From:
The Department of Oral Microbiology,
University of Umeå,
Sweden.

SIDEROCHROMES IN SALIVA IN PERSONS WITH HIGH AND LOW CARIES FREQUENCY

by

F. NORDH

INTRODUCTION

In recent years bacterial metabolic products have been isolated which are characterised by an iron complex with an absorption maximum at 430 mμ. They have been called siderochromes (*Bickel et al.*, 1960). They have fairly different biological properties and have been divided into three groups: sideromycins (antibiotics), sideramines (growth factors) and other siderochromes (biologically not yet differentiated). The sideramine activity has often been demonstrated in metabolic products of bacteria (*Keller-Schierlein et al.*, 1964). Such activity has also been demonstrated in extracts from *e.g.* tomatoes and liver (*Zähner et al.*, 1960) and inflammed tissue (*Wöhler*, 1964). The sideramines that have been studied most, the so-called ferrioxamines, are formed by Actinomycetes (*Zähner et al.*, 1962).

Siderochromes are complex formations between organic thrihydroxaminic acids and trivalent Fe (Fig. 1).

Of the sideromycins the chemical structure of ferrimycin A (*Bickel et al.*, 1965) is known only; that of succinimycin, of interest in the present investigation, is only partly understood (*Haskell et al.*, 1963). Of the sideramines, ferrioxamine B is the one that has been studied most. It occurs also without chelated iron (desferrioxamine) and then has great affinity for Fe^{3+}. The

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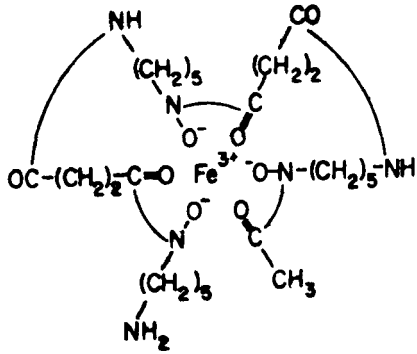

Fig. 1. The chemical structure of ferrioxamin B. (*Keller-Schierlein et al.*, 1964)

stability constant of this complex is $10^{30.7}$, while that of transferrin, the iron-transporting globulin in serum, is 10^{27-29}. Theoretically, this means that desferrioxamine can release iron from transferrin (*Moeschlin & Schnider*, 1964).

Because of its strong ability to chelate Fe^{3+} ferrioxamine B has been used in medicine to eliminate pathologically increased iron from the body (*Moeschlin*, 1962; *Wöhler*, 1963; *Fielding et al.*, 1966; *Hallberg et al.*, 1967 and others).

According to *Zähner et al.* (1962), the wide spread of the sideramine activity suggests that these substances are essential for the maintenance of cell function and that they act as coenzymes in hemin synthesis.

In a previous investigation the transferrin value in the blood serum in a group of persons with high caries frequency and a large number of lactobacilli in the saliva was found to be higher than in another group with a low caries frequency and low lactobacillus count (*Nordh*, 1965). The higher transferrin value in the former group was interpreted as a consequence of a chronic iron deficiency. In the light of our present knowledge of siderochromes, however, it cannot be excluded that persons with high caries activity have desferrisideramines in the oral cavity which chelate iron and prevent its uptake or utilisation by the body. It was therefore considered worthwhile to study the saliva for siderochromes and sideramines.

MATERIAL AND METHODS

Bacteria. Oxford strain Staphylococcus aureus (S 209) was used as test bacterium for sideramine activity. As possible producers of sideramine the following bacteria were examined: *Actinomyces*, isolated from the oral cavity,

Leptotrichia: 3 strains: Nos 18, 19, 20 (all courteously placed at disposal by ass. professor Göran Frostell, Department of Cariology Stockholm); *Lactobacillus casei, L. plantarum, L. fermenti, L. Leichmanni* (from the State Bacteriological Laboratory, Stockholm); *Bacillus subtilis*; *Streptococcus salivarius, S. mitis, S. faecalis, S. zymogenes.* All were isolated from the oral cavity.

Media. Beef Broth (7.5 g Beef Broth +10 g Peptone +1 g Dextrose); Brewer-Thioglycollate Medium (Oxoid); Brewer-Thioglycollate Medium (Oxoid) + serum; Bacto-Dextrose-Broth (Difco); Trypticase Soy Broth (BBL); Medium according to Zähner et al. (1960): 2 % Soy flour + 2 % mannite + water, pH 7.3.

Determination of siderochromes in saliva. 40 ml of saliva was obtained from a group of 19 persons with high caries frequency and a high lactobacillus count ($\geqslant$ 600,000 lb/ml)*, and from a group of 9 persons with low caries frequency and a low count ($\geqslant$ 10,000 lb/ml)**. The flow of saliva was stimulated by chewing of paraffin. Samples were obtained in the morning before breakfast and before the teeth were brushed. The amount was reduced to about 15 ml in a vacuum. The siderochromes were extracted with benzyl alcohol by the method of *Keberlé* (1964). To a 15 ml centrifuge tube was added 6 ml concentrated saliva plus 6 ml of trichloracetic acid, which were then shaken vigorously and centrifuged. Of the supernatant fluid, 5 ml was transferred to each of two centrifuge tubes. To the one tube was added 0.15 ml of 0.6 % $FeCl_3$-solution. The tube was allowed to stand for at least 5 minutes, after which 3 ml of saturated $NaHCO_3$, 1.5 g NaCl and 5 ml benzyl alcohol were added. The tube was then vigorously shaken for at least 1/2 minute and centrifuged. The bottom layer was pipetted off. Washing was repeated a further 2 times. Of the benzyl alcohol, 4 ml was transferred to a serum tube, and 1 ml ethyl alcohol was added.

The same procedure was performed with the other test tube but without addition of $FeCl_3$-solution. The two benzyl alcohol solutions were read in a spectrophotometer(Unicam SP 500) at 430 mμ. From the difference between the two values read the siderochrome-quantity was determined with desferrioxamine B as a standard.

Determination of sideramine activity in saliva and culture

I. *Saliva.* A. 40 ml of saliva was mixed with an equal volume of 90 % methanol, filtered and evaporated in a vacuum to half its volume. Equal

* Hereafter called »Lactobacill-mill.»

** Hereafter called »Lactobacill-O»

parts of acetone were added and the solution was centrifuged and evaporated in vacuum to about 4 ml. The concentrate was tested immediately for sideramine (ferrioxamine B) activity.

B. With a fourfold volume of ether sideramines were extracted from the benzyl alcohol from the quantitative determination method, and the extract was dissolved in water and tested for ferrioxamine B activity.

The testing for ferrioxamine B activity was performed essentially according to the specific »antagonist test» according to *Bonifas* (1952) and *Zähner et al.* (1960). A blood agar plate inoculated with a 24-hour culture of Oxford strain Staphylococcus aureus (S 209) (*Eriksson*, 1960) was prepared. A strip of filter paper soaked with ferrimycin (Succinimycin, CIBA A 22765) was first placed on the plates and then transverse strips with the bacteria or organic suspension to be tested for sideramine activity. After the paper strips had been applied to the plate, the latter was kept at room temperature for 3 hours. After 3—5 hours' incubation at 37°C an inhibition zone was observed around the paper containing ferrimycin. If the papers placed at right angles to this strips (zone) contained ferrioxamine B, the part of the inhibition zone nearest the papers became narrower. The degree of narrowing varying with the concentration of the ferrioxamine (Fig. 2).

II. *Culture medium.* Bacteria from plates were cultured in 10 ml broth for 2—3 days. When growth was good a further 40 ml was inoculated of the same medium. The culture was incubated at 37°C for 6—8 days.

To the medium were added 5 ml of 0.6 % $FeCl_3$ and 2 % Hyflosupercel (*Zähner et al.*, 1960). The flask was carefully shaken and then allowed to stand for half an hour. The bacteria were filtered off and to the clear, filtered

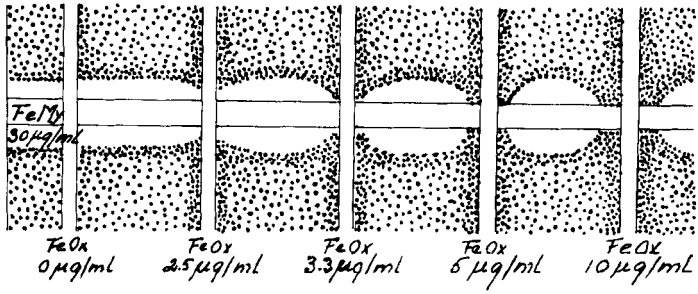

Fig. 2. A drawing of a blood agar plate inoculated with Staph. aur. (S 209) as test bacterie. In the horizontal paperstrip succinimycin (FeMy, 30 µg/ml). In the vertical strips ferrioxamin B of different concentrations (FeOx).

At the vertical strips one can see different degrees of inhibition of the antibiotic effect of succinimycin.

medium was added 10 ml of a 65 % phenol-chloroform solution. This was shaken for one minute and centrifuged at 4,000 r.p.m. for half an hour. The phenol-chloroform part was washed with water, after which a fourfold volume of ether was added. The activity factor was dissolved in half a volume of water. The mixture was evaporated to about 2 ml in vacuum.

RESULTS

Quantitative determinations of siderochromes

Determinations were made in a group of 19 »Lactobacill-mill». Of these, 4 determinations were made in 4 persons, 2 in 10 pers. and 1 in 5 pers. In the group of »Lactobacill-0» determinations were made in 9 persons: 3 in 3 pers., 2 in 1 pers., and 1 in 5 pers.

The 2 groups were not studied separately according to sex. The first group consisted mainly of males; the latter, mainly of females.

Means (M) and standard error of the Means ε(M) of the 2 groups were:

»*Lactobacill-mill.*»: M = 7.1 μg/ml
ε(M) $\pm$ 0.56
n = 41

»*Lactobacill-O*»: M = 1.8 μg/ml
ε(M) $\pm$ 0.23
n = 16

The difference between the 2 groups showed a t-value of 8.5 ($p < 0.001$).

Table I.

Qualitative sideramine activity determinations in saliva. Antagonist: 30 μg/ml succinimycin (CIBA A 22765)

	$\pm$ 0	+	2+	3+	4+
Ferrioxamine B 2.5 μg/ml Reference n = 70	0	6	33	52	9
»Lactobacill-mill.» n = 23	0	56	26	9	9
»Lactobacill-O» n = 43	26	30	33	11	0

Qualitative determinations in saliva of sideramine activity.

The determinations are given in Table I., where the competitive effect of 70 antagonist-test determinations with 2.5 μg/ml ferrioxamine B and 30 μg/ml succinimycin graded from 0 to 4+ and with Staph. aureus S 209 as a test strain was used as reference (Fig. 3).

On comparison between the reference series and the group »Lactobacill-mill» and »Lactobacill-0» both test groups had a lower effect than the reference series. The largest difference between the two test groups was that the »Lactobacill-mill» group had the largest number of high values and the »Lactobacill-O» group the largest number of O-values.

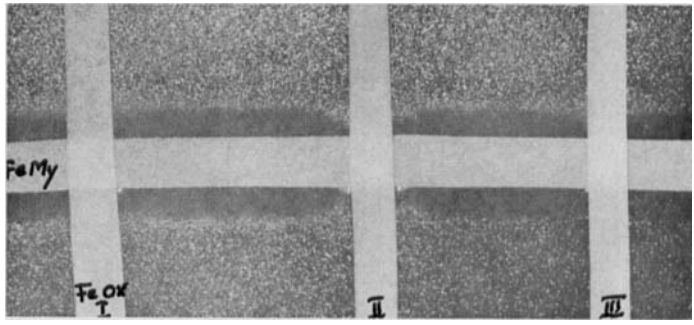

Fig. 3. A blood agar plate with Staph. aur. (S 209) as test bacterie. In the horizontal paperstrip succinimycin (FeMy, 30 μg/ml). In the first (I) vertical strip ferrioxamin B (FeOx, 2,5 μg/ml) as reference. In the second (II) strip saliva extract from the »Lactobacill-mill.»-group and in

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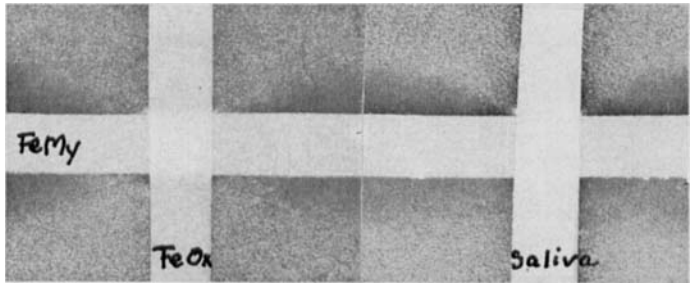


Fig. 4. A blood agar plate with Staph. aureus as test bacterie, cultured from the oral cavity. In the horizontal paper strip succinimycin (FeMy, 30 μg/ml.) In the vertical strip to the left (FeOx) ferrioxamin B (2,5 μg/ml) as reference and to the right saliva extract from the »Lactobacill-milj.»-group.
The inhibitory effect is similar at both vertical strips.

The results from an antagonist test with 2.5 μg/ml ferrioxamine B and 30 μg/ml succinimycin as reference and »Lactobacill-mill.»-saliva with a strain of Staph. aureus from the oral cavity as test strain are given in Fig. 4.

Qualitative determinations of sideramine activity in bacterial media

The results of the qualitative determination of sideramine activity in bacterial media is apparent from Fig. 5, 6, 7 and 8. with text.

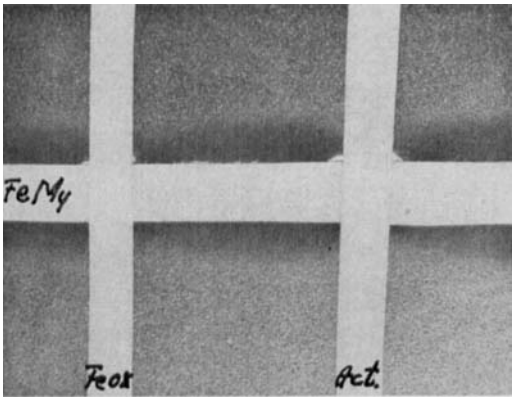


Fig. 5. Results from »antagonist test» with Actinomyces, cultured from the oral cavity. As test bacterie on the blood agar plate: Staph. aur. (S 209). In the horizontal paperstrip succinimycin (FeMy, 30 μg/ml), in the vertical strip to the left ferrioxamin B (FeOx, 2,5 μg/ml) and in the vertical strip to the right culture extract of Actinomyces (Act.).

The inhibitory effect of the Actinomyces extract is greater than that of 2,5 μg/ml ferrioxamin B.

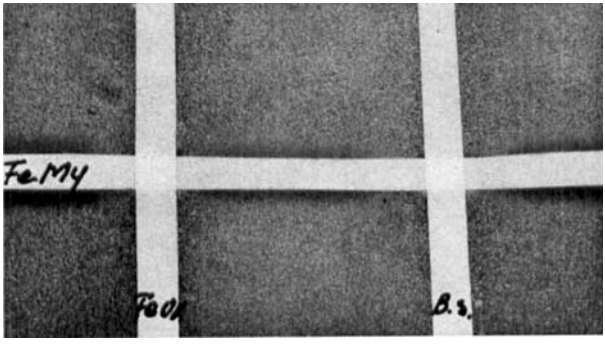
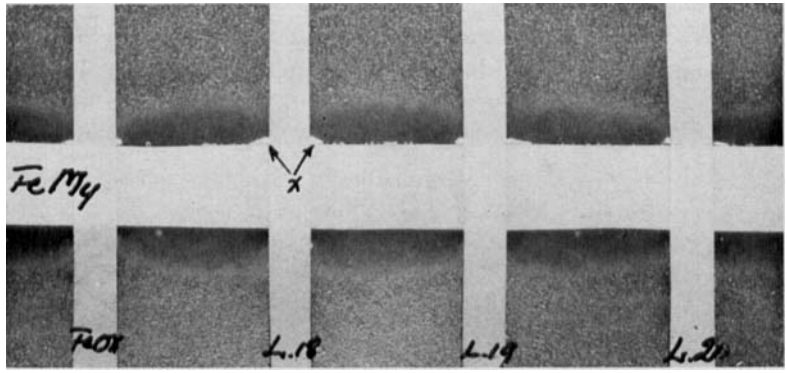


Fig. 6. Results from »antagonist test» with Bacillus subtilis, cultured from the oral cavity. As test bacterie on the blood agar plate: Staph. aur. (S 209). In the horizontal paperstrip succinimycin (FeMy, 2,5 μg/ml), in the vertical strip to the left ferrioxamin B (FeOx, 2,1 μg/ml) and in the vertical strip to the right culture extract of Bacillus subt. (B.s.).

The inhibitory effect of the B.s.-extract is greater than that of 2,1 μg/ml ferrioxamin B.



Fg. 7. Results from »antagonist test» with Leptotrichia 18, 19, 20, according to G. Frostell (see text), As test bacterie on the blood agar plate: Staph. aur. (S 209). In the horizontal paper-strip succinimycin (FeMy, 30 μg/ml), in the vertical strip to the left ferrioxamin B (FeOx, 2,5 μg/ml) and in the strips to the right culture extract from Leptotrichia 18, 19, 20 (L. 18, L. 19, L. 20).

The inhibitory effect is similar to that of ferrioxamin B, 2,5 μg/ml, of L.-18 and of L.-19-extract, smaller of L.-20-extract.

(At »X» in the picture light reflexes at photography.)

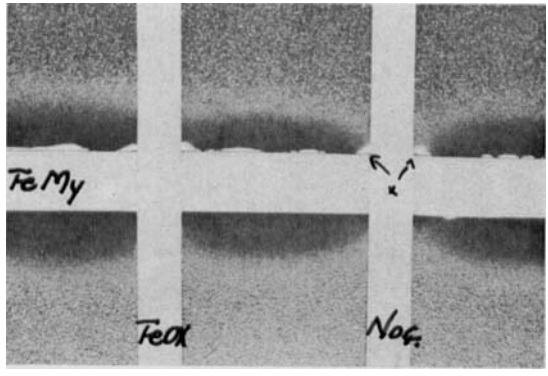


Fig. 8. Results from »antagonist test» with Nocardia, cultured from the oral cavity. As test bacterie on the blood agar plate: Staph. aur. (S 209). In the horizontal paperstrip succinimycin (FeMy, 30 μg/ml) in the vertical strip to the left ferrioxamin B (FeOx, 2,5 μg/ml) and in the vertical strip to the right culture extract of Nocardia (No.).

The inhibitory effect of the Noc.-extract is greater than that of 2,5 μg/ml ferrioxamin B.

(At »X» in the picture light reflexes at photography.)

DISCUSSION

Siderochromes were demonstrated in the saliva. In the group »Lactobacill-mill.» the amount was larger than in the group »Lactobacill-O». The difference was highly significant. In the group »Lactobacill-mill.» there were some siderochromes in the form of desferrioxamine, which could not be demonstrated in the group »Lactobacil -O». However, in control test with parotid saliva obtained under sterile conditions from 3 of the persons in the latter group it was possible to demonstrate siderochromes (average 2.8 μg/ml), and some of these occurred as desferrisiderochromes.

The difference between the two groups may be due to several factors. First of all, one might assume an addition of sideramines from the bacteria in the oral cavity in the group »Lactobacill-mill» with its abundance of plaques. To check this assumption the sideramine activity in the bacterial cultures was determined. Of these, it was found that strains of Actinomyces, Leptotrichia, Nocardia and Bacillus subtilis were good producers of sideramines. Strains of Lactobacillus and Streptococcus showed only weak sideramine activity. Other differentiating factors may be the rate of secretion of saliva, filtration from serum and supplementary iron from the food.

Since the Actinomyces, Leptotrichia and Nocardia, which usually occur in the plaques, are producers of desferrisideramines, it cannot be excluded that Fe^{3+} is released from the ferritin in the enamel surface and in this way exposes apoferritin to proteinolysis. Chelation requires a low pH and phosphate ions in excess (Keberlé 1964). Also in the qualitative determination a difference was found between the two groups: a larger number of O-values in the »Lactobacill-O»-group. Agreement between the two methods was, however, not to be expected because Keberlé's method measures for the whole amount of siderochromes, while the antagonist test shows the presence of sideramines inhibited by the ferrimycin succinimycin.

That the test papers also contained several sideramines was apparent from that part of the outlines nearest of the inhibition zones. They were often diffuse, presumably because of a different degree of rate of diffusion and relative activity of the sideramines (*Keller-Schierlein et al.*, 1964).

A question of interest is the significance of the occurrence of desferrisideramines in saliva for the turnover of iron in the body. The mechanism of the absorption of iron in the intestinal mucosa is not properly understood, nor is it known why iron accumulates in the reticuloendothelial system in inflammatory conditions (*Wheby et al.*, 1963; *Crosby*, 1963; *Karabus et al.*, 1967.). Because of the high stability constant of sideramines, the latter may exert a certain influence on this mechanism, especially on the transfer of iron from the intestinal mucosa to the plasma. An excess of desferrisideramines

in the serum would, by chelation of Fe^{3+} and excretion to the kidneys, also result in higher values for transferrin in the serum because of iron deficiency (*Brendstrup*, 1950; *Laurell*, 1960, 1963).

As previously mentioned, the growth factors of sideramines constitute growth factors for some bacteria. One must therefore expect sideramines in plaques and saliva being a governing factor for certain types of bacteria and thereby influence the ecology of the oral cavity.

The synthesis and biological function of siderochromes is not properly understood. The occurrence of sideramines in the oral cavity may prove useful in the investigation of caries.

SUMMARY

Siderochromes and Sideramines, complex formations between organic trihydroxamic acids and Fe^{3+} have been demonstrated in saliva. The amount was significantly larger in a group with high caries frequency and high lactobacillus count than in a group with low caries frequency and low lactobacillus count. The difference may be due to differences in the oral flora because Actinomyces, Leptotrichia and Nocardia, which are common in dental plaques, proved to be good producers of sideramines. But sideramine activity was demonstrated also in parotid saliva collected under sterile conditions from persons with a low caries frequency and with low lactobacillus count. Sideramines are growth factors for certain bacteria and may thus constitute a governing factor in the ecological conditions in the oral cavity. The affinity of siderochromes for Fe^{3+} and the high stability constant of this compound may perhaps shed new light on the pathogenesis of caries.

RÉSUMÉ

LES SIDÉROCHROMES DE LA SALIVE CHEZ LES PERSONNES PRÉSENTANT UNE FRÉQUENCE DE LA CARIE ÉLEVÉE OU BASSE

Des sidérochromes et des sidéramines, combinaisons entre les acides organiques trihydroxamiques et Fe^{3+} ont été mis en évidence dans la salive. On les trouvait en quantité significativement plus abondante dans un groupe présentant une fréquence de carie élevée et chez qui la numération des lactobacilles donne des valeurs élevées que dans un groupe ayant une faible fréquence de carie et des valeurs peu élevées à la numération des lactobacilles. Cette différence peut être due à des différences entre les flores buccales, puique les Actinomyces, les Leptotrichia et les Nocardia, qui sont courants

dans la plaque bactérienne dentaire, sont apparus comme de bons producteurs de sidéramines. Cependant, l'activité sidéramine a aussi été constatée dans la salive parotidienne recueillie dans des conditions stériles chez des personnes ayant une faible fréquence de carie et des valeurs peu élevées à la numération des lactobacilles. Les sidéramines sont des facteurs de croissance pour certaines bactéries et peuvent ainsi constituer un facteur décisif pour les conditions écologiques dans la cavité buccale. L'affinité des sidérochromes envers Fe^{3+} et la constante de stabilité élevée de ce composé permettront peut-être d'éclaircir de nouveaux aspects de la pathogénese de la carie.

ZUSAMMENFASSUNG

SIDEROCHROME IM SPEICHEL VON VERSUCHSPERSONEN MIT HOHER UND MIT NIEDRIGER KARIESFREQUENZ

Siderochrome und Sideramine, komplexe Bildungen zwischen organischen Trihydroxamsäuren und Fe^{3+} konnte man im Speichel finden. Die Menge Menge war signifikant grösser in der Gruppe mit hoher Kariesfrequenz und hoher Lactobazillenzahl als in der Gruppe mit niedriger Kariesfrequenz und niedriger Lactobazillenzahl. Der Unterschied kan möglicherweise damit erklärt werden, dass in der Gruppe mit hoher Kariesfrequenz u.a. Actinomyces, Leptorichia und Nocardia vorkommen. Die angegebenen Organismen findet man reichlich in dentalen Plaques und sind gute Sideraminbilder. Aber auch im Speichel, gewonnen unter sterilen Verhältnissen von Versuchspersonen mit niedriger Kariesfrequenz und niedriger Lactobazillenzahl konnte man Sideraminaktivitet beweisen. Die Sideraminen sind ein Wachstumsfaktor für gewisse Bakterien und können solcherart einen Steuerungsfaktor für die ekologischen Verhältnisse in der Mundhöhle bilden. Die Selektivität der Siderochrome zum Fe^{3+} und dessen hoher Stabilitätskonstante können ausserdem der Pathogenesis des Kariesprozesses neue Aspekte zuführen.

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Address:

Department of Oral Microbiology,
School of Dentistry,
University of Umeå,
Umeå, Sweden