BioMetals **17:** 271–278, 2004.

Both lactoferrin and iron influence aggregation and biofilm formation in *Streptococcus mutans*

Berlutti Francesca[1], Maria Ajello[2], Pietro Bosso[2], Clara Morea[1], Petrucca Andrea[1], Antonini Giovanni[3] & Valenti Piera[2,*]

[1]*Department of Public Health Sciences, University of Rome 'La Sapienza';* [2]*Department of Experimental Medicine II University of Naples;* [3]*Department of Biology, University of Rome Tre, Italy;* [*]*Author for correspondence (Tel: +39 335 5403965; Fax +39 6 49914626; E-mail: piera.valenti@uniroma1.it)*

Key words: aggregation, biofilm, caries, lactoferrin, *S. mutans*

Abstract

Streptococcus mutans, a Gram-positive immobile bacterium, is an oral pathogen considered to be the principal etiologic agent of dental caries. Although some researches suggest that trace metals, including iron, can be associated with dental caries, the function of salivary iron and lactoferrin in the human oral cavity remains unclear. The data reported in this study indicates that iron-deprived saliva ($Fe^{3+} < 0.1$ μM) increases *S. mutans* aggregation and biofilm formation in the fluid and adherent phases as compared with saliva (Fe^{3+} from 0.1 to 1 μM), while iron-loaded saliva ($Fe^{3+} > 1$ μM) inhibits both phenomena. Our findings are consistent with the hypothesis that *S. mutans* aggregation and biofilm formation are negatively iron-modulated as confirmed by the different effect of bovine lactoferrin (bLf), added to saliva at physiological concentration (20 μg/ml) in the apo- or iron-saturated form. Even if saliva itself induces bacterial aggregation, iron binding capability of apo-bLf is responsible for the noticeable increase of bacterial aggregation and biofilm development in the fluid and adherent phases. On the contrary, iron-saturated bLf decreases aggregation and biofilm development by supplying iron to *S. mutans*. Therefore, the iron-withholding capability of apo-Lf or native Lf is an important signal to which *S. mutans* counteracts by leaving the planktonic state and entering into a new lifestyle, biofilm, to colonize and persist in the human oral cavity. In addition, another function of bLf, unrelated to its iron binding capability, is responsible for the inhibition of the adhesion of *S. mutans* free, aggregated or biofilm on abiotic surfaces. Both these activities of lactoferrin, related and unrelated to the iron binding capability, could have a key role in protecting the human oral cavity from *S. mutans* pathogenicity.

Introduction

Iron is an essential element for living cells. In the human host, extracellular iron is bound to iron transport proteins, as transferrin in serum and lactoferrin in mucosal secretions, while intracellular iron is mainly sequestered by ferritin or heme compounds (Weinberg 1978).

In the extracellular environment the concentration of free iron (10^{-18} μM) is far lower than that required for the growth of bacteria (10^{-7} μM) (Weinberg 1999) which acquire iron from the host synthesizing different iron-transport systems (Wooldridge & Williams 1993). Moreover, iron limitation can be considered an important signal of shift from high to low iron availability for pathogenic bacteria invading the host. Bacterial iron acquisition systems and virulence determinants are often co-modulated by iron (Litwin & Calderwood 1993). The importance of iron-dependent regulation in pathogenic Gram-negative bacteria has been well known, but only few papers have considered its role in the pathogenicity of Gram-positive bacteria (Ratledge & Dover 2000). For instance, although some clinical studies suggest that trace metals, including iron, can be associated with dental caries caused by

Streptococcus mutans, the mechanism of salivary iron and lactoferrin remains unclear (Spatafora *et al.* 2001).

S. mutans, a Gram-positive immobile bacterium, is an oral pathogen considered to be the principal etiological agent of dental caries and to be associated with endocarditis (Kitten *et al.* 2000). The human oral cavity is the natural habitat of this bacterium and its pathogenesis depends on bacterial aggregation and adhesion. Saliva induces bacterial aggregation through an interaction between salivary components including agglutinin and cell-wall associated adhesin P1 (Demuth *et al.* 1990, Senpuku *et al.* 1996). The adhesion is also an interaction between *S. mutans* and salivary components adsorbed on tooth surfaces. In the presence of dietary sugar, it is well known that adhesion is mediated by an extracellular polysaccharide synthesized by *S. mutans* from sucrose via glucosyltransferase (Hamada & Slade 1980). In the absence of sugar, the bacterial adhesins, including adhesin P1, play an important role in the adhesion process to abiotic surfaces (Lee & Boran 2003). In addition, saliva of caries-resistant patients shows a high bacterial aggregation efficiency and a very low adhesion-promoting activity (Rosan *et al.* 1982).

In human saliva the iron content ranges from 0.1 to 1.0 μM depending on meals (Spatafora *et al.* 2001) and the physiological level of human lactoferrin (hLf) varies from 5 to 20 μg/ml, reaching 60 μg/ml during infections and inflammations. No data is available on the influence of the saliva iron content on *S. mutans* virulence, while the studies on the role of Lf, in the apo- or iron-saturated form, against the aggregation of this bacterium show conflicting results. Soukka *et al.* (1993) demonstrated that apo-bLf induces bacterial aggregation, while Mitoma *et al.* (2001) reported that bLf inhibits bacterial aggregation by its binding with salivary agglutinin, independently on iron saturation. These different results could be ascribed to different experimental conditions.

In this study, we investigated the influence of both iron and bLf on non-adherent aggregates and biofilm of *S. mutans* floating in fluid phase and on *S. mutans* biofilm adherent onto abiotic surfaces. For this purpose, a whole saliva pool with defined iron and hLf content was used to better mimic the *in vivo* environment.

The results obtained clearly showed that even if saliva itself induces bacterial aggregation, iron binding capability of bLf is responsible for the noticeable increase of *S. mutans* aggregates and biofilm floating in fluid phase before the adhesion on abiotic surfaces and for the increased layer of adherent biofilm. In addition, another function of bLf, unrelated to its iron binding capability, is responsible for the inhibition of the adhesion of *S. mutans* free, aggregated or biofilm on abiotic surfaces.

Materials and methods

Bacterial strain, media and culture conditions

S. mutans ATCC25175^T was maintained in Trypticase Soy broth (Difco Laboratories, MD, USA) with glycerol (25%) at -80 °C and checked for purity on Columbia Agar with 5% red sheep cells before the experiments. Brain Heart Infusion broth (BHI), containing 20 μM ferric ions, low-iron BHI (5 μM ferric ions) or iron-deprived BHI (1 μM ferric ions) were used to prepare the bacterial inoculum for the experiments. Low-iron BHI was obtained after an overnight dialysis at 4 °C against 1 mg of bovine apo-lactoferrin (apo-bLf) per 1 ml of medium and iron-deprived BHI was obtained by dialyzing low-iron BHI against 1 mg of apo-bLf per ml for 48 h at 4 °C. Iron concentration was detected by atomic absorption spectrophotometry (Perkin-Helmer 360)

Saliva

Human saliva was collected from several healthy volunteers 1 h after meals, pooled, and centrifuged for 10 min at 15,000 *g*. The supernatants were sonicated (Soniprep MSE 150, Sanyo) (three impulses for 10 sec, frequency 23 kHz; amplitude 15 μm), sterilized by filtration (0.45 μ filter, Millipore, Millipore Corporation, MA, USA), and stored at -80 °C until use. All experiments were carried out using the same saliva pool (pH 7.0) containing 5 μg/ml of human lactoferrin as detected by Elisa test (Marchetti *et al.* 1998). Iron-deprived saliva was obtained after an overnight dialysis at 4 °C against 0.1 mg of apo-bLf per 1 ml of saliva. After this treatment, the saliva iron content decreased to an undetectable level (<0.1 μM) as determined by atomic absorption spectrophotometry (Perkin-Helmer 360). Iron-loaded saliva was prepared by adding ferric ions at physiological level (0.1 or 1 μM $Fe_2(SO_4)_3$) or at higher concentrations (>1 μM $Fe_2(SO_4)_3$) to iron-deprived saliva.

Bovine lactoferrin

Apo- and iron-saturated bLf, kindly supplied by Dicofarm (Rome, Italy), were obtained and checked for purity according to Rossi *et al.* (2002). Apo- and iron-saturated bLf were added to the saliva pool at a non-antibacterial concentration (determined by counting colony forming units) corresponding to 20 μg/ml. Native bLf resulted to be about 20% iron-saturated.

Quantitative detection of free, aggregated, biofilm, and adherent S. mutans

The capability of *S. mutans* to acidify the culture medium was used in order to determine the number of free, aggregated, biofilm and adherent bacteria. This analytical procedure correlates the time required for the color switch of the pH indicator with the concentration of bacteria initially present in a sample, by a correlation line specific for *S. mutans* (y= -0.3167x $+8.5529$ and r = 0.9983).

Epifluorescence staining method

The BacLight® live/dead viability probe (Molecular Probes) (Boulos *et al.* 1999) was prepared and used according to the instructions. A suspension of *S. mutans* (0.1 ml) was washed twice with distilled water and then stained in 20 μl of work solution containing the fluorescent dyes. After 15 min of incubation in the dark, viable (stained green) and non-viable cells (stained red) were observed by using fluorescent optical microscopy (Leitz, Dialux 20 EB).

Aggregation assay

S. mutans, grown for 18 h at 37 °C in BHI, was washed three times in phoshate-buffered saline without Ca^{2+} and Mg^{2+} (PBS) and suspended at a concentration of 10^7 cells/ml in iron-deprived or iron-loaded saliva and incubated different times at 37 °C. Bacterial aggregation was checked by monitoring the changes at A_{570} nm with a recording spectrophotometer. The percentage of aggregation was calculated as $100 \times [(a - b)/a]$ where **a** is the mean value at A_{570} nm of the bacterial inoculum suspended in PBS and **b** is the mean value at A_{570} nm of bacterial suspensions at different iron concentrations.

Biofilm detection

Biofilm formation was detected according to a minor modification of the procedure described by Huber *et al.* (2001). Briefly, *S. mutans* (10^7 cells/ml) grown in BHI overnight at 37 °C was inoculated in 1.5 ml-tubes containing 1 ml of iron-deprived or iron-loaded saliva for different times at 37 °C without shaking. Thereafter, the mixtures were removed and 1 ml of aqueous solution of crystal violet (1% w/v) was added to each sample. After 20 min of incubation at room temperature, the dye was removed, the tubes were thoroughly washed and then air-dried for 3 h. The biofilm was quantified by solubilizing crystal violet with a mixture of ethanol and acetone (80:20 v/v) and by determining the absorbance of the samples at 570 nm.

Adhesion of S. mutans *onto dental polymers in the presence of apo- and iron-saturated bLf*

Dental polymer (2-hydroxyethyl methacrylate) disks (diameter = 6 mm, depth = 1 mm) were incubated for different times with *S. mutans* free, aggregated and biofilm obtained by growing the bacterium for 6 h in BHI, in low-iron BHI (5 μM) or in iron-deprived BHI (1 μM), respectively. *S. mutans*, in different morphological forms, was quantitatively determined as described in Materials and methods and the inoculum was adjusted at the same concentration (10^7 cells/ml) for all the experiments in the presence or in the absence of bLf in the apo- and iron-saturated form at 37 °C. After each time, polymer disks were thoroughly washed and tested to detect the number of adherent bacteria. The assay was capable of reliably counting the number of bacterial cells without removing them from the polymer disk surfaces.

Results

Saliva iron content influences S. mutans *aggregation*

The whole iron-deprived saliva after the addition of different iron concentrations, from <0.1 to 1 μM, was inoculated with 10^7 *S. mutans*/ml and incubated at 37 °C for different times. Until 6 h of incubation, a large number of *S. mutans* cells was floating in fluid phase, thereafter bacterial cells were adsorbed onto hard surfaces. Figure 1 summarises bacterial aggregation in fluid phase after 6 h of incubation in saliva as a function of iron concentration. The highest aggregation efficiency was induced by iron-deprived saliva ($Fe^{3+} < 0.1$ μM); saliva containing physiological concentrations of iron (from 0.1 to 1.0 μM),

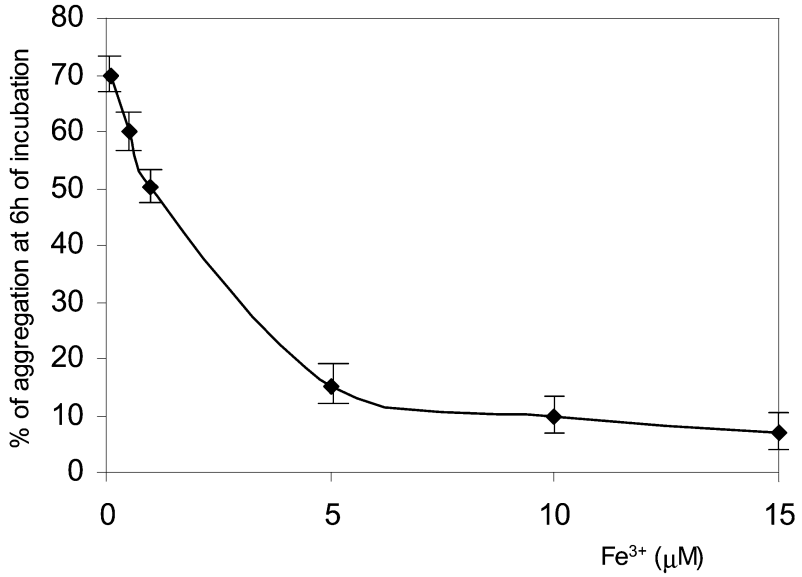

Fig. 1. Influence of saliva iron content on *S. mutans* aggregation. Inoculum of *S. mutans* corresponded to 10^7 cells/ml. The percentage of aggregation was obtained by measuring the reduction in A_{570} nm after 6 h of incubation. The percentage of aggregation was calculated as $100 \times [(a - b)/a]$ where **a** is the mean value at A_{570} nm of bacterial inoculum suspended in PBS and **b** is the mean value at A_{570} nm of bacterial suspensions at each iron concentration. Values are given as the means ± S.D. of triplicate assays.

induced aggregation to a lower extent. Iron concentrations higher than those detected in saliva (>1 μM) inhibited *S. mutans* aggregation. From these results, *S. mutans* aggregation appears to be negatively iron-modulated. The microscopic examinations (Figure 2), after BacLight® live/dead epifluorescence staining, demonstrated that iron-deprived saliva (<0.1 μM) induced non-adherent *S. mutans* aggregates in fluid phase which increased in number and size during the incubation (from 2 to 6 h) as compared with those observed in saliva (1 μM Fe^{3+}). These observations confirm that the induction of *S. mutans* aggregation is negatively iron-modulated.

Lactoferrin in apo- and iron-saturated form differently influences S. mutans *aggregation*

Iron-deprived saliva and saliva (1 μM Fe^{3+}), added with non-antibacterial concentration of bLf (20 μg/ml) in the apo- or iron-saturated form, were inoculated with 10^7 *S. mutans* /ml and incubated for 6 h at 37 °C in order to detect floating *S. mutans*. As shown in Figure 3, apo-bLf induced bacterial aggregation in fluid phase both in iron-deprived and iron-loaded saliva even if the number and size of *S. mutans* aggregates increased under iron stress conditions. Moreover, iron-saturated bLf inhibited *S. mutans* aggregation such that bacteria were free-floating in iron-loaded saliva, but formed very little aggregates in iron-deprived saliva. The addition of 20 μg/ml of native bLf (about 20% iron-saturated) induces bacterial aggregation similarly to that observed with apo-bLf (data not shown). The results of these experiments confirm the data, reported above, which indicate that *S. mutans* aggregation is negatively iron-modulated.

Saliva iron and lactoferrin content influences S. mutans *biofilm development*

In Figures 2 and 3 some *S. mutans* aggregates are highlighted with a white line. In iron-deprived saliva, these aggregates formed in fluid-phase a thin layer of non-adherent biofilm, already after 2 h and up to 6 h, while adherent biofilm was undetectable by the crystal violet assay ($A_{570} < 0.01$).

From these microscopic observations it could be argued that also biofilm development was negatively iron-modulated. In order to confirm this hypothesis bLf, in the apo- or iron-saturated form, was added at physiological non-antibacterial concentration (20 μg/ml), to iron-deprived and iron-loaded saliva infected with 10^7vcells/ml of *S. mutans* in the presence of sucrose (1%). The addition of sucrose and prolonged times of incubation were carried out in order to verify the putative iron-modulated increase of the adherent biofilm formation. After an overnight incub-

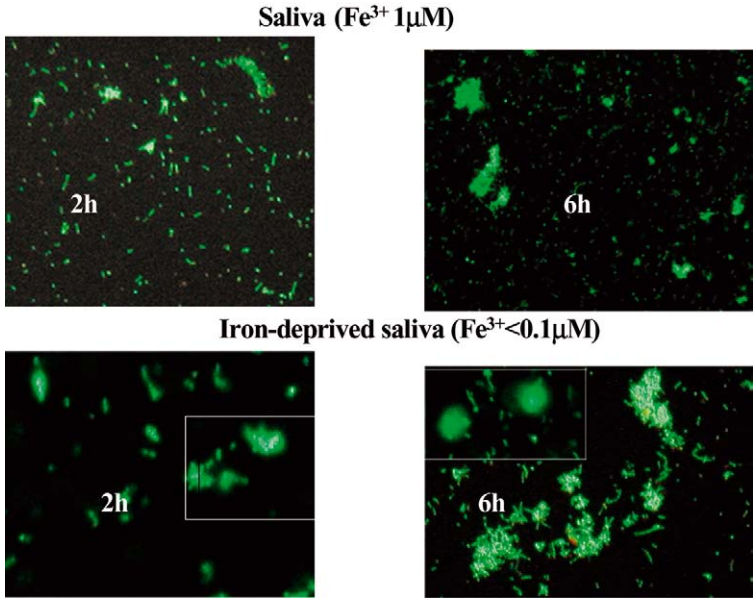

Fig. 2. Micrographs of *S. mutans* aggregates in saliva (1.0 μM) or in iron-deprived saliva (< 0.1 μM) after 2 h and 6 h of incubation at 37 °C (epifluorescence staining). Magnification $\times$ 600.

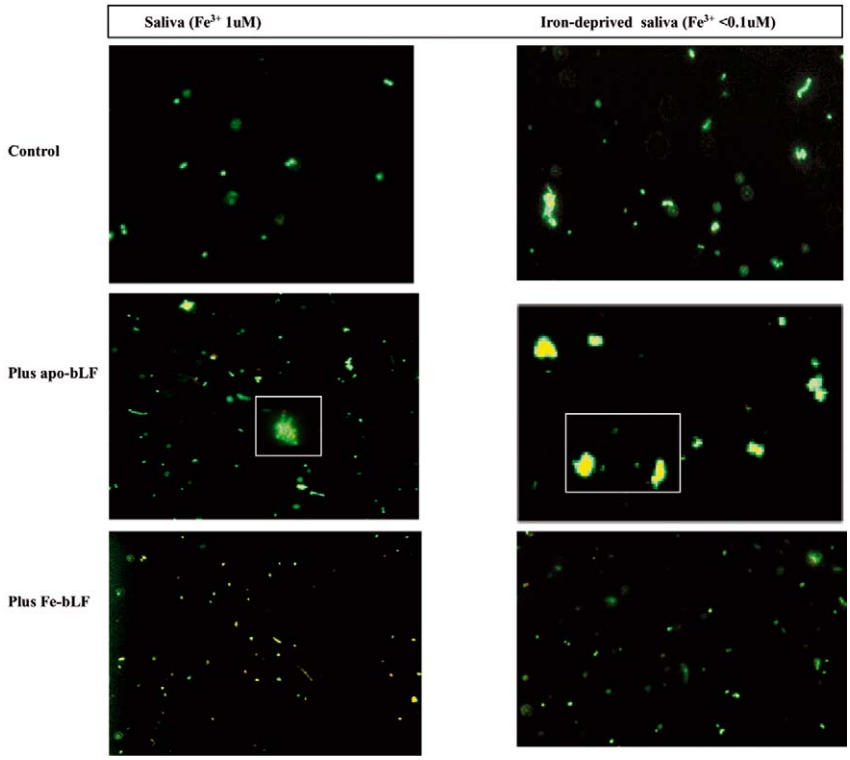

Fig. 3. Micrographs of bacterial aggregation in the presence of apo- and iron-saturated bovine lactoferrin (20 μg/ml) (epifluorescence staining). Magnification $\times$600.

Table 1. S. mutans biofilm, detected by crystal violet assay, after 18 h of incubation in iron-deprived saliva (<0.1 μM) or in saliva (Fe^{3+} 1.0 μM). Bovine lactoferrin was added in the apo- or iron-saturated (Fe-bLf) form at a non-antibacterial concentration (20 μg/ml). The bacterial inoculum corresponded to 10^7 cells/ml. Values are given as the means ±S.D. of triplicate assays.

	Biofilm detection at A_{570} nm	
	Without 1% sucrose	With 1% sucrose
Saliva	0.12 ± 0.02	0.56 ± 0.03
Saliva plus apo-bLf	0.18 ± 0.01	0.95 ± 0.04
Saliva plus Fe-bLf	0.10 ± 0.01	0.51 ± 0.04
Iron-deprived saliva	0.23 ± 0.03	1.12 ± 0.06
Iron-deprived saliva plus apo-bLf	0.38 ± 0.02	1.43 ± 0.08
Iron-deprived saliva plus Fe-bLf	0.14 ± 0.01	0.65 ± 0.03

ation at 37 °C, *S. mutans* biofilm adherent onto abiotic surfaces was quantitatively detected by the crystal violet assay. The results summarised in Table 1 indicate that adherent biofilm development in the presence of sucrose was very high. In particular it strongly increased under iron-stress conditions induced by iron-deprived saliva or by the presence of 20 μg/ml apo-bLf. On the contrary, the iron availability due to iron-loaded saliva or to the presence of iron-saturated bLf decreased the adherent biofilm formation. In the absence of sucrose similar results were obtained under all experimental conditions, even if the layer of adherent biofilm was thinner than that observed with added sucrose (see biofilm detection in Table 1). The addition of native bLf (20% iron-saturated) induced adherent biofilm development at a similar extent than that observed by adding apo-bLf (data not shown).

Anti-adhesive activity of lactoferrin

It is well known that the functions of Lf can be distinguished between those related to its iron binding capability and those unrelated to the iron chelating property. The induction of bacterial aggregation and biofilm development in fluid and adherent phase by bLf is related to its iron binding ability, while bLf inhibition of bacterial adhesion onto surfaces is iron-independent (Longhi *et al.* 1993, Longhi *et al.* 1994, Antonini *et al.* 1997, Qiu *et al.* 1998, Oho *et al.* 2002). To test the influence of bLf on *S. mutans* adhesion, the crucial event to colonise the oral cavity and to exert pathogenicity, the adhesion efficiency of this

Table 2. Adhesion efficiency of different forms of *S. mutans* on dental polymer disks detected by MRT. Bovine lactoferrin (bLf) in the apo- or iron-saturated (Fe-bLf) form was added at 20 μg/ml. The bacterial inoculum corresponded to 10^7 cells/ml. Values are given as the means ±S.D. of triplicate assays.

	% Adherent bacteria		
	Free form	Aggregated form	Biofilm form
None	60 ± 5	73 ± 7	85 ± 9
Plus apo-bLf	25 ± 8	15 ± 5	30 ± 3
Plus Fe-bLf	20 ± 6	14 ± 3	32 ± 5

bacterium on dental polymer disks, in the presence or in the absence of bLf in apo- or iron-saturated form, has been investigated. The experiments were carried out by incubating dental polymer disks with *S. mutans* in the free, aggregated and biofilm floating forms at the same concentrations. Already after 2 h of incubation at 37 °C, the maximal adhesion ability of *S. mutans* in all morphological forms was observed (data not shown). Consequently, 10^7 bacteria/ml added to dental polymer disks were incubated only for 2 h with or without differently iron-saturated bLf (20 μg/ml). The results are reported in Table 2. The quantitative evaluation of adherent bacteria, demonstrated that the highest adhesion ability is possessed by biofilm, followed by bacterial aggregates and lastly by free bacteria. Both apo- and iron-saturated bLf exerted a noticeable inhibition of bacterial adhesion against free and aggregated forms and to a lower extent towards biofilm.

Discussion

In this paper we report that the induction of *S. mutans* aggregation by saliva is dependent on its iron content. The highest aggregation efficiency was observed in iron-deprived saliva ($Fe^{3+} < 0.1$ μM) and in normal saliva (Fe^{3+} from 0.1 μM to 1.0 μM) while higher iron concentrations inhibited *S. mutans* aggregation (Figure 1). Therefore, the bacterial aggregation efficiency is inversely proportional to saliva iron concentration. The microscopic examinations (Figure 2) support these results clearly indicating that in fluid phase *S. mutans* aggregation is negatively iron-modulated. The involvement of iron availability on *S. mutans* aggregation is also consistent with the data obtained by adding to the whole saliva pool, bLf at a physiological non-antibacterial dose (20 μg/ml), in the apo- or in iron-saturated form. Apo-bLf added to

saliva and to iron-deprived saliva, by chelating iron from *S. mutans* cells, increased the number and size of the non-adherent aggregates as compared with those observed in the whole saliva pool (containing 5 μg/ml of hLf). Iron-saturated bLf, by supplying ferric ions to *S. mutans* (Brown & Holden 2002), noticeably decreased non-adherent *S. mutans* aggregates in both iron-deprived and iron-loaded saliva. It is likely that the iron deprivation induced *S. mutans* aggregation represents an early microbial adaptive response to stress conditions that allows the bacterium to survive and persist in a harmful habitat.

Mitoma *et al.* (2001) reported that bLf inhibits bacterial aggregation by binding to the salivary agglutinin in apparent conflict with the results presented here. It should be stressed that Mitoma *et al.* did not determine the saliva iron content. Iron-saturated bLf inhibits the aggregation by supplying iron to bacteria (Brown & Holden 2002), while apo- or native bLf by chelating iron induce a stress condition which is counteracted by *S. mutans* by increasing aggregation in fluid phase. It would be reasonable to assume that the ability of *S. mutans* to form aggregates and biofilm, involves gene regulation systems, including quorum sensing, but at the moment, these are unknown.

Our findings are consistent with the hypothesis that bacterial density, as well as bacterial aggregation, can be considered an important and early signal for bacteria which leave the planktonic state and enter into biofilm to colonize and persist in the human oral cavity. Moreover, we report that iron-deprivation produces a stress environmental condition, which rapidly induces non-adherent bacterial aggregates evolving in a thin layer of non-adherent biofilm (Figures 2 and 3), before the development of a thick adherent biofilm (Table 1). Similarly, bLf, in the apo- or native-form, by chelating iron, produces a harmful condition to which bacteria answer by forming non-adherent aggregates, developing early in thin non-adherent biofilm and successively in thick adherent biofilm (Table 1). On the contrary, iron availability does not induce noticeable bacterial aggregation and free-floating bacteria do not form biofilm, except with added sucrose. However, the induction of biofilm by sucrose is considerably lower in iron-loaded saliva or in the presence of iron-saturated bLf as compared with that observed in iron-deprived saliva or in the presence of apo-bLf (Table 1).

Finally, it should be recalled that Lf not only may modulate *S. mutans* aggregation and biofilm development by chelating available iron, but that it is also capable of inhibiting bacterial adhesion to abiotic surfaces. This latter function, unrelated to the Lf iron-binding ability, depends on the intrinsic characteristics of the protein itself. Both these Lf activities, related and unrelated to iron binding capability, could have a key role in protecting the human oral cavity from *S. mutans* pathogenicity.

Acknowledgements

This paper is dedicated to the memory of our unforgettable mentor Professor Eraldo Antonini, deceased prematurely on March 19, 1983, who introduced us to the study of ovotransferrin and lactoferrin.

This work was supported in part by grants from the Ministero Istruzione Università e Ricerca, Project "Stress ossidativo e metalloproteine in infezioni da batteri intracellulari facoltativi" and from CNR, PF Materiali speciali per tecnologie avanzate II.

References

Antonini G, Catania MR, Greco R, Longhi C, Pisciotta MG, Seganti L, Valenti P. 1997 Antiinvasive activity of bovine lactoferrin towards *Listeria monocytogenes*. *J Food Protection* **1**, 60–72.

Boulos L, Prevost M, Barbeau B, Coallier J, Desjardins R. 1999 LIVE/DEAD BacLight: application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J Microbiol Methods* **37**, 77–86.

Brown JB, Holden DW. 2002 Iron acquisition by Gram-positive bacterial pathogens. *Micro Infect* **4**, 1149–1156.

Demuth DR, Lammey MS, Huck M, Lally ET, Malamud D. 1990 Comparison of *Streptococcus mutans* and *Streptococcus sanguis* receptors for human salivary agglutinin. *Microb Pathog* **9**, 199–211.

Hamada S, Slade HD. 1980 Biology, immunology, and cariogenicity of *Streptococcus mutans. Microbiol Rev* **44**, 331–384.

Huber B, Riedel K, Hentzer M, Heydorn A, Gotschlich A, Givskov M, Molin S, Eberl L. 2001 The cep quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. *Microbiology* **147**, 2517–2528.

Kitten T, Munro CL, Michalek SM, Macrina FL. 2000 Genetic characterization of a *Streptococcus mutans* LraI family operon and role in virulence. *Infect Immun* **68**, 4441–4451.

Lee SF, Boran TL. 2003 Roles of sortase in surface expression of the major protein adhesin P1, saliva-induced aggregation and adherence, and cariogenicity of Streptococcus mutans. *Infect Immun* **71**, 676–681.

Litwin CM, Calderwood SB. 1993 Role of iron in regulation of virulence genes. *Clin Microbiol Rev* **6**, 137–149.

Longhi C, Conte MP, Seganti L, Polidoro M, Alfsen A, and Valenti P. 1993 Influence of lactoferrin on the entry process of *E. coli* HB101 (pRI203). *Med Microbiol Immunol* **182**, 25–35.

Longhi C, Conte MP, Bellamy W, Seganti L, and Valenti P. 1994 Effect of lactoferricin B, a pepsin-generated peptide of bovine lactoferrin, on *E. coli* HB101(pRI203) entry into HeLa cells. *Med Microbiol Immunol* **183**, 77–85.

Marchetti M, Pisani S, Antonini G, Valenti P, Seganti L, and Orsi N 1998 Metal complexes of bovine lactoferrin inhibit *in vitro* replication of Herpes simplex virus type1 and 2. *Biometals* **11**, 89–94.

Mitoma M, Oho T, Shimazaki Y, Koga T. 2001 Inhibitory effect of bovine milk lactoferrin on the interaction between a streptococcal surface protein antigen and human salivary agglutinin. *J Biol Chem* **276**, 18060–18065.

Oho T, Mitoma M, Koga T. 2002 Functional domain of bovine milk lactoferrin which inhibits the adherence of *Streptococcus mutans* cells to a salivary film. *Infect Immun* **70**, 5279–5282.

Qiu J, Hendrixson DR, Baker EN, Murphy TF, St Geme JW 3rd, Plaut AG. 1998 Human milk lactoferrin inactivates two putative colonization factors expressed by *Haemophilus influenzae. Proc Natl Acad Sci USA* **95**, 12641–12646.

Ratledge C, Dover LG. 2000 Iron metabolism in pathogenic bacteria. *Annu Rev Microbiol* **54**, 881–941.

Rosan B, Appelbaum B, Golub E, Malamud D, Mandel ID 1982 Enhanced saliva-mediated bacterial aggregation and decreased bacterial adhesion in caries-resistant versus caries-susceptible individuals. *Infect Immun* **38**, 1056–1059.

Rossi P, Giansanti F, Boffi A, Ajello M, Valenti P, Chiancone E, Antonini G 2002 Ca^{2+} binding to bovine lactoferrin enhances protein stability and influences the release of bacterial lipopolysaccharide. *Biochem Cell Biol* **80**, 1–8.

Senpuku H, Kato H, Todoroki M, Hanada N, Nisizawa T 1996. Interaction of lysozyme with a surface protein antigen of *Streptococcus mutans. FEMS Microbiol Lett* **139**, 195–201.

Soukka T, Tenovuo J, Rundegren J. 1993 Agglutination of *Streptococcus mutans* serotype C cells but inhibition of *Porphyromonas gingivalis* autoaggregation by human lactoferrin. *Arch Oral Biol* **38**, 227–232.

Spatafora G, Moore M, Landgren S, Stonehouse E, Michalek S. 2001 Expression of *Streptococcus mutans* fimA is iron-responsive and regulated by a DtxR homologue. *Microbiology* **147**, 1599–1610.

Weinberg ED. 1978 Iron and infection. *Microbiol Rev* **42**, 45–66.

Weinberg ED. 1999 Iron loading and disease surveillance. *Emerg Infect Dis* **5**, 346–352.

Wooldridge KG, Williams PH. 1993 Iron uptake mechanisms of pathogenic bacteria. *FEMS Microbiol Rev* **12**, 325–348.