



Iron Oxidation and Deposition in the Biofilm Covering *Montacuta ferruginosa* (Mollusca, Bivalvia)

DAVID C. GILLAN
MICHEL WARNAU

Marine Biology Laboratory
Free University of Brussels
Brussels, Belgium

ELISABETH W. DE VRIND-DE JONG

Department of Biochemistry
Leiden University
Leiden, The Netherlands

FRÉDÉRIC BOULVAIN

Associated Laboratories of Geology, Petrology, and Geochemistry
Faculty of Sciences
Liège University
Liège, Belgium

ALAIN PRÉAT

Department of Earth Sciences and Environment
Free University of Brussels
Brussels, Belgium

CHANTAL DE RIDDER

Marine Biology Laboratory
Free University of Brussels
Brussels, Belgium

The shell of the bivalve Montacuta ferruginosa is covered with a rust-colored biofilm. This biofilm includes filamentous bacteria and protozoa encrusted with a mineral, rich in ferric ion and phosphate. The aim of this research was to study two possible microbial iron precipitation pathways in the biofilm, namely, microbial iron oxidation and microbial degradation of organic Fe(III) complexes. The iron-oxidizing activity was assayed spectrophotometrically by monitoring the formation of the dye Wurster blue in

Received 4 October 1999; accepted 15 December 1999.

This research was supported by a FRIA grant to D.G. (ref. 940733) and a FRFC grant to C.D.R. (ref. 2-4515-99). This article is a contribution of the Centre Interuniversitaire de Biologie Marine (CIBIM).

Address correspondence to Dr. David C. Gillan, Laboratoire de Biologie Marine, CP 160/15, Université Libre de Bruxelles, 50 av. F. D. Roosevelt, 1050 Brussels, Belgium. E-mail: dgillan@ulb.ac.be

biofilm extracts. Iron-oxidizing activity was effectively detected in extracts obtained by oxalic acid treatment of biofilm fragments. Extracts obtained without oxalic acid treatment, heated extracts, or extracts supplemented with HgCl_2 did not show any activity. This suggests that an iron-oxidizing factor (IOF), possibly an enzyme, coprecipitated with the mineral. Additional information gathered by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gel-filtration chromatography, and UV spectrophotometry indicate that the IOF would be a small peptide or glycopeptide (1,350 Da). Microbial degradation of organic Fe(III) complexes was assayed with biofilm fragments incubated in a medium containing ferric citrate. Analysis of the supernatants after various intervals revealed that the complex was degraded by living microorganisms much faster than in the heat-killed negative controls. We conclude that ferric iron precipitation in the biofilm may proceed by way of microbial Fe(II) oxidation as well as microbial degradation of organic Fe(III) complexes.

Keywords bacteria, biofilm, bivalve, Fe(III), iron, oxidation

Intimate associations of microorganisms with Fe(III) mineral deposits are widespread in nature (Ghiorse 1984; Ehrlich 1990; Tazaki et al. 1997). In marine environments, such associations occur in marine snow (Cowen and Silver 1984; Heldal et al. 1996), in ferromanganese concretions (Burnett and Neelson 1981), at deep-sea hydrothermal vents (Karl et al. 1989; Juniper and Tebo 1995), and as epibiotic biofilms on marine animals (Jannash and Wirsén 1981; Baross and Deming 1985; Gillan and De Ridder 1997). Although the fine structure of most of these communities has been elucidated, very little is known about the role of microorganisms in Fe(III) deposition processes. In oxygenated seawater, Fe(III) is highly insoluble (Wells et al. 1995) and Fe(II) oxidation is spontaneous (Roekens and Van Grieken 1983). As a consequence, biological Fe(III) deposition is hard to differentiate from nonbiological spontaneous Fe(III) deposition. Investigators have frequently proposed that seawater microorganisms simply adsorb colloidal Fe oxyhydroxides in their exopolymeric substances (EPS) or passively accumulate the Fe ions resolubilized within microenvironments (Cowen and Silver 1984). In contrast to freshwater environments, microbial (enzymatic) Fe(II) oxidation in seawater environments is not a well-studied process. Only recently was the occurrence of such a process demonstrated in anaerobic cultures of marine phototrophic bacteria (Ehrenreich and Widdel 1994) and in anaerobic cultures of marine nitrate-reducing bacteria (Straub et al. 1996). To our best knowledge, that process remains undemonstrated at marine anoxic-oxic transition zones using O_2 as electron acceptor. Nevertheless, microbial Fe(II) oxidation at transition zones, as for the bacterium *Gallionella ferruginea* (Hanert 1989), could be widespread in marine sediment burrows and other microenvironments where water is slowly moving from oxic to anoxic zones.

Montacuta ferruginosa is a marine bivalve living inside the burrow of the echinoid *Echinocardium cordatum* (Gage 1966). A typical rust-colored coating covers the shell of the bivalve, and the species epithet refers to this coating. The coating is a partially mineralized biofilm (amorphous ferric minerals rich in phosphate) featuring filamentous bacteria that resemble members of the Beggiatoales and ciliates of the suctorial group (Gillan and De Ridder 1995, 1997). The phylogenetic diversity of the biofilm eubacteria was determined by denaturing gradient gel electrophoresis of polymerase chain reaction-amplified gene fragments coding for 16S rRNA, as well as by cloning and sequencing the 16S rRNA genes (Gillan et al. 1998). These molecular analyses revealed a diverse consortium, finding close relatives of *Pseudoalteromonas*, *Colwellia*, *Oceanospirillum*, and *Flexibacter maritimus* in the biofilm. Traditional cultivation methods were also used to study the biofilm. Among others, the heterotrophic strains obtained were tentatively classified in the genera *Cytophaga*, *Flexibacter*, and *Leucothrix* (Gillan and De Ridder, unpublished).

The aim of the present research was to look for two active processes of Fe(III) deposition in the biofilm: microbial Fe(II) oxidation and microbial degradation of Fe(III) organic

complexes. The burrow where *M. ferruginosa* is living is a good place to look for these processes because it is an oxic–anoxic transition zone that appears to be very rich in Fe(II) and organic matter (Gillan and De Ridder 1995). As explained in the discussion, the study of living Fe(III) microbial communities can give important information to sedimentologists studying ancient Fe(III) microbial ecosystems.

Materials and Methods

Samples

Specimens of *M. ferruginosa* (Montagu) were collected intertidally from the burrows of *E. cordatum* (Pennant) (Echinoidea, Spatangoida) at Wimereux (Pas-de-Calais, France) during 1995, 1996, 1997, and 1999. Bivalves were briefly rinsed in sterile artificial seawater (SASW; Sigma) before use in iron oxidation assays or iron deposition assays.

Assay for Iron Oxidation

Biofilms were scraped off 12 to 50 bivalve shells and suspended in SASW. The biofilms were then centrifuged and resuspended in 3 ml of oxalic acid (0.2 M, pH 3.0) to dissolve the iron precipitates. Some biofilms were resuspended in 3 ml of SASW only and not treated with oxalic acid. The suspensions were placed on ice, sonicated (3×15 s) at about 120 μ m of amplitude (setting 4 of the output control, on a VibraCell 375 ultrasonic processor fitted with a 5-mm microtip), incubated 20 min at 4°C, and again sonicated. The suspensions were then centrifuged 15 min at 10,000g (at 4°C), and 2.5 ml of the supernatant was desalted on Sephadex G-25 columns (PD-10; Pharmacia) equilibrated with MilliQ water as follows: After the sample had run through the column, 0.5 ml of water was added; then the high-molecular-mass components were eluted with 2.5 ml of MilliQ water. This treatment removed iron and oxalic acid from extracts. Negative controls consisted of 3 ml of oxalic acid without biofilm fragments, or 3 ml of oxalic acid plus 5 mg of oxidized iron powder (Fe₂O₃ pro analysi). Controls were also sonicated and desalted.

The iron-oxidizing activity of the extracts was then tested by the method described in De Vrind-de Jong et al. (1990) for detection of Fe(III) formation. To 395 μ l of extracts placed in a spectrophotometer cuvette is added 5 μ l of 10 mM potassium ferrocyanide [source of Fe(II)], 50 μ l of 10 mM 2-(*N*-morpholino)ethanesulfonic acid (MES buffer), pH 6.0, and 50 μ l of 2 mM fresh (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD). TMPD is oxidized in Wurster blue (oxidized TMPD generated in the extract) by the Fe(III) ions generated during Fe(II) oxidation. The formation of Wurster blue in the cuvette is monitored by recording the increase in absorbance at 620 nm with a double-beam spectrophotometer for 3 min (cuvettes are placed in the spectrophotometer immediately after the addition of reagents). The blank cuvette contained MES and TMPD but not Fe (to show that any increase in absorbance depends on the presence of Fe and is not caused by other TMPD-oxidizing compounds). To denature any iron-oxidizing proteins, some extracts were heated 10 min at 100°C before the addition of Fe, MES, and TMPD. The enzymatic inhibitors HgCl₂ and KCN (5 μ l of different concentrations) were also added to some cuvettes before the other reagents were added.

Characterization of the Iron-Oxidizing Factor

Extracts of biofilms were prepared and desalted as for the iron-oxidation assays. Protein contents were determined by the assay of Bradford (Bradford 1976), with bovine serum albumin as a standard. Sugar contents were determined by the assay of Dubois et al. (1956), with D-glucose as a standard.

For spectrophotometry, absorption spectra of extracts were recorded from 200 to 800 nm on a Philips PU 8700 spectrophotometer in Hellma Quartz Suprasil cuvettes.

For sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), extracts were lyophilized and dissolved in Laemmli sample buffer (Laemmli 1970), and the SDS-PAGE was conducted by the method of Laemmli (1970). Gels were stained for proteins with Coomassie brilliant blue R250. For iron-oxidizing activity, gels were rinsed in MES buffer and incubated in $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$, as described by Corstjens et al. (1992). Some gels were silver-stained.

For gel-filtration chromatography, extracts were lyophilized and dissolved in 500 μl of MES-NaCl (10 mM MES, pH 6.0; 0.02 M NaCl) with 1% saccharose. Samples were then deposited on Econo-Columns (1.5 \times 30 cm; Bio-Rad) packed with Sephadex G-200 and eluted with MES-NaCl at a flow rate of 2.3 ml/h/cm² (controlled with a Minipuls-2 peristaltic pump) at 4°C. The bed volume was 47.1 ml. Columns were connected to a UV detector set at 280 nm (Gilson 115). The UV detector was connected to a chart recorder (a Macintosh running chart v3.2.8) through a MacLab/2e interface. Fractions of 600 μl were collected with a fraction collector (Gilson FC 204), and the iron-oxidizing activity of each fraction was determined as described above. Nonlyophilized extracts were also deposited on Sephadex G-25 columns (PD-10). Here, the sample volume was set to 250 μl , the eluent was MilliQ water, and gravity was used to pump the eluent to the UV detector. To determine the molecular mass of the iron-oxidizing factor, a calibration curve was constructed with a calibration kit (HMW kit; Pharmacia).

Assay for Microbial Degradation of Organic Fe(III) Complexes

Biofilms were scraped off 40 bivalve shells and suspended in SASW. The suspension was divided among 12 sterile 3-cm-diameter tubes. The samples were centrifuged and the biofilm fragments in each tube were resuspended in 9 ml of SASW. Six of the tubes (control tubes) were heated 15 min at 100°C to kill the bacteria. One milliliter of filtered Fe(III) citrate (0.2- μm -pore filtration), 10 mM in SASW, was then added to each tube. For three experimental and three control tubes, the pH of their contents was adjusted to 6.5 with concentrated NaOH; in the remaining tubes the pH was adjusted to 8.2. All samples were aerated on a gyratory shaker at 20°C and 140 rpm. At timed intervals, the tubes were centrifuged for 5 min at 160g, and 300- μl portions of the supernatant were pipetted into Eppendorf tubes. The iron in these fractions was analyzed by atomic absorption spectrometry (GBC 906 AAS) after acid digestion of the samples (HNO_3 65%, Suprapur; Merck) for 48 h. Each sample was treated in triplicate. The reproducibility of the method was checked by using an internal standard (FeNO_3).

Results

Iron Oxidation Assay

The iron oxidation assay was performed in April 1995 (30 biofilms), May 1995 (15 biofilms), October 1995 (50 biofilms), February 1996 (40 biofilms), March 1996 (25 biofilms), April 1996 (25 biofilms), and February 1999 (12 biofilms). All extracts of biofilm were tested at least three times. All extracts tested showed an iron oxidation activity. The rate of iron oxidation varied from 0.060 $\Delta A/\text{min}$ to 0.094 $\Delta A/\text{min}$ (Figure 1). There was no iron oxidation in the negative controls, in extracts supplemented with 1 mM HgCl_2 , or in extracts prepared without the oxalic acid treatment (a rate $<0.005 \Delta A/\text{min}$ was considered negative). The iron oxidation activity was not inhibited by 0.1 mM HgCl_2 or 0.1 mM KCN. In extracts previously heated to 100°C for 10 min, the rate of iron oxidation was reduced to zero.

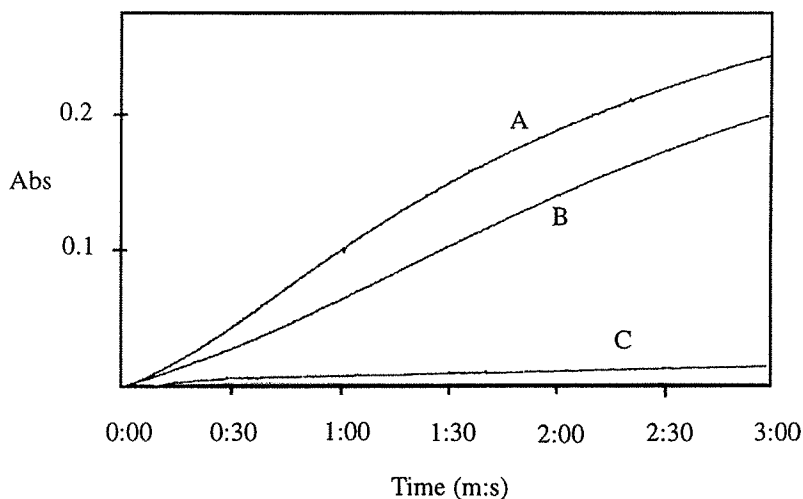


FIGURE 1 Rate of iron oxidation in biofilm extracts after addition of Fe(II), TMPD, and MES buffer at time zero. (A, B) Two extracts of biofilm; (C) negative control (heated extract). Iron oxidation in extracts was measured by recording the increase in the absorbance (Abs) at 620 nm in a spectrophotometer. This increase occurs as the dye Wurster blue is formed from TMPD by Fe(III) generated in the extract.

Characterization of the Iron-Oxidizing Factor (IOF)

The absorption spectrum of a desalted biofilm extract is shown in Figure 2. The absorption was very strong in the ultraviolet region of the spectrum, from 200 nm to about 400 nm. Negative controls absorbed less UV light and only from 200 nm to ~230 nm (Figure 2). No proteins were detected in desalted extracts by the Bradford assay. Sugars were detected

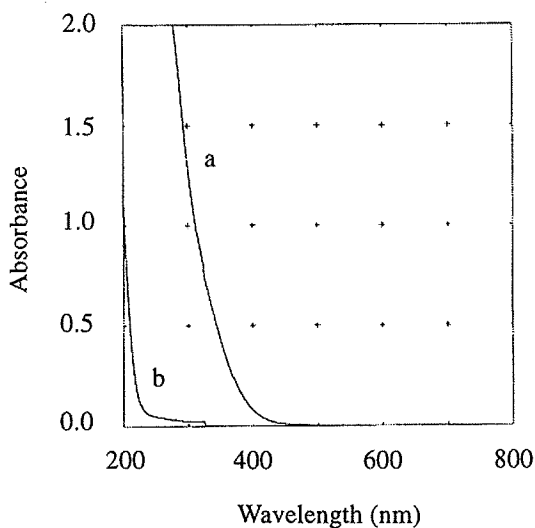


FIGURE 2 Absorption spectrum of a desalted (Sephadex G-25) biofilm extract (a) and a desalted negative control (b). The biofilm extract was prepared with biofilms suspended in oxalic acid; before desalting, the suspension was sonicated and then centrifuged. The negative control consisted of oxalic acid and 5 mg of Fe_2O_3 .

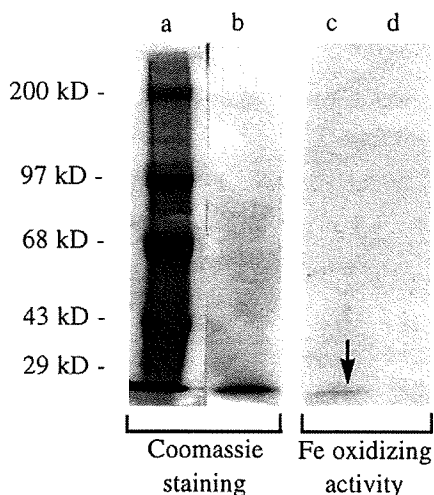


FIGURE 3 SDS-PAGE of biofilm extracts. Lane a, molecular mass markers; lanes b and c, biofilm extracts; lane d, negative control (oxalic acid and Fe_2O_3). For lanes a and b the gel was stained for proteins with Coomassie brilliant blue R250. For lanes c and d the gel was stained for iron-oxidizing activity; after electrophoresis, gels were rinsed in MES buffer and incubated in $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$. The arrow shows the band of precipitated iron oxide corresponding to the IOF (see text for more details).

at 15 to 20 $\mu\text{g}/\text{ml}$ by the Dubois assay. After separation of the extracts with SDS-PAGE and coloration with Coomassie brilliant blue, no bands, apart from the migration front, could be detected (Figure 3, lane b). On gels incubated in the presence of $\text{Fe}(\text{II})$, a band of precipitated iron oxide formed at the migration front for biofilm extracts only (lane c, arrow); this band was absent from the control lanes (lane d). These results suggest that the IOF was a small protein with an apparent molecular mass of <29 kDa. No bands could be detected on silver-stained gels because a smear was produced. Chromatographic studies showed the IOF to be a small molecule of $\sim 1,350$ Da because it eluted at the same time as 1,356 Da cyanocobalamin on the chromatographic media used.

Assay for Microbial Degradation of Organic Fe(III) Complexes

At different intervals, the amount of iron in supernatants was monitored by atomic absorption spectrometry. For the samples adjusted at pH 6.5, the amount of iron in the supernatant fluid decreased with time much more quickly than in the control samples (Figure 4). After 120 h of incubation, only 5% of the starting iron content was detected in the supernatants of the treated samples, whereas the control samples still contained 90% of their iron. For the samples adjusted at pH 8.2, the levels of iron decreased quickly and similarly for the experimental and control tubes, and only 8% of iron remained in supernatants after only 48 h of incubation (data not shown).

Discussion

We concluded from our $\text{Fe}(\text{II})$ oxidation assays that an extracellular IOF was coprecipitated with $\text{Fe}(\text{III})$ minerals in the biofilm. The IOF has an apparent molecular mass of $\sim 1,350$ Da and is thus in the same molecular mass range as the pyoverdins/pseudobactin class of siderophores (1,200–1,400 Da) isolated from various bacteria (Reid and Butler

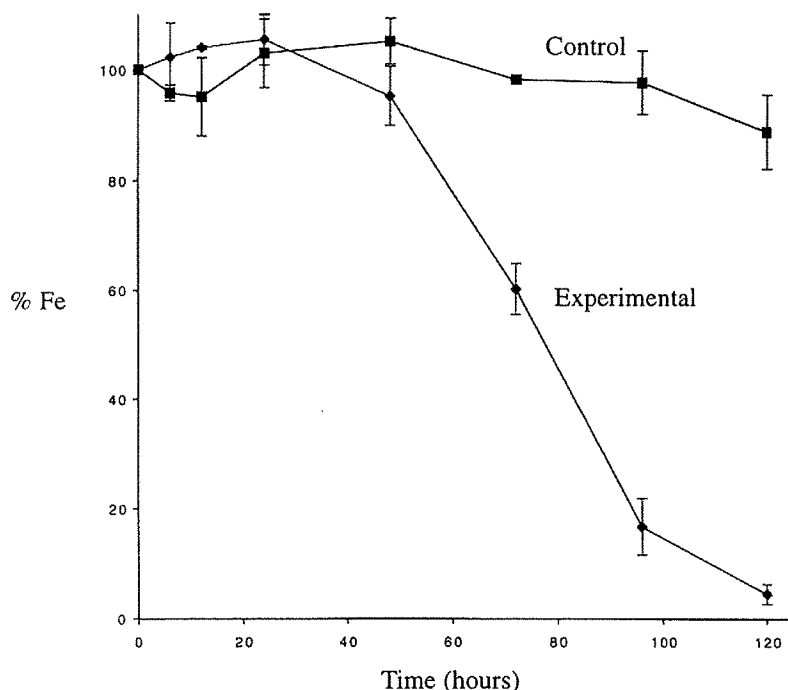


FIGURE 4 Variation of Fe concentration in control tubes (heat-killed biofilms) and experimental tubes (living biofilms) according to time (mean \pm SD, $n = 3$). Biofilms are suspended in SASW and 1 mM Fe(III)-citrate. The pH was adjusted to 8.2. Fe concentrations were determined by atomic absorption spectrometry and are expressed as percentages of Fe remaining in the supernatant.

1991). However, siderophores are not iron-oxidizing compounds. To our knowledge, extracellular iron-oxidizing compounds from neutrophilic bacteria have been described only in the freshwater bacteria *Leptothrix discophora* SS-1, as 150-kDa proteins (Corstjens et al. 1992).

The molecular nature of the IOF is still uncertain. The first possibility would be that it is a small peptide. Although proteins were not detected in extracts by the assay of Bradford, an iron-oxidizing band was detected on SDS-PAGE gels, and extracts strongly absorbed UV radiation (suggesting the presence of tyrosine and tryptophan residues as well as peptide bonds). To explain the fact that the IOF was not revealed by Coomassie brilliant blue (in the Bradford assay and on SDS-PAGE gels), peptides <3 kDa do not always produce color in the presence of acidified Coomassie brilliant blue (Sedmark and Grossberg 1977). The presence of sugars in desalted extracts would be explained by the abundance of exopolymeric substances in biofilms (Cooksey and Wigglesworth-Cooksey 1995), substances that originate from sheaths and capsules of microorganisms. The other possibility would be that the IOF is a glycopeptide and that high absorbance in the UV is caused by carboxyls of the glycan part of the molecule in addition to the proteic part. The IOF would probably not be a pure polysaccharide because that would not migrate well in SDS-PAGE gels and form a sharp band (SDS binds strongly only to proteins). Moreover, polysaccharides are not expected to be heat-sensitive.

The question of enzymatic nature should be posed with caution. The IOF behaves like an enzyme because it is inhibited by heat and HgCl_2 . However, enzymes are large proteins

(usually >10 kDa) and are generally inhibited by moderate concentrations of KCN and HgCl₂. For example, the activity of the *Leptothrix discophora* iron-oxidizing protein is ~3.3 times less in the presence of 0.1 mM HgCl₂ and ~4.5 times less in the presence of 0.2 mM NaCN (De Vrind-de Jong et al. 1990). In the present case, inhibition occurs only with higher concentrations of HgCl₂. If not an enzyme itself, perhaps the IOF is a breakdown product of a former iron-oxidizing enzymatic protein (possibly a glycosylated oligopeptide) and is generated during the sample preparation procedure. Although the IOF nature awaits further research, the IOF still can be described as a catalyst of iron oxidation.

Another procedure for Fe(III) precipitation has been demonstrated in the biofilm: microbial degradation of Fe(III) organic complexes. This process was demonstrated here with Fe(III) citrate at pH 6.5 and 20°C in well-oxygenated seawater, but not at pH 8.2, probably because of the rapid chemical degradation of complexes under these experimental conditions. The exact process of Fe(III) precipitation from complexes is unknown. It may have been brought about indirectly as a consequence of metabolism that changed the environmental conditions (e.g., pH) and the degree of complexation (Gerke 1997), or it could happen directly by way of enzymatic degradation of citrate. Bacteria and protozoans capable of Fe(III) organic complexes degradation are well known (Ehrlich 1990; Harding and Royt 1990), and Fe(III) organic complexes are probably abundant in seawater (Gledhill and van den Berg 1994). However, this way of iron precipitation needs heterotrophic microorganisms and, indeed, close relatives of heterotrophic eubacteria were detected in the biofilm by molecular and cultivation methods (Gillan et al. 1998; Gillan and De Ridder, unpublished). In addition, cultivation studies indicate that the number of colony-forming units per biofilm (per bivalve) are significantly higher (*t* test, *P* = 0.045) when the culture media contains iron complexed to citrate than in culture media without complexed iron (Gillan and De Ridder, unpublished). It is thus possible that this process of iron deposition occurs in the biofilm.

In summary, we believe that Fe(III) deposition in the biofilm covering *M. ferruginosa* may be explained by at least four different processes: microbial Fe(II) oxidation by an extracellular IOF; degradation of Fe(III) organic complexes; binding of Fe(II) to anionic groups of exopolymeric substances, followed by nonbiological autooxidation; and direct accumulation of colloidal Fe oxyhydroxides from seawater. Although only the two first types of processes have been studied here, the two other processes are more classical and have already been suggested or demonstrated for other bacteria associated with Fe(III) deposits (Macrae and Edwards 1972; Ghiorse 1984; Ferris 1989). Accordingly, these two last processes could also occur in the biofilm. The two first processes are active processes, requiring metabolically active specific microbes, such that without such microbes, the composition and abundance of Fe(III) minerals on the bivalve would probably be very different. Moreover, chemical conditions in biofilms may be radically different from those of the bulk water (Little et al. 1997). The two first processes, therefore, demonstrated here at pH 6.0–6.5, should be considered seriously.

Fe(III)-encrusted microbial communities are frequently observed in deposits (biolaminated or not) and iron formations from Early Proterozoic to modern ages (Muir 1978; Knoll and Awramik 1983; Dahanayake and Krumbein 1986; Bourque and Boulvain 1993; Mamet et al. 1997; Pr  at et al. 1999a, 1999b). In the geological literature, microfossils of these strata are frequently presumed to be petrified remains of iron- and manganese-oxidizing bacteria, similar as those found today in freshwater and terrestrial environments (Muir 1978). Such observations are frequently used to infer paleometabolisms and paleoenvironmental conditions such as oxygen levels. However, as illustrated in this study with living microorganisms, the simple presence of precipitated Fe(III) on microbes (or microfossils) cannot be taken as diagnostic for any particular metabolism or any Fe(III) precipitation pathway;

other (paleo)environmental data are necessary to give clues about the kind of microbial processes involved. For example, Fe(III)-encrusted microfossils have been found in red matrices of different European Paleozoic and Mesozoic series (Mamet et al. 1997; Pr at et al. 1999a, 1999b). Sedimentological data suggested these were from deep environments (> 100 m, well below the photic zone), microaerophilic conditions, low organic-matter content, and paucity of benthic macrofauna. Under such conditions, microbial Fe(II) oxidation could have been more significant in comparison with other Fe(III) deposition processes. The most we can conclude is that in the present day environment, Fe(III)-encrusted microbial communities such as the biofilm of *M. ferruginosa* deserve better attention because they could constitute living models of ancient Fe(III) microbial ecosystems, which seem to be abundant in the geological record.

References

- Baross JA, Deming JW. 1985. The role of bacteria in the ecology of black-smoker environments. *Biol Soc Wash Bull* 6:355–371.
- Bourque PA, Boulvain F. 1993. A model for the origin and petrogenesis of the red stromatolite limestone of Paleozoic carbonate mounds. *J Sediment Petrol* 63:607–619.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 72:248–254.
- Burnett BR, Neelson KH. 1981. Organic films and microorganisms associated with manganese nodules. *Deep-Sea Res* 28A:637–645.
- Cooksey KE, Wigglesworth-Cooksey B. 1995. Adhesion of bacteria and diatoms to surfaces in the sea: review. *Aquat Microb Ecol* 9:87–96.
- Corstjens PLAM, De Vrind JPM, Westbroek P, De Vrind-de Jong EW. 1992. Enzymatic iron oxidation by *Leptothrix discophora*: identification of an iron-oxidizing protein. *Appl Environ Microbiol* 58:450–454.
- Cowen JP, Silver MW. 1984. The association of iron and manganese with bacteria on marine macroparticulate material. *Science* 224:1340–1342.
- Dahanayake K, Krumbein WE. 1986. Microbial structures in oolitic iron formations. *Miner Deposita* 21:85–94.
- De Vrind-de Jong EW, Corstjens PLAM, Kempers ES, Westbroek P, De Vrind JPM (1990). Oxidation of manganese and iron by *Leptothrix discophora*: use of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine as an indicator of metal oxidation. *Appl Environ Microbiol* 56:3458–3462.
- Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. *Anal Chem* 28:350–356.
- Ehrenreich A, Widdel F. 1994. Anaerobic oxidation of ferrous iron by purple bacteria, a new type of phototrophic metabolism. *Appl Environ Microbiol* 60:4517–4526.
- Ehrlich HL. 1990. Geomicrobiology of iron. In: *Geomicrobiology*, 2nd ed. New York: Marcel Dekker Inc, p 283–346.
- Ferris FG. 1989. Metallic ion interactions with the outer membrane of Gram-negative bacteria. In: TJ Beveridge, RJ Doyle, editors. *Metal ions and bacteria*. New York: J Wiley and Sons, p 295–323.
- Gage J. 1966. Observations on the bivalves *Montacuta substriata* and *M. ferruginosa*, “commensals” with spatangoids. *J Mar Biol Assoc UK* 46:49–70.
- Gerke J. 1997. Aluminium and iron (III) species in the soil solution including organic complexes with citrate and humic substances. *Z Pflanzenernahr Bodenk* 160:427–432.
- Ghiorse WC. 1984. Biology of iron- and manganese-depositing bacteria. *Annu Rev Microbiol* 38:515–550.
- Gillan DC, De Ridder C. 1995. The microbial community associated with *Montacuta ferruginosa*, a commensal bivalve of the echinoid *Echinocardium cordatum*. In: RH Emson, AB Smith, AC Campbell, editors. *Echinoderm Research 1995*. Rotterdam, The Netherlands: Balkema, p 71–76.
- Gillan DC, De Ridder C. 1997. Morphology of a ferric iron-encrusted biofilm forming on the shell of a burrowing bivalve (Mollusca). *Aquat Microb Ecol* 12:1–10.

- Gillan DC, Speksnijder AGCL, Zwart G, De Ridder C. 1998. Genetic diversity of the biofilm covering *Montacuta ferruginosa* (Mollusca, Bivalvia) as evaluated by denaturing gradient gel electrophoresis analysis and cloning of PCR-amplified gene fragments coding for 16S rRNA. *Appl Environ Microbiol* 64:3464–3472.
- Gledhill M, van den Berg CMG. 1994. Determination of complexation of iron(III) with natural organic complexing ligands in seawater using cathodic stripping voltammetry. *Mar Chem* 47:41–54.
- Hanert HH. 1989. Genus *Gallionella* Ehrenberg 1838. In: JT Staley, editor. *Bergey's manual of systematic bacteriology*, Vol 3. Baltimore: Williams and Wilkins, p 1974–1979.
- Harding RA, Royt PW. 1990. Acquisition of iron from citrate by *Pseudomonas aeruginosa*. *J Gen Microbiol* 136:1859–1867.
- Heldal M, Fagerbakke KM, Tuomi P, Bratbak G. 1996. Abundant populations of iron and manganese sequestering bacteria in coastal water. *Aquat Microb Ecol* 11:127–133.
- Jannasch HW, Wirsén CO. 1981. Morphological survey of microbial mats near deep-sea thermal vents. *Appl Environ Microbiol* 41:528–538.
- Juniper SK, Tebo BM. 1995. Microbe–metal interactions and mineral deposition at hydrothermal vents. In: DM Karl, editor. *The microbiology of deep-sea hydrothermal vents*. New York: CRC Press, p 219–253.
- Karl DM, Brittain AM, Tilbrook BD. 1989. Hydrothermal and microbial processes at Loihi Seamount, a mid-plate hot-spot volcano. *Deep-Sea Res* 36:1655–1673.
- Knoll AH, Awramik SM. 1983. Ancient microbial ecosystems. In: W Krumbein, editor. *Microbial geochemistry*. Oxford: Blackwell Scientific, p 287–315.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Little BJ, Wagner PA, Lewandowski Z. 1997. Spatial relationships between bacteria and mineral surfaces. In: JF Banfield, KH Nealson, editors. *Geomicrobiology: interactions between microbes and metals*. *Rev Mineral* 35:123–159.
- Macrae IC, Edwards JF. 1972. Adsorption of colloidal iron by bacteria. *Appl Microbiol* 24:819–823.
- Mamet B, Préat A, De Ridder C. 1997. Bacterial origin of the red pigmentation in the Devonian Slivenec limestone, Czech Republic. *Facies* 36:173–188.
- Muir MD. 1978. Microenvironments of some modern and fossil iron- and manganese-oxidizing bacteria. In: WE Krumbein, editor. *Environmental biogeochemistry and geomicrobiology*. Ann Arbor, MI: Ann Arbor Science, p 937–944.
- Préat A, Mamet B, Bernard A, Gillan D. 1999a. Rôle des organismes microbiens dans la formation des matrices rougeâtres Paléozoïques: exemple du Dévonien, Montagne Noire. *Rev Micropaléontologie* 42:161–182.
- Préat A, Mamet B, Bernard A, Gillan D. 1999b. Bacterial mediation, red matrices diagenesis, Devonian, Montagne Noire (southern France). *Sediment Geol* 126:223–243.
- Reid RT, Butler A. 1991. Investigation of the mechanism of iron acquisition by the marine bacterium *Alteromonas luteoviolaceus*: characterization of siderophore production. *Limnol Oceanogr* 36:1783–1792.
- Roekens EJ, Van Grieken RE. 1983. Kinetics of iron (II) oxidation in seawater of various pH. *Mar Chem* 13:195–202.
- Sedmark JJ, Grossberg SE. 1977. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Anal Biochem* 79:544–552.
- Straub KL, Benz M, Schink B, Widdel F. 1996. Anaerobic, nitrate-dependent microbial oxidation of ferrous iron. *Appl Environ Microbiol* 62:1458–1460.
- Tazaki K. 1997. A new world in the science of biomineralization—environmental biomineralization in microbial mats in Japan. *Sci Rep Kanazawa Univ* 42(1, 2).
- Wells ML, Price NM, Bruland KW. 1995. Iron chemistry in seawater and its relationship to phytoplankton: a workshop report. *Mar Chem* 48:157–182.