JOURNAL OF BACTERIOLOGY, Dec. 1986, p. 1096–1099
0021-9193/86/121096-04$02.00/0

Vol. 168, No. 3

Ferrous Iron Transport In *Streptococcus mutans*

S. L. EVANS,† J. E. L. ARCENEAUX, B. R. BYERS,* M. E. MARTIN,‡ AND H. ARANHA§

Department of Microbiology, University of Mississippi Medical Center, Jackson, Mississippi 39216-4505

Received 11 August 1986/Accepted 5 September 1986

Radioiron uptake from $^{59}FeCl_3$ by *Streptococcus mutans* OMZ176 was increased by anaerobiosis, sodium ascorbate, and phenazine methosulfate (PMS), although there was a 10-min lag before PMS stimulation was evident. The reductant ascorbate may have provided ferrous iron. The PMS was reduced by the cells, and the reduced PMS then may have generated ferrous iron for transport; reduced PMS also may have depleted dissolved oxygen. We conclude that *S. mutans* transports only ferrous iron, utilizing reductants furnished by glucose metabolism to reduce iron prior to its uptake.

Streptococcus mutans is considered the major odontopathogen of human dental caries (11). However, the presence of *S. mutans* in the oral cavity does not imply disease in a simple cause-and-effect relationship; caries results from a complex interaction between host, microbial, and dietary factors (11). Some trace metals may shift this equilibrium to favor either the host or the microbe, and the presence and concentrations of these metals may be cariogenic or cariostatic (reviewed in reference 2). *S. mutans* has a superoxide dismutase that uses manganese as a cofactor but can substitute iron for manganese to produce active superoxide dismutase if manganese is absent (12, 13). Neither metal is required for anaerobic growth of *S. mutans* (13). Aerobic metabolic roles for iron in addition to its function as a cofactor for superoxide dismutase are suggested by experiments showing iron stimulation (nearly threefold) of *S. mutans* steady-state growth in manganese-containing medium (2). Iron-containing cytoplasmic fractions (other than superoxide dismutase) have been demonstrated in *S. mutans* (12). In other studies, iron was required for *S. mutans* growth (3), and an increase in colony size was obtained by adding iron to a solidified minimal medium (10). Lactoferrin (but not iron-saturated lactoferrin) increased the length of the *S. mutans* lag phase (5), suggesting that the organism is unable to obtain iron bound by lactoferrin.

Transport of iron by *S. mutans* has not been extensively investigated. For efficient iron acquisition, many microorganisms produce siderophores that bind highly insoluble ferric iron, making it available for transport (14). Intracellular utilization of iron by *S. mutans* and the existence of specialized transport systems for iron in other organisms imply that *S. mutans* has membrane-associated iron uptake mechanisms. In the present studies, phenolate or hydroxamate siderophore production by *S. mutans* OMZ176 was not detected. The organism appeared to transport only reduced (ferrous) iron.

MATERIALS AND METHODS

Bacterial strain and culture medium. *S. mutans* OMZ176 and procedures for maintenance of the culture have been described previously (2). The chemically defined FMC medium, with modifications (2), was treated with Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.) to lower trace metal contamination (2). The sterile medium was supplemented before use with filter-sterilized solutions of high-purity (Johnson-Matthey and Co., Inc., New York, N.Y.) $MgSO_4$ (391 μM magnesium) and (where indicated) $MnSO_4$ (173 μM manganese) and $FeSO_4$ (17 μM iron). All cultures were incubated at 35°C.

Uptake of radioiron. For the radioiron uptake assays, the cells were grown aerobically without shaking in an atmosphere of 95% air–5% CO_2 by the following procedure. A 0.1-ml amount of a 24-h culture of *S. mutans* OMZ176 in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) was transferred to 10 ml of Chelex-100-treated FMC medium supplemented with magnesium, manganese, and iron. After 24 h of incubation, the entire 10-ml culture was transferred to 1 liter of Chelex-100-treated FMC medium supplemented only with magnesium. The resulting iron and manganese concentrations of this culture were 0.0002 and 0.002 μM, respectively. The 1-liter culture, contained in a 2-liter Teflon bottle, was incubated (usually for 24 h) until it reached an A_{600} of 0.2. Cells were collected by centrifugation at 15,000 × *g* for 10 min at 4°C. The cells were washed once in ice-cold uptake buffer (described below) and finally suspended in 36.4 ml of uptake buffer. The cell suspension was placed in an ice bath and used immediately. The uptake buffer contained (in grams per liter): sodium acetate (6.0), dibasic sodium phosphate (3.15), monobasic sodium phosphate (2.05), ammonium sulfate (0.60), monobasic potassium phosphate (0.442), and dibasic potassium phosphate (0.232). A 10× solution containing all ingredients at 10 times the above concentrations was treated with Chelex-100 by the methods used to treat the FMC medium (2). The Chelex-100-treated 10× solution of uptake buffer was frozen and stored in 100-ml portions. To prepare 1 liter of uptake buffer, 100 ml of the 10× solution was thawed and supplemented with 391 μM magnesium and 10 mmol of potassium hydroxide to give a final potassium concentration of 15 mM and pH of 7.3. The volume was brought to 1 liter with high-purity water that was prepared by reverse osmosis and charcoal demineralizer treatment (2). Samples (2.4 ml) of the cells (grown aerobically as described above) in this buffer were transferred to 50-ml Erlenmeyer flasks, and 0.2 ml of a Chelex-100-treated glucose solution (1.2 M) usually was added. The final A_{600} of the assay cell suspension was 1.5 (dry weight of 1.56 mg/ml). If desired, additions (in 0.1-ml portions, to give a final concentration of 5 mM) of sodium ascorbate or phenazine

* Corresponding author.
† Present address: Department of Chemistry, University of California, Berkeley, CA 94720.
‡ Present address: Department of Microbiology, University of Texas, Austin, TX 78712.
§ Present address: Caius Laboratory for Interdisciplinary Research, St. Xavier's College, Bombay, India.

methosulfate (PMS) (Sigma Chemical Co., St. Louis) were made. The volume then was brought to 4.9 ml with uptake buffer. Most of the assays were done under aerobic conditions, in which the vessels were placed in air at the desired assay temperature (usually 35°C) in a shaking water bath. Some radioiron uptake assays were done under anaerobic conditions in the previously described (12, 13) anaerobic chamber. This chamber was warmed to 35°C and contained the necessary equipment (e.g., vacuum lines, filter manifold, gyratory shaker, colorimeter) for all operations to be done in an atmosphere of 90% N_2–5% CO_2–5% H_2. In the anaerobic chamber, the aerobically grown cells were resuspended in uptake buffer that had been equilibrated with the anaerobic atmosphere to a dissolved oxygen concentration of less than 0.04 μl/liter (13). In both aerobic and anaerobic conditions, the assay was initiated after 10 min by addition of 0.1 ml of radioiron (ICN, Irvine, Calif.) as $^{59}FeCl_3$ at a usual specific activity of 6 to 25 mCi/mg of Fe. Alternatively, additions of compounds to determine their effects on radioisotope uptake were made simultaneously with radioisotope input. At timed intervals, 0.1 ml of the uptake assay was removed, and the cells were collected by filtration on 0.45-μm-pore-diameter filters. The filters were rinsed three times with ice-cold uptake buffer and then placed in 5-ml plastic scintillation counting vials. After being dried overnight at 35°C, the filters were solubilized (in the dark) with 5 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) by vigorous shaking for 1 min. Radioactivity in each sample was determined by liquid scintillation counting.

Assays for possible siderophore excretion. Cell-free supernatants, obtained by centrifugation (15,000 × g for 15 min) of *S. mutans* cultures grown in the Chelex-100-treated FMC medium supplemented with magnesium and manganese (above concentrations) and either high (17 μM) or low iron (no added iron, <0.7 μM), were examined for phenolate or hydroxamate substances that might represent a siderophore. Steady-state cultures grown in the Teflon chemostat by previously described methods (2), as well as maximal stationary-phase batch cultures, were used; uninoculated FMC medium was included as a control. The Arnow reagents (9) were used to assay for possible phenolate excretion; 2,3-dihydroxybenzoic acid (Aldrich Chemical Co., Milwaukee, Wis.) was added to some samples as a positive control. Supernatants (600 ml) were also extracted with ethyl acetate, and the extracts were concentrated (100-fold) by vacuum evaporation by previously used methods (18). The concentrated material was assayed with the Arnow reagents. The Csaky reagents (8) were used to assay for hydroxamates. The possible presence in the culture supernatant of a siderophorelike substance that would reverse growth inhibition by the chelating agent ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA) was assayed by previously described methods (13).

RESULTS AND DISCUSSION

Lack of hydroxamate or phenolate siderophore production. Dental plaque, the normal habitat of *S. mutans*, is considered anaerobic (4) because oxygen is rapidly consumed by plaque microorganisms. Despite its lack of the enzymes of oxygen metabolism (including respiratory cytochromes and catalase), *S. mutans* displays a high rate of oxygen consumption (16). The presence of oxygen in the environment of *S. mutans* would favor the formation of profoundly insoluble ferric iron, invoking a special system for iron acquisition. Many microorganisms have solved the iron uptake problem

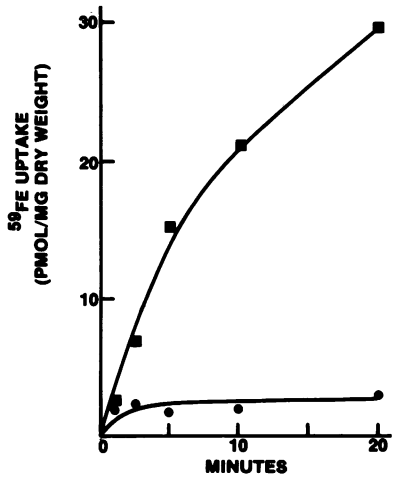

FIG. 1. Radioiron uptake by aerobically grown *S. mutans* OMZ176 (from 0.57 μM ^{59}Fe added as ferric chloride) under aerobic (●) and anaerobic (■) (90% N_2, 5% H_2, 5% CO_2) conditions.

by producing ferric-solubilizing and -transporting siderophores. The possibility of phenolate or hydroxamate siderophore excretion by *S. mutans* OMZ176 was investigated. No apparent color change was produced by the addition of drops of a 1% ferric chloride solution to supernatants of high- (17 μM) and low-iron (<0.7 μM) aerobic cultures growing at steady state in the Teflon chemostat or to supernatants of maximal stationary-phase aerobic batch cultures grown at the same iron levels in the same medium, as well as to the uninoculated medium. Assays with the Arnow reagents (9) showed no detectable phenolates in any of the above samples, although the phenolic acid 2,3-dihydroxybenzoic acid (when added to culture supernatant and uninoculated medium) could be quantitatively assayed at 0.9 μg/ml, and lower concentrations could be qualitatively visualized. Although at least two phenolate siderophores that cannot be extracted into ethyl acetate have been reported (1; D. Liles, R. Byers, P. Byers, J. Arceneaux, and C. Lobb, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, K179, p. 201), most of the phenolate siderophores can be recovered by ethyl acetate extraction of culture supernatants (14). Ethyl acetate extraction of 600 ml of the low-iron culture supernatant, by previously used methods (18), and concentration (100-fold) of the extract by vacuum evaporation failed to demonstrate Arnow reagent-reactive substances in the concentrated extract. The Csaky reagents (8) may be used to assay secondary hydroxamates; however, components (including histidine and arginine) of the chemically defined FMC medium gave a positive Csaky reaction. There was no significant difference in the Csaky reactions of any of the above samples.

Siderophores can sometimes be assayed by their ability to reverse growth inhibition by the chelating agent EDDA. Previously published (13) results show that EDDA inhibits aerobic (but not anaerobic) growth of *S. mutans* OMZ176. EDDA inhibition of *S. mutans* OMZ176 was reversed by culture supernatants; however, uninoculated medium (when the medium was supplemented with manganese) was also effective. Manganese, but not iron or several siderophores, reversed EDDA inhibition of *S. mutans* (13), suggesting that residual manganese in the culture supernatants was responsible for their activity. While these results do not completely disprove siderophore production by *S. mutans*, the data

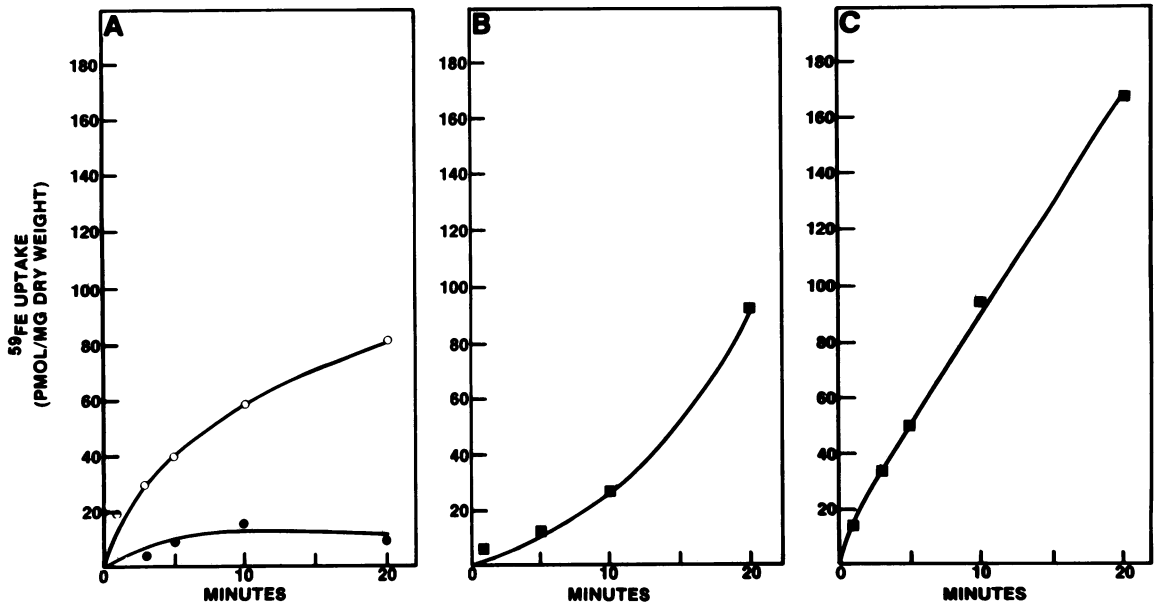

FIG. 2. Aerobic radioiron uptake by aerobically grown *S. mutans* OMZ176 (from 0.57 μM ^{59}Fe added as ferric chloride) with (A) no addition (●) or 5 mM sodium ascorbate added simultaneously with radioiron (○); (B) 5 mM PMS added simultaneously with radioiron, and (C) 5 mM PMS added 10 min before radioiron addition.

indicate that this organism does not excrete a readily detectable, classic phenolate or hydroxamate siderophore.

Uptake of radioiron: stimulation by anaerobiosis, ascorbate, and PMS. Cells used in all radioiron uptake assays were grown aerobically in the chemically defined medium at a low iron concentration (see Materials and Methods) in unshaken (static) cultures. During growth in such cultures, oxygen consumption by the organism lowers the dissolved oxygen level to about 0.1 μl/liter, and maximum expression of superoxide dismustase activity implies full response of the organism to oxygen (12, 13). Aerobic cultivation was selected because oxygen fluxes in plaque should require the organism to consume oxygen in its normal habitat. Although the organism will grow in aerobic shaken cultures in rich media (such as Todd-Hewitt or brain-heart infusion), it was unable to grow in aerobic shaken cultures in the chemically defined medium. Therefore, static cultures were used. Possibly, the reducing capacity of the complex media allowed growth at the higher oxidation level. Iron uptake was assessed by addition of $^{59}FeCl_3$ to cells in a glucose-salts suspension. In assays done aerobically, low-level uptake of radioiron (from 0.57 μM ^{59}Fe at 45 min) was similar in both viable cells and cell preparations autoclaved prior to testing, 14 and 13 pmol/mg (dry weight) of cells, respectively. This suggests that ferric iron was being deposited on the surface of the cells. Addition of 5 mM sodium citrate to the assays reduced this apparent nonspecific binding (5 and 3 pmol/mg for viable and autoclaved cells, respectively), possibly by chelating the ferric iron. However, the reductant sodium ascorbate (5 mM) markedly increased uptake of radioiron by viable cells (111 pmol/mg) but not by autoclaved cells (12 pmol/mg), suggesting that the reducing capacity of ascorbate was responsible for uptake. In assays done at 0°C, ascorbate stimulation of uptake was not apparent, indicating the temperature dependence of the ascorbate effect. At the concentration used, ascorbate did not alter the pH of the uptake assay. Ascorbate probably reduced the radioiron to the ferrous state, which was then transported by the cells.

Radioiron uptake was assessed in an anaerobic, reducing atmosphere (90% N_2, 5% H_2, 5% CO_2) with uptake buffer that was equilibrated with the anaerobic atmosphere to produce a dissolved oxygen concentration of less than 0.04 μl/liter. Anaerobic uptake of radioiron (added as ferric iron) by aerobically grown cells occurred more rapidly and to a greater extent than uptake by the same cell preparation under aerobic conditions (Fig. 1). The data suggest that *S. mutans* transports only ferrous iron. Either the anaerobic atmosphere reduced the iron or (because of the absence of oxygen) anaerobiosis allowed the cells to more readily reduce iron by using reductants furnished by glucose metabolism, possibly via a membrane or cytoplasmic flavoprotein dehydrogenase system. *S. mutans* is able to reduce externally located compounds (16); therefore, the ability of the cell suspensions to reduce ferric iron was determined.

Ferric chloride (5 μM) was added to an aerobic uptake cell suspension that contained the ferrous iron chelator ferrozine (20 μM). Since ferrozine is sulfonated, it would not be expected to readily traverse the cell membrane, and only reduction of external iron should be visualized. Cell suspensions reduced ferric iron, as indicated by the appearance of the magenta color of the ferrous-ferrozine complex (15) within 25 min after addition. The delay before apparent iron reduction may represent the time required for depletion of oxygen. No spontaneous reduction of iron was observed in cell-free controls.

As reduction of ferric iron prior to its transport by *S. mutans* may be obligatory, the effect of the electron acceptor PMS, added as oxidized PMS, on radioiron uptake was determined. PMS stimulated radioiron accumulation, but stimulation was apparent only after a lag of about 10 min (Fig. 2B and C). In contrast, stimulation by ascorbate was immediate; adding ascorbate to the cell suspension 10 min before radioiron input did not alter the uptake curve shown in Fig. 2A. Ascorbate is capable of rapid iron reduction, whereas reduction of PMS by the cells apparently preceded the reduction of iron. Reduction of PMS by the cells was

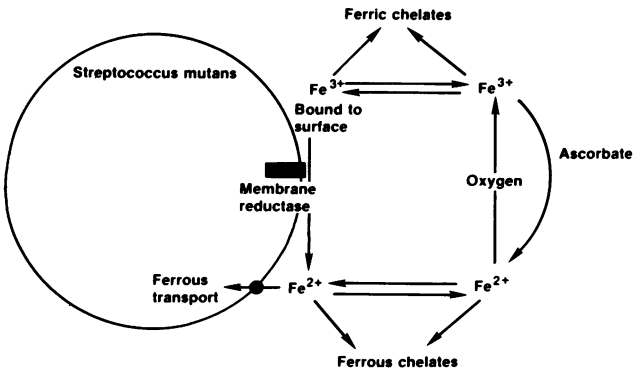

FIG. 3. Model for reductive assimilation of iron by *S. mutans*, showing the suggested effect of an environmental reductant (sodium ascorbate) on iron uptake, as well as possible chelation and withholding of iron by certain ferric or ferrous chelators.

evidenced by the appearance of the characteristic green color of the partially reduced, semiquinoid form of the compound (19). This reduction did not occur in cell-free controls or in assays done with autoclaved cells. The stimulatory effect of PMS may have been due to its reduction by the cell suspension; the reduced PMS may in turn reduce ferric iron for ferrous transport. The linear uptake kinetics and the delay before initiation of high-level radioiron uptake in the presence of PMS also might be partly explained by reaction of reduced PMS with oxygen, acting to deplete the system of oxygen.

Model of reductive iron assimilation in *S. mutans*. The apparent absence of a ferric chelating and transporting siderophore in *S. mutans* and the stimulation of iron uptake by agents and conditions favoring formation of ferrous iron suggest that iron transport by this organism in its normal habitat (where ferric iron is probably present) may be a three-step ferrous-transporting process (Fig. 3). Ferric polymers initially may be precipitated (bound) onto cell surface layers. Iron binding by the cell surface of siderophore-deficient microorganisms may be a general occurrence (17). Subsequent reduction of the metal by the cells (perhaps via a membrane flavin reductase) would solubilize iron from the polymers and generate the appropriate form for the final step, transport by a membrane ferrous translocating system. Dissolved oxygen would interfere by converting the reduced iron to its ferric state. Certain environmental reductants (such as ascorbate) might assist transport by increasing the level of ferrous iron. Cellular reductive mechanisms will reduce certain external substances (for example, PMS used in the experiments reported here), which might then in turn reduce the iron or deplete dissolved oxygen, or both, enhancing iron uptake. The phenazine pigment pyocyanin produced by *Pseudomonas aeruginosa* may have a similar function in iron uptake (7). Production of an external reducing agent that facilitates transport of iron has been suggested for *Listeria monocytogenes* (6). Finally, certain ferrous or ferric chelating agents might impede *S. mutans* iron uptake. The data suggest that *S. mutans* is capable of transporting only ferrous iron.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant DE-04903 from the National Institute of Dental Research.

We are grateful to June Lascelles, University of California, Los Angeles, for advice.

LITERATURE CITED

1. **Actis, L. A., J. H. Crosa, K. Kellerman, S. R. Ellenberger, F. M. Hauser, and J. Sanders-Loehr.** 1986. Characterization of anguibactin, a novel siderophore from *Vibrio anguillarum* 775(pJM1). J. Bacteriol. **167**:57–65.
2. **Aranha, H., R. C. Strachan, J. E. L. Arceneaux, and B. R. Byers.** 1982. Effect of trace metals on growth of *Streptococcus mutans* in a Teflon chemostat. Infect. Immun. **35**:456–460.
3. **Arnold, R. R., J. E. Russell, W. J. Champion, M. Brewer, and J. J. Gauthier.** 1982. Bactericidal activity of human lactoferrin: differentiation from the stasis of iron deprivation. Infect. Immun. **35**:792–799.
4. **Cole, J. A.** 1977. A biochemical approach to the control of dental caries. Biochem. Soc. Trans. **5**:1232–1239.
5. **Cole, M. F., R. R. Arnold, J. Mestecky, S. Prince, R. Kulhavy, and J. R. McGhee.** 1976. Studies with human lactoferrin and *Streptococcus mutans*, pp. 359–373. *In* H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), Proceedings: microbial aspect of dental caries (a special supplement to Microbiology Abstracts), vol. II. Information Retrieval, Inc., Washington, D.C.
6. **Cowart, R. E., and B. G. Foster.** 1985. Differential effects of iron on the growth of *Listeria monocytogenes*: minimum requirements and mechanisms of acquisition. J. Infect. Dis. **151**:721–730.
7. **Cox, C. D.** 1986. Role of pyocyanin in the acquisition of iron from transferrin. Infect. Immun. **52**:263–270.
8. **Csaky, T. Z.** 1948. On the estimation of bound hydroxylamine in biological material. Acta Chem. Scand. **2**:450–459.
9. **Evans, W. C.** 1947. Oxidation of phenol and benzoic acid by some soil bacteria. Biochem. J. **41**:373–382.
10. **Fujiwara, S., S. Kobayashi, and N. Nakagawa.** 1978. Development of a minimal medium for *Streptococcus mutans*. Arch. Oral Biol. **23**:601–602.
11. **Hamada, S., and H. D. Slade.** 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**:331–384.
12. **Martin, M. E., B. R. Byers, M. O. J. Olson, M. L. Salin, J. E. L. Arceneaux, and C. Tolbert.** 1986. A *Streptococcus mutans* superoxide dismutase that is active with either manganese or iron as a cofactor. J. Biol. Chem. **261**:9361–9367.
13. **Martin, M. E., R. C. Strachan, H. Aranha, S. L. Evans, M. L. Salin, B. Welch, J. E. L. Arceneaux, and B. R. Byers.** 1984. Oxygen toxicity in *Streptococcus mutans*: manganese, iron, and superoxide dismutase. J. Bacteriol. **159**:745–749.
14. **Neilands, J. B.** 1981. Iron absorption and transport in microorganisms. Annu. Rev. Nutr. **1**:27–46.
15. **Stookey, L. L.** 1970. Ferrozine—a new spectrophotometric reagent for iron. Anal. Chem. **42**:779–781.
16. **Thomas, E. L., and K. A. Pera.** 1983. Oxygen metabolism of *Streptococcus mutans*: uptake of oxygen and release of superoxide and hydrogen peroxide. J. Bacteriol. **154**:1236–1244.
17. **Winkelmann, G.** 1979. Surface iron polymers and hydroxy acids. I. Model of iron supply in sideramine-free fungi. Arch. Microbiol. **121**:43–51.
18. **Young, I. G.** 1976. Preparation of enterochelin from *Escherichia coli*. Prep. Biochem. **6**:123–131.
19. **Zaugg, W. S.** 1964. Spectroscopic characteristics and some chemical properties of *N*-methylphenazinium methyl sulfate (phenazine methosulfate) and pyocyanin at the semiquinoid oxidation level. J. Biol. Chem. **239**:3964–3970.