditions. Biofilm assays showed that all strains tested attached to and formed biofilms on plastic under iron-rich and iron-chelated conditions. However, scanning electron microscopy showed that smooth strains formed simpler biofilms than rough isolates. Furthermore, the incubation of rough cells in the presence of FeCl₃ or haemin resulted in the formation of more aggregates and microcolonies compared with the fewer cell aggregates formed when cells were grown in the presence of the synthetic iron chelator dipyriddy. These cell responses to changes in extracellular iron concentrations may reflect those that this pathogen expresses under the conditions it encounters in the human oral cavity.

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INTRODUCTION

Actinobacillus actinomycetemcomitans has been associated with a variety of infections, but mainly with localized aggressive periodontitis (LAP) (Araujo, 2002; Armitage, 1999), a disease that causes progressive destruction of dental tissue (Meyer & Fives-Taylor, 1997; Slots & Genco, 1984; Zambon, 1985). *A. actinomycetemcomitans* requires iron to grow under the host iron-limiting conditions, which are imposed by the production of iron-binding compounds such as ferritin, haemin, lactoferrin and transferrin. Bacteria have responded to this nutrient limitation by expressing high-affinity iron-acquisition systems, which in the case of this oral pathogen does not involve the production of siderophores (Winston *et al.*, 1993) or direct interaction with the iron-binding protein transferrin (Hayashida *et al.*, 2002; Winston *et al.*, 1993). The interaction of *A. actinomycetemcomitans* with lactoferrin seems to be more

variable among different isolates and could involve the production of lactoferrin-binding proteins (Alugupalli *et al.*, 1995; Winston *et al.*, 1993). However, none of the strains tested so far is able to use lactoferrin as an iron source when plated on chelated brain heart infusion (BHI) agar (Hayashida *et al.*, 2002). The latter study and that reported by Grenier *et al.* (1997) have shown that *A. actinomycetemcomitans* uses haemoglobin as an iron source under iron limitation, when the cells harbour and express an active *hgpA* gene (Hayashida *et al.*, 2002). Haemin is another compound that this oral pathogen could bind and use as an iron source to grow under iron-limiting conditions (Graber *et al.*, 1998; Grenier *et al.*, 1997; Rhodes *et al.*, 2005), although the nature of its receptor on the bacterial cell surface has not been determined.

Iron is also an important signal that controls the differential expression of a large number of genes, some of which code for important bacterial virulence factors (Wandersman & Delepelaire, 2004). Biofilms are among these factors, the formation of which could be controlled by iron, as has been shown for pathogens such as *Acinetobacter baumannii*

Abbreviations: DIP, dipyriddy; DMB, 3,3'-dimethoxybenzidine dihydrochloride; HRP, horseradish peroxidase; LAP, localized aggressive periodontitis; SEM, scanning electron microscope/microscopy.

(Tomaras *et al.*, 2003), *Pseudomonas aeruginosa* (Banin *et al.*, 2005; Singh, 2004), *Staphylococcus aureus* (Johnson *et al.*, 2005) and *Streptococcus mutans* (Berlutti *et al.*, 2004). This regulation is mediated through Fur-dependent as well as Fur-independent regulatory circuits. Currently, very little is known about the regulatory mechanisms and environmental signals that could control the formation of biofilms by *A. actinomycetemcomitans*.

Based on all these considerations we decided to study in more detail the ability of *A. actinomycetemcomitans* smooth and rough isolates to interact with and use different compounds as iron sources, as well as the effects of these compounds on cell attachment and biofilm formation on plastic surfaces.

METHODS

Bacterial strains, plasmids and media. The bacterial strains and plasmids used in this work are listed in Table 1. The strain DF2200N, a clinical isolate that preserved the aggregative and rough phenotypes observed after its initial isolation from an LAP patient (Fine *et al.*, 1999), was used for further analyses because of its amenability to genetic manipulations, such as site-directed mutagenesis by allelic exchange mediated by homologous recombination (Rhodes *et al.*, 2005). The *A. actinomycetemcomitans* strains were cultured in *A. actinomycetemcomitans* growth medium (AAGM) broth or agar (Goncharoff *et al.*, 1993) in 5% CO₂ at 37 °C. *Escherichia coli* DH5 α was grown in Luria-Bertani (LB) broth or agar (Sambrook & Russell, 2001) at 37 °C. The iron content of AAGM medium was 1–3 μ M, as determined with a Variant Liberty 150 Inductive Coupled Plasma (ICP) atomic emission spectrometer using standard conditions and a set of appropriate standards.

Iron source-utilization assays. Each *A. actinomycetemcomitans* strain was spread over the entire surface of an AAGM agar plate

containing 250 μ M dipyriddy (DIP). This condition created an environment that did not allow any bacterial growth without an additional iron source. Paper filter disks were saturated with 10 μ l human transferrin (2 mg ml⁻¹), human lactoferrin (5 mg ml⁻¹), human haemoglobin (1 mg ml⁻¹), FeCl₃ (100 μ M), haemin (10 mg ml⁻¹) or sterile water and then placed on the plate. After 48 h incubation at 37 °C in the presence of 5% CO₂, the plates were observed for growth around the disks. Human haemoglobin, lactoferrin and transferrin, haemin and DIP were purchased from Sigma. Iron-saturated lactoferrin (Sigma L-3770) and lactoferrin containing 0.01% iron (low-iron lactoferrin; Sigma L-0520) were used in these assays.

Haemoglobin, lactoferrin and transferrin binding assays. The *A. actinomycetemcomitans* strains were cultured in AAGM supplemented with 100 μ M FeCl₃ (iron-rich condition, final concentration 101–103 μ M) or 100 μ M DIP (iron-chelated condition) to induce the differential expression of iron-regulated genes during cell growth. The concentration of 100 μ M FeCl₃ was selected based on the observation that the mean iron content of crevicular fluid of patients with periodontitis is 5.2 mg ml⁻¹ (93 μ M) (Mukherjee, 1985).

After incubation, the cells were collected by centrifugation, washed in Tris-buffered saline (TBS; 10 mM Tris-HCl, 0.15 M NaCl, pH 7.5) and suspended to an OD₆₀₀ of approximately 6.0. A 20 μ l aliquot of each sample was collected on a nitrocellulose membrane and allowed to dry at room temperature. The membranes were blocked with 5% (w/v) skimmed milk in NIBB (50 mM Tris-HCl, 0.15 M NaCl, 5.0 mM EDTA, 0.05% Triton X-100, pH 7.4) for 1 h at room temperature. The blots were washed three times with either NIBB (haemoglobin and lactoferrin assays) or TBS (transferrin assay) for 15 min at room temperature. For testing transferrin and lactoferrin binding, the membranes were incubated with horseradish peroxidase (HRP)-labelled human transferrin or human lactoferrin for 1 h with agitation at room temperature and then rinsed with TBS. The complexes bound to bacterial cells were detected with a chemiluminescence detection system (SuperSignal ULTRA substrates, Pierce Chemical). The binding of haemoglobin was tested by incubating the filters with biotin-labelled

Table 1. Bacterial strains and plasmids used in this work

Strain/plasmid	Relevant characteristics*	Source/reference
Strains		
<i>A. actinomycetemcomitans</i>		
HK1651	Clinical isolate, genome project, serotype b, JP2 clone, rough phenotype	ATCC
SUNY465	Invasion prototype strain, smooth phenotype, serotype b	P. Fives-Taylor
CU1000	Clinical isolate, rough phenotype, serotype f	Fine <i>et al.</i> (1999)
CU1060	Smooth derivative of CU1000	Fine <i>et al.</i> (1999)
DF2200N	Clinical isolate, rough phenotype, serotype a	D. Fine
MB1237	Artificially inducible competent derivative of DF2200N, Cm ^r	D. Figurski
MB1237-MU14	MB1237 with <i>hmsF</i> ::EZ-Tn5<R6K γ oriKan-2>, Kan ^r	This work
<i>E. coli</i>		
DH5 α	General gene cloning host	Gibco-BRL
Plasmids		
pCR-Blunt II-TOPO	Used to clone amplicons, Km ^r , Zeo ^r	Invitrogen
pMB78	USS-containing cloning vector, Amp ^r , suicide vector used for allelic exchange	D. Figurski
pMU606	DF2200N amplicon harbouring <i>hmsF</i> cloned in pCR-Blunt II-TOPO	This work
pMU608	Insert of pMU606 subcloned in pMB78, Amp ^r	This work
pMU620	pMU608 with an EZ-Tn5<R6K γ oriKan-2> insertion in <i>hmsF</i> , Amp ^r , Kan ^r	This work

*Abbreviations: Amp, ampicillin; Cm, chloramphenicol; Kan, kanamycin; Zeo, zeocin.

human haemoglobin in 30 ml NIBB for 1 h at room temperature. The presence of biotin-labelled human haemoglobin bound to cells was detected with StrepTactin-HRP conjugates (Bio-Rad) and the aforementioned chemiluminescence detection system. Biotin-labelled human haemoglobin was prepared using the EZ-Link Sulfo-NHS-Biotinylation kit (Pierce), following the manufacturer's instructions. The binding assays were done at least three times using fresh biological samples each time, and the results of a representative experiment are shown in Fig. 1.

Haemin binding assays. Each bacterial strain was grown in AAGM containing 50 μ M haemin. The cells were washed in 0.1% NaCl and the pellets were observed for greyish to dark-brown colouring indicating haemin binding to the cells. The binding of haemin was also detected by immobilizing cells grown under iron-chelated and iron-rich conditions onto a nitrocellulose membrane, as described above, and detecting the haemin-associated peroxidase activity with 3,3'-dimethoxybenzidine dihydrochloride (DMB) (Fouz *et al.*, 1996). The membranes were blocked with 5% (w/v) skimmed milk in NIBB for 1 h and then incubated for 12 h in TBS containing 10 μ M haemin, immersed for 30 min in 12.5% TCA, and then washed with water for 30 min. The binding of haemin was detected by incubating the blots with the benzidine derivative DMB, as previously described (Fouz *et al.*, 1996). The binding assays were done at least three times using fresh biological samples each time, and the results of a representative experiment are shown in Fig. 1.

Fig. 1. Binding of iron-containing compounds by bacterial cells. The binding of HRP-lactoferrin (a), biotin-haemoglobin (b) and haemin (c) was tested with dot-blot assays. Haemin binding was also determined by visual inspection of cells washed and collected by centrifugation. *E. coli* DH5 α (1) and *A. actinomycetemcomitans* HK1651 (2), CU1000 (3), CU1060 (4) and SUNY465 (5) cells cultured under iron-chelated conditions were collected, washed and spotted onto nitrocellulose. The binding of HRP-lactoferrin and biotin-haemoglobin was detected using the appropriate chemiluminescence substrates, while the binding of haemin was detected with DMB.

Congo red binding assays. The binding of Congo red was tested by culturing the *A. actinomycetemcomitans* strains in AAGM agar or broth containing 30 μ g Congo red ml^{-1} at 37 $^{\circ}\text{C}$ in 5% CO_2 . Plates were examined after 48 h incubation and the results were recorded with a dissection microscope at $\times 40$ magnification. Cells from broth cultures containing 30 μ g Congo red ml^{-1} were collected by centrifugation, washed with TBS, and then suspended in DMSO to dissolve the stain bound to the cells, which were collected by centrifugation. The cleared supernatants were used to determine the absorbance at 488 nm. The cell pellets were used to determine total proteins using the Bradford method (Bradford, 1976), and the ratio of eluted Congo red to the amount of total protein was calculated. This experiment was repeated three times using fresh biological samples each time.

Detection, expression and analysis, and mutagenesis of *hms* genes. The presence of the predicted HK1651 genes *hmsH* and *hmsF* in the strains CU1000 and DF2200N was tested by PCR, using total DNA isolated from each strain and the primers HmsH-up (5'-CGTTTCCGACCATATTCG-3') and 00493-down (5'-AAATACGC-CTCCGGTTCC-3'). These primers were designed using the genomic data available for the HK1651 strain (<http://www.oralgen.lanl.gov/> and <http://www.genome.ou.edu/act.html>) and used as described previously (Rhodes *et al.*, 2005). The transcriptional expression of these two coding regions was tested by RT-PCR using total RNA isolated from HK1651, CU1000 and DF2200N as templates, and the primers *hmsH*.3' (5'-TATGCGCTGATTCTGACC-3') and *hmsF*.5' (5'-GCGTATCCAACCAACTGC-3') as described for the analysis of the *afeABCD* operon (Rhodes *et al.*, 2005). The *hmsH*.3' and *hmsF*.5' primers anneal at the 3' end of *hmsH* and the 5' end of *hmsF*, respectively. PCR of total RNA that was not reverse transcribed, and which did not produce a detectable amplicon, was used as a control for DNA contamination.

The chromosomal region encompassing *hmsF* was PCR-amplified with primers *hmsH*.3' and 00492-5' (5'-TCTCAGCTGAAGCAAGTG-3') using DF2200N total DNA as a template and *Pfu* DNA polymerase (Stratagene), as described previously (Rhodes *et al.*, 2005). The amplicon was cloned into pCR-BluntII-TOPO using the Zero Blunt TOPO PCR cloning kit (Invitrogen) to obtain pMU606. Once confirmed by nucleotide sequencing with the M13 forward and reverse primers provided with the cloning kit, the insert of pMU606 was subcloned into pMB78 to obtain pMU608. pMB78, a pUC19 derivative that contains DNA-uptake sequences and does not replicate in *A. actinomycetemcomitans*, allows allelic exchange of mutated cloned fragments after transformation of *A. actinomycetemcomitans* MB1237 artificially induced competent cells (M. K. Bhattacharjee and others, personal communication). pMU608 was mutagenized *in vitro* with the EZ-Tn5<R6K γ oriKan-2> (Epicentre), as described previously (Rhodes *et al.*, 2005). Transposon insertions within *hmsF* were mapped by PCR with the M13 forward and reverse primers and confirmed by automated nucleotide sequencing with the forward and reverse primers supplied with the transposon insertion kit. The insert of a pMU608 derivative with an insertion in *hmsF* (pMU620) was amplified with the M13 forward and reverse primers, and the amplicon was used to transform MB1237 competent cells, as described previously (Rhodes *et al.*, 2005). Total DNA was isolated from a Kan r *A. actinomycetemcomitans* transformant, which was named MB1237-MU14, and used as a template to confirm the appropriate allelic exchange by PCR with the primers *hmsH*.3' and 00492-5'. Gel electrophoresis and nucleotide sequencing with the forward and reverse primers supplied with the transposon insertion kit confirmed the transposon disruption of *hmsF* in MB1237-MU14. AAGM plates containing 50 μ M haemin or 30 μ g Congo red ml^{-1} were used to compare the ability of the parental strain and the *hmsF* isogenic insertion derivative to bind these compounds after incubation at 37 $^{\circ}\text{C}$ in 5% CO_2 for 48 h.

Electrophoretic protein analysis. Total and outer-membrane fractions were isolated by high-speed centrifugation and differential detergent solubilization, as described previously (Actis *et al.*, 1985). Proteins were size-fractionated by SDS-PAGE using 12.5 % polyacrylamide gels and detected by staining with Coomassie blue, as described previously (Actis *et al.*, 1985).

Biofilm assays and electron microscopy. Duplicate cultures of each strain were grown in AAGM broth containing 100 µM FeCl₃, 100 µM DIP or 50 µM haemin in polystyrene tubes (12 × 75 mm) for 2 days at 37 °C in 5 % CO₂ without shaking. The culture medium was aspirated and the tubes were rinsed thoroughly with distilled water. The cells attached to the tube walls were stained with crystal violet, as described previously (Tomaras *et al.*, 2003). The amount of stain retained by the biofilms was quantified colorimetrically after solubilization with ethanol/acetone (Tomaras *et al.*, 2003). All assays were repeated three times using fresh samples each time.

For scanning electron microscopy (SEM) analysis, 5 ml non-supplemented AAGM broth or broth containing 100 µM FeCl₃, 50 µM haemin or 100 µM DIP was poured into 50 ml plastic conical tubes and inoculated with 0.05 ml of 2-day-old cultures of the *A. actinomycetemcomitans* strains. A sterile plastic cover slip was added to each tube immediately after inoculation, and incubated for 48 h at 37 °C in 5 % CO₂ without shaking. The cover slips were then removed and immediately submerged in a solution containing 2 % paraformaldehyde and 2 % glutaraldehyde in 0.05 M sodium cacodylate and incubated at room temperature for 2 h. The fixative was removed and rinsed immediately with distilled water, and then samples were dehydrated with increasing concentrations of ethanol, ranging from 25 to 100 %. The dehydrated samples were then CO₂ critical-point dried, gold-coated and visualized with a JEOL JSM-840A SEM. These assays were done twice using fresh samples each time, and representative fields are shown in Figs 5 and 6. The large SEM images shown in these figures were analysed with Image-Pro Plus (MediaCybernetics). A minimum aggregate size value of 2 µm² was established to avoid counting individual cells attached to the plastic surfaces. Class 1 and class 2 objects were defined as aggregates whose size was from 2 to 300 µm² and >300 µm², respectively.

RESULTS AND DISCUSSION

Utilization and binding of different iron-containing compounds

We initiated these studies by testing the ability of *A. actinomycetemcomitans* HK1651, CU1000, CU1060, SUNY465 and DF2200N to use different iron-containing compounds to grow under iron-chelated conditions. All these strains, which represent different serotypes and colony morphologies (Table 1), were able to produce 5–7 mm growth haloes on AAGM agar containing 250 µM DIP around filter disks saturated with haemin or FeCl₃ after 48 h incubation. In contrast, no cell growth was detected when the disks were impregnated with human haemoglobin, iron-saturated lactoferrin, low-iron lactoferrin, transferrin or sterile water. Dot-blot binding assays showed that none of the strains was able to bind transferrin (data not shown), while all of them bound HRP-labelled lactoferrin and biotin-labelled haemoglobin (Fig. 1a, b, respectively). The cell pellets of the HK1651, CU1000, CU1060 and SUNY465 strains were stained when they were cultured in AAGM containing haemin (Fig. 1c, top panel), an observation

suggesting that the cells of these strains bind haemin. Such a possibility was confirmed by detecting the haemin-associated peroxidase activity with DMB (Fouz *et al.*, 1996) in dot-blot assays. These assays showed that cells of all four strains produced an intense signal with DMB (Fig. 1c, dot blot). The binding of these iron-containing compounds was similar in cells grown under iron-rich and iron-chelated conditions (data not shown), and none of the compounds tested was bound by *E. coli* DH5α cells, which were used as a negative control [compare the tube samples and dot blots shown in both panels of Fig. 1(c)], when tested under similar experimental conditions.

Taken together, these results indicate that although capable of binding lactoferrin, haemoglobin and haemin, all *A. actinomycetemcomitans* strains tested use haemin alone as an iron source under chelated conditions. The inability of *A. actinomycetemcomitans* to bind transferrin is consistent with the observation that all strains examined so far do not harbour genes encoding functional transferrin-binding proteins (Hayashida *et al.*, 2002). This observation suggests that transferrin may not be a relevant iron source in the human oral cavity for this bacterial pathogen. The binding of lactoferrin without using it as an iron source, which is in accordance with the lack of HK1651 genomic determinants encoding a complete and active transport system, may reflect the interaction of *A. actinomycetemcomitans* with a human protein that could serve as a line of non-specific defence rather than as an iron source in the oral cavity. Such a defence could be mediated by the bactericidal activity of this glycoprotein (Kalmar & Arnold, 1988), although this potential effect is not conclusive considering the observation that apo- and holo-lactoferrin do not affect the growth and survival of strains expressing clinical phenotypes when tested under laboratory conditions (Fine & Furgang, 2002). An alternative is the potential capacity of lactoferrin to affect important host–pathogen interactions, considering the inhibitory effects that this protein has on the attachment of *A. actinomycetemcomitans* to buccal epithelial cells when used in the iron-saturated form (Fine & Furgang, 2002).

The ability of all tested strains to bind and use haemin could reflect the availability of this iron-containing compound during the colonization and infection of the host. It has been proposed that free haemin compounds could be available to bacteria of dental plaque after the haemolysis of erythrocytes present in the crevicular fluid (Mukherjee, 1985). Such a possibility is consistent with the recently reported haemolytic activity associated with the LtxA toxin produced by this pathogen (Balashova *et al.*, 2006) and the observation that haemoglobin but not inorganic iron enhances the virulence of *A. actinomycetemcomitans* when tested in guinea pigs (Mukherjee *et al.*, 1988). It is not known whether this pathogen uses haemin and haemoglobin by direct binding or by the production of a haemophore, which could scavenge haemin from haemoglobin, as described for other bacteria, such as *Serratia marcescens* (Wandersman & Delepelaire, 2004). Unfortunately, the annotation of the

HK1651 genome does not provide a clear indication of the mechanisms that this pathogen expresses to acquire and use these compounds as an iron source under iron-limiting conditions.

Binding of haemin and Congo red

It has been shown for other pathogens, such as *Shigella flexneri* (Stugard *et al.*, 1989) and *Yersinia pestis* (Perry *et al.*, 1990), that the ability of bacterial cells to bind and store haemin is associated with their ability to bind Congo red. Such a correlation was observed when *A. actinomycetemcomitans* strains were plated on AAGM agar containing this stain. Fig. 2(a) shows that CU1000 cells form small, rough, dark-red-stained colonies on the surface of the agar. In contrast, CU1060 forms smooth and bigger colonies that are light-red stained. The HK1651 strain displays an intermediate phenotype, with colonies that are similar in size but less rough and stained than those formed by CU1000. Colorimetric assays, which measured the amount of stain eluted from cells cultured in broth containing Congo red (Fig. 2b), confirmed the microscopy observations. The

smooth strain SUNY465 and the rough isolate DF2200N showed behaviours similar to those displayed by the CU1060 and CU1000 strains, respectively, when tested under similar experimental conditions (data not shown).

Several attempts failed to detect the binding of Congo red to outer-membrane proteins size-fractionated by PAGE under denaturing and non-denaturing conditions. As an alternative, we examined the HK1651 genome annotation data (<http://www.oralgen.lanl.gov/>) for genes encoding haemin and Congo red binding functions, such as those described in *Y. pestis* (Lillard *et al.*, 1997). This approach showed that the HK1651 genome includes a 4.3 kb region harbouring the predicted coding regions AA00490 and AA00491. These genes were annotated as *hmH* and *hmsF*, respectively, due to the significant similarity of their products to those encoded by the cognate *Y. pestis* genes located within the pigmentation (*pgm*) locus (Lillard *et al.*, 1997). These two genes are part of the *hmsHFRS* operon involved in the expression of the exogenous haemin-storage phenotype expressed by *Y. pestis* cells cultured at 26 °C (Lillard *et al.*, 1997; Perry *et al.*, 1990). Although this phenotype is not essential for virulence in mammals, it plays a role in the transmission of plague by fleas (Hinnebusch *et al.*, 1996).

PCR analysis showed that the genomes of the HK1651, CU1000 and DF2200N strains harboured the *hmsH* and *hmsF* genes, which proved to be expressed as a bicistronic transcript when total RNA was tested with RT-PCR using primers expanding the *hmsH*–*hmsF* intergenic region (data not shown). These observations prompted us to generate the MB1237-MU14 isogenic derivative harbouring the *hmsF*::EZ-Tn5<R6K_{Yori}Kan-2> allele. Fig. 3 shows that the interruption of *hmsF* results in a drastic reduction in the ability of mutant cells to bind haemin as well as Congo red compared with the parental cells. It is important to note that the MB1237-MU14 cells did not display an apparent growth defect on AAGM medium. Furthermore, the mutant cells formed growth haloes around haem-impregnated filter disks on iron-chelated AAGM plates similar to those detected with the parental strain (data not shown). The latter observation suggests that, as in *Y. pestis* (Perry *et al.*, 1993), this gene is involved in exogenous haemin storage rather than the utilization of this compound as an iron source under chelated conditions. The annotation data available at the Oralgen database and the significant similarity with the *Y. pestis* HmsF protein, which is a surface-exposed outer-membrane protein (Pendrak & Perry, 1993), predict that the *A. actinomycetemcomitans* *hmsF* gene encodes a protein located in the bacterial cell-membrane fraction. Such a prediction was confirmed by SDS-PAGE analysis, which showed that the absence of a 71 kDa outer-membrane protein was the most apparent difference between the protein profiles of the parental and *hmsF* mutant cells (Fig. 3c). Taken together, these results indicate that *A. actinomycetemcomitans* expresses cell-surface haemin and Congo red binding functions, as described for *Y. pestis*.

Fig. 2. Congo red binding assays. (a) Cells were cultured on AAGM agar plates containing Congo red ($30 \mu\text{g ml}^{-1}$). Pictures of the plates were taken with a dissection microscope at $\times 40$ magnification. Bar, $250 \mu\text{m}$. (b) Quantification of the Congo red (CR) retained by cells cultured in AAGM broth containing $30 \mu\text{g ml}^{-1}$ of this stain. The amount of stain eluted was normalized to the protein content as described in the Methods. Error bars represent 1SD.

Fig. 3. Comparative analysis of the DF2200N parental strain and the *hmsF* isogenic insertion derivative. DF2200N (1) and MB1237-MU14 (2) cells were streaked on AAGM agar containing either haemin (a) or Congo red (b). (c) SDS-PAGE of outer-membrane proteins isolated from DF2200N (1) and MB1237-MU14 (2) cells. The arrow identifies a 71 kDa protein that is absent in the *hmsF* insertion mutant cells.

Based on the HK1651 genome annotation and the structure of the *Y. pestis* *hmsHFRS* operon, it is possible to predict that the AA00490 coding region, which is located upstream of *hmsF* and is predicted to code for a protein related to the HmsH protein, also plays a role in the binding of haemin and Congo red to the cell surface. Unfortunately, several attempts failed to generate an *hmsH* insertion derivative using an approach similar to that used with *hmsF*. Currently, we are in the process of cloning a chromosomal fragment that encompasses *hmsH* and *hmsF* as well as the downstream predicted coding regions AA00492 and AA00493. Although all four genes are apparently transcribed in the same direction, there is no experimental evidence supporting the transcription of the last two genes as a bicistronic message or as part of a larger transcript that would include all four coding regions. If the latter proves to be the case, this *A. actinomycetemcomitans* chromosomal region would resemble the *Y. pestis* *hmsHFRS* operon. Interestingly, the product of AA00492 has been annotated as a hypothetical protein related to glycosyltransferases, potentially involved in cell wall biogenesis, and to *Y. pestis* HmsR, while the product of AA00493 is significantly related to an *Actinobacillus pleuropneumoniae* hypothetical protein (<http://www.oralgen.lanl.gov/>). Our current transcriptional and mutagenesis work should elucidate some of these uncertainties and provide novel information on the function of these uncharacterized genes and their products, which could play

a role in the interactions among bacterial cells as well as in host–pathogen interactions.

Taken together, these results indicate that *A. actinomycetemcomitans* expresses cell-surface haemin and Congo red binding functions, as described for *Y. pestis*. However, the biological significance of these functions is not apparent at the moment, considering that this oral pathogen interacts only with human and Old World primate cells (Fine *et al.*, 2005), and it is not known to be transmitted by a vector in which surface-haemin-binding functions could play a role similar to those described for the interaction of *Y. pestis* with infected fleas (Hinnebusch *et al.*, 1996). Nevertheless, it is tempting to speculate that haemin could be an important source of iron as well as a means to enhance the interactions of *A. actinomycetemcomitans* with the host and other members of the oral microbial community. Our observations open a new direction of study in which the role of these *A. actinomycetemcomitans* cellular functions could be characterized and tested with an animal model that mimics the oral infection process in humans (Schreiner *et al.*, 2003).

Biofilm studies

Iron is an important environmental signal that controls gene expression. Although there are some reports that describe the production of the Fur iron-dependent transcriptional regulator and the effect of iron on protein synthesis (Fong *et al.*, 2003; Graber *et al.*, 1998; Haraszthy *et al.*, 2002; Rhodes *et al.*, 2005; Spitznagel *et al.*, 1995; Willemsen *et al.*, 1997; Winston *et al.*, 1993), the extent and the components of the *A. actinomycetemcomitans* iron regulon and Fur regulon remain virtually unexplored. Therefore, we decided to test the effects of iron-rich and iron-chelated conditions on the formation of biofilms on abiotic surfaces.

Biofilm assays showed that the rough strains CU1000 and DF2200N attach to and form similar robust biofilms on polystyrene tubes when cultured statically in AAGM broth (Fig. 4). In contrast, the surface adherence and biofilm formation by cells of the smooth derivative CU1060 were significantly reduced compared with those of the rough strains. Such a behaviour is in accordance with the earlier observation that the formation of long bundle fibrils is needed for surface adherence and autoaggregation (Kachlany *et al.*, 2000). These assays also showed that the supplementation of AAGM with FeCl₃, haemin or DIP does not result in significant changes in the amount of biofilm formed by the rough and smooth strains (Fig. 4).

SEM was used to examine in more detail the biofilms formed on plastic coverslips. Cells of the DF2200N rough strain attached and formed tightly packed aggregates, whose number and size were affected by the culture conditions (Fig. 5a–d). Computer analysis of these micrographs showed that the addition of either FeCl₃ or haemin to AAGM increased by more than twofold the total number of aggregates compared with the number of aggregates formed

Fig. 4. Detection and quantification of biofilms formed on polystyrene tubes. The crystal violet retained by biofilms formed by CU1000, CU1060 and DF2200N cells cultured in AAGM or AAGM supplemented with FeCl₃ (+Fe), haemin (+He) or DIP (+DIP) was eluted and quantified photometrically. Error bars represent 1SD.

by cells incubated in AAGM with no further supplementation (Table 2). In contrast, the addition of DIP produced a 1.5-fold reduction in the number of aggregates. Most of the aggregates formed under all conditions belonged to class 1,

with class 2 aggregates representing only 2–7 %. The cell aggregates formed in the presence of FeCl₃ were the biggest, with the largest of them having an area of 2739 μm². The percentage of covered area (Table 2) reflects the variations in the number and size of the aggregates formed under the different conditions used in this work. Interestingly, some of the aggregates formed in the presence of FeCl₃ were either connected by or attached to filaments made of amorphous material (Fig. 5b). These filaments were rarely observed when the cells were cultured in AAGM or AAGM supplemented with haemin or DIP (Fig. 5a, c, d, respectively). The addition of haemin also promoted the formation of very dense and tightly packed aggregates, some of which seemed to fold on themselves (Fig. 5c). Such structures were not seen when the cells were incubated under the other experimental conditions used in this study. Similar results were obtained with strain CU1000 (data not shown).

Cells of the CU1060 smooth derivative also attached to and formed biofilms on the plastic coverslips (Fig. 6). However, these cell aggregates were simpler – mostly a monolayer of attached cells with some aggregates containing only a few cells – and less dense than those formed by the DF2200N and CU1000 rough strains (note that the scale bars in this figure are one-tenth of those shown in Fig. 5). These observations are in agreement with those made during the analysis of the *tad* genes, the products of which are needed for this oral pathogen to aggregate and form tenacious biofilms on glass (Kachlany *et al.*, 2000). However, the iron content of the medium has an effect on the interaction of CU1060 cells with a plastic surface. The addition of FeCl₃ to the medium resulted in the fewest (Table 2) and simplest aggregates

Fig. 5. SEM of DF2200N cells attached to plastic coverslips. Cells were cultured in AAGM (a) or AAGM supplemented with FeCl₃ (b), haemin (c) or DIP (d). Bars, 100 μm; bars in insets, 10 μm. Open arrows identify amorphous filaments formed between cell aggregates and the plastic surface or between cell aggregates. Large and small black arrows identify large and small cell aggregates, respectively.

Table 2. Quantification of biofilm formation under different iron conditions

Strain	Supplement*	Object class†	No. of objects (%)‡	Coverage (%)
DF2200N	None	1	520 (93)	19.8
		2	39 (7)	
			<i>559</i>	
	Fe	1	1627 (98)	23.1
		2	31 (2)	
			<i>1658</i>	
	Haemin	1	1257 (97)	23.1
		2	39 (3)	
			<i>1296</i>	
	DIP	1	346 (95)	10.6
		2	20 (5)	
			<i>366</i>	
CU1060	None	1	<i>1152</i>	8.0
	Fe	1	<i>277</i>	3.5
	Haemin	1	<i>1646</i>	23.1
	DIP	1	<i>1238</i>	9.4

*None, no addition; Fe, 100 µM FeCl₃; Haemin, 50 µM haemin; DIP, 100 µM DIP.

†Class 1, 2–300 µm²; class 2, >300 µm². All CU1060 objects were <300 µm².

‡The total no. of objects is given in italic type.

formed on the coverslips (Fig. 6b, compared to a, c, d). Furthermore, the presence of inorganic iron induced the formation of smaller and more rounded cells (Fig. 6b, inset) compared with those formed under the other experimental conditions tested (Fig. 6a, c, d, insets). In contrast, the addition of haemin to the medium increased the number of attached cells and aggregates formed (Fig. 6c), an observation

that is reflected by the percentage area coverage (Table 2). It is interesting to note that although the biofilms formed in the presence of DIP were similar in density to those formed when the cells were cultured in AAGM (Fig. 6a versus d; Table 2), the presence of this synthetic iron chelator induced the formation of chains in which the cells remained attached to each other (see Fig. 6d, inset).

Fig. 6. SEM of CU1060 cells attached to plastic coverslips. Cells were cultured in AAGM (a) or AAGM supplemented with FeCl₃ (b), haemin (c) or DIP (d). Bars, 10 µm; bars in insets, 1 µm. Open arrows identify chains of cells attached to each other.

The increased coverage induced by the presence of haemin, which is opposite to the effect seen with the addition of inorganic iron, is the most noticeable response detected with the smooth strain CU1060. Although these changes reflect the ability to respond to and adapt to changes in extracellular iron concentrations, this response by the smooth derivative may not be very significant considering that rough strains are normally isolated from patients and are the only strains to colonize and reproduce the clinical symptoms in an animal model (Schreiner *et al.*, 2003).

The significant increase in the number of aggregates in response to the addition of inorganic iron or haemin and the apparent decrease in the number of aggregates and surface coverage when an iron chelator was present in the medium are the most striking effects observed with the rough strain DF2200N. The fact that the overall amount of biofilm formed by the rough strains CU1000 and DF2200N was similar under all conditions tested (Fig. 4) indicates that the changes in biofilm structures are due to a specific differential response in attachment and aggregation on an abiotic surface rather than significant variations in growth rates under the culture conditions used in this study. Considering this response and the capacity of this oral pathogen to bind haemin, it may be hypothesized that the iron and haemin content of the crevicular fluid plays a role in the ability of *A. actinomycetemcomitans* to colonize and cause oral disease in the human host. This hypothesis is supported by the observation that the ability of *Strep. mutans* to aggregate and form biofilms on abiotic surfaces is influenced by the iron content of saliva (Berlutti *et al.*, 2004). Iron-responsive regulation also plays a role in the ability of *Staph. aureus* to form biofilms, the formation of which is enhanced under low-iron conditions (Johnson *et al.*, 2005).

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