Geobacter species enhances pit depth on 304L stainless steel in a medium lacking with electron donor

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A B S T R A C T

Geobacter sulfurreducens bacteria increased the open circuit potential of 304L stainless steel by around 320 mV in only a few hours after inoculation. This represents a significant increase in the corrosion risk. In contrast, the oxidation of acetate, which is catalysed by well-established biofilms, shifted the pitting potential towards positive values. In acetate-lacking media, pitting occurred with and without bacteria in the same range of potential values, but the presence of bacteria drastically increased the size of pits. AFM showed pits more than 10 times broader and deeper due to the presence of bacteria.

In the absence of acetate, the masking effect due to acetate oxidation disappeared and the full corrosive effect of the biofilm was revealed.

This also fully explains why pitting was predominantly observed close to surface areas where bacterial settlement was the densest.

Keywords:
Microbial corrosion
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Stainless steel
Electrochemically active biofilms
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1. Introduction

The implication of sulphate reducing bacteria (SRB) in the anaerobic corrosion of steels is well acknowledged [1]. Nevertheless, studies are starting to indicate that SRB may not be the only cause of anaerobic corrosion [2]. Recently, it has been demonstrated that *Geobacter* species can be involved in microbial corrosion, through direct electron exchange between microbial cells and the material surface. *Geobacter* is a bacterial genus quite ubiquitous in soils and sediments [3], it may consequently be a source of microbial corrosion for numerous types of buried industrial equipment.

Some *Geobacter* species have been shown to be able to connect their metabolism directly to solid electrodes [4–6]. *Geobacter sulfurreducens* can oxidize organic substrates (acetate, benzoate, toluene...) to carbon dioxide by transferring the electrons produced directly to graphite [3] or metallic anodes [7] thanks to periplasmic and outer membrane c-type cytochromes [8], On the other hand, *G. sulfurreducens* has also been shown to reduce nitrate to nitrite or fumarate to succinate with a graphite [9] or stainless steel [10] cathode as electron donor.

Our previous work has brought to light the crucial role that direct microbial electron transfer can play in anaerobic biocorrosion of steel [11]. The presence of *G. sulfurreducens* cells led to a drastic increase in the open circuit potential of 304L steel in a few hours only after inoculation. In contrast, after several days, well-established *G. sulfurreducens* biofilms shifted the pitting potential towards positive values. Well-established biofilms were able to catalyse the oxidation of acetate and thus provided the material with electrons that modified the behaviour of the passive layer with respect to pitting. Pitting was thus delayed by the presence of mature biofilms but paradoxically, it has been observed that pitting occurred preferentially in the areas of the material surface where biofilm was the densest.

The purpose of this work was to determine whether mature biofilms of *G. sulfurreducens* promoted pitting or not. To this end, it was necessary to finely determine how the biofilm-catalysed oxidation of acetate affected the material behaviour.

2. Experimental

Working electrodes were 2-cm-diameter cylinders of 304L stainless steel embedded in resin (Resipoly Chrysor). Elemental composition of 304L stainless steel by weight percentage is as follow: 9.68 Ni; 0.02 C; 1.43 Mn; 0.35 Cu; 0.35 Si; 0.03 S; 0.03 P; 0.40 Mn; 18.26 Cr. Electrical connections were made through titanium wire protected with resin. Coupons were abraded successively with SiC papers P120, P180, P400 and P800 (Lam Plan) and rinsed with distilled water. Electrochemical tests were performed in 0.5L reactors under N_2/CO_2 (80/20) flux with an Ag/AgCl reference electrode. Open circuit potentials were monitored using a

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multipotentiostat (VMP-Bio-Logic) and pitting curves were recorded at 0.5 mV s^{-1} with a platinum grid as counter electrode.

G. sulfurreducens strain PCA (ATCC 51573) purchased from DSMZ was grown at 30 °C for 5 days in the standard medium pH 7.2, which contained 28 mM NH_4Cl, 5 mM NaH_2PO_4, 1.3 mM KCl, 29.7 mM $NaHCO_3$, 10 mM sodium acetate (electron donor), 25 mM fumarate fumarate (electron acceptor), 10 mL L^{-1} vitamin mix (ATCC MD-VS) and 10 mL L^{-1} trace mineral mix (ATCC MD-TMS). Accordingly, the medium contained 29.3 mM chloride and 5 mM phosphate. Neither nitrates nor sulphates were present [12]. The bacteria were incubated in the growth medium for 5 days under anaerobic conditions at 30 °C. The number of planktonic cells (cell forming units per millilitre, CFU mL^{-1}) was evaluated through the absorbance at 620 nm [12]:

$$[\text{CFU mL}^{-1}] = \text{OD}_{620\text{ nm}} * 472,067$$

Epifluorescent microscopy was achieved with a Leica confocal microscope. Biofilms were stained for 10 min with acridine orange (0.03%), then rinsed with distilled water and air dried in the dark. SEM was performed with a LEO 435 VP-Carl Zeiss SMT and topographic pictures were recorded with an atomic force microscope (AFM) (Veeco) in contact mode using silicon nitride Si_3N_4 cantilevers (Park Scientific) with a scan rate of 0.5 Hz.

3. Results and discussion

G. sulfurreducens was grown for 5 days in the ATCC culture medium, which contained 10 mM acetate as electron donor and 25 mM fumarate as electron acceptor. This culture was used to inoculate different electrochemical reactors (5% v/v or 10% v/v i.e. 142,000 CFU mL^{-1} or 284,000 CFU mL^{-1}) that contained 1–4 304L stainless steel coupon(s). Inoculation was performed around 24 h after the immersion of the coupons. The solution in the electrochemical reactors was the same as the culture medium but with different concentrations of acetate: 0, 1, 5 and 10 mM. The open circuit potential E_{oc} of each coupon started to increase as soon as the reactor was inoculated, while control experiments run without bacteria showed no potential increase. The shape of E_{oc} evolution during the first 10 days depended on both the concentration of acetate and the amount of bacteria in the inoculum but, after around 10 days, each coupon reached the same E_{oc} value, around −10 mV vs. Ag/AgCl, which corresponded to a potential increase of around

Table 1
E_{pit} measured after 20 days in media inoculated with *G. sulfurreducens* (5% v/v i.e. 142000 CFU mL^{-1}) and containing different concentrations of acetate. pH and concentration of planktonic cells were measured at the end of the experiments. E_{pit} values were determined when the current reached 0.1 mA. Control experiments were carried out in the same media, using the culture medium without bacteria as inoculum.

Acetate (mM)	E_{pit} with *Geobacter* (V vs Ag/AgCl)	pH	CFU mL^{-1}	E_{pit} control (V vs Ag/AgCl)
0	0.79; 0.81; 0.90 (3)	6.97	70 810	0.83
1	0.84; 0.86; 1.09 (3)	7.03	94 413	0.79; 0.80; 0.81 (3)
5	1.09 average from seven experiments in the range (1.07; 1.11)	7.05	122 737	0.84 average from five experiments in the range (0.76; 0.95)
10	1.05	7.12	207 709	0.86

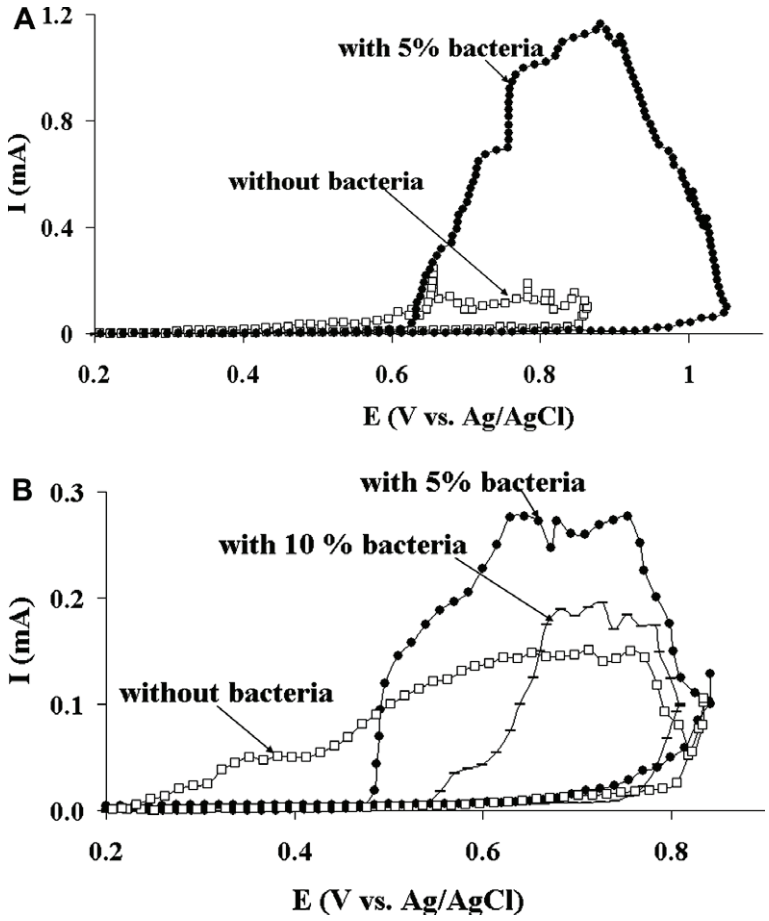

Fig. 1. Pitting curves (scan rate 0.5 mV s^{-1}) after 20 days immersion in an anaerobic medium inoculated with bacteria (5% v/v or 10% v/v i.e. 142,000 CFU mL^{-1} or 284,000 CFU mL^{-1}) or in the absence of bacteria. (A) Acetate 10 mM and (B) acetate 0 mM.

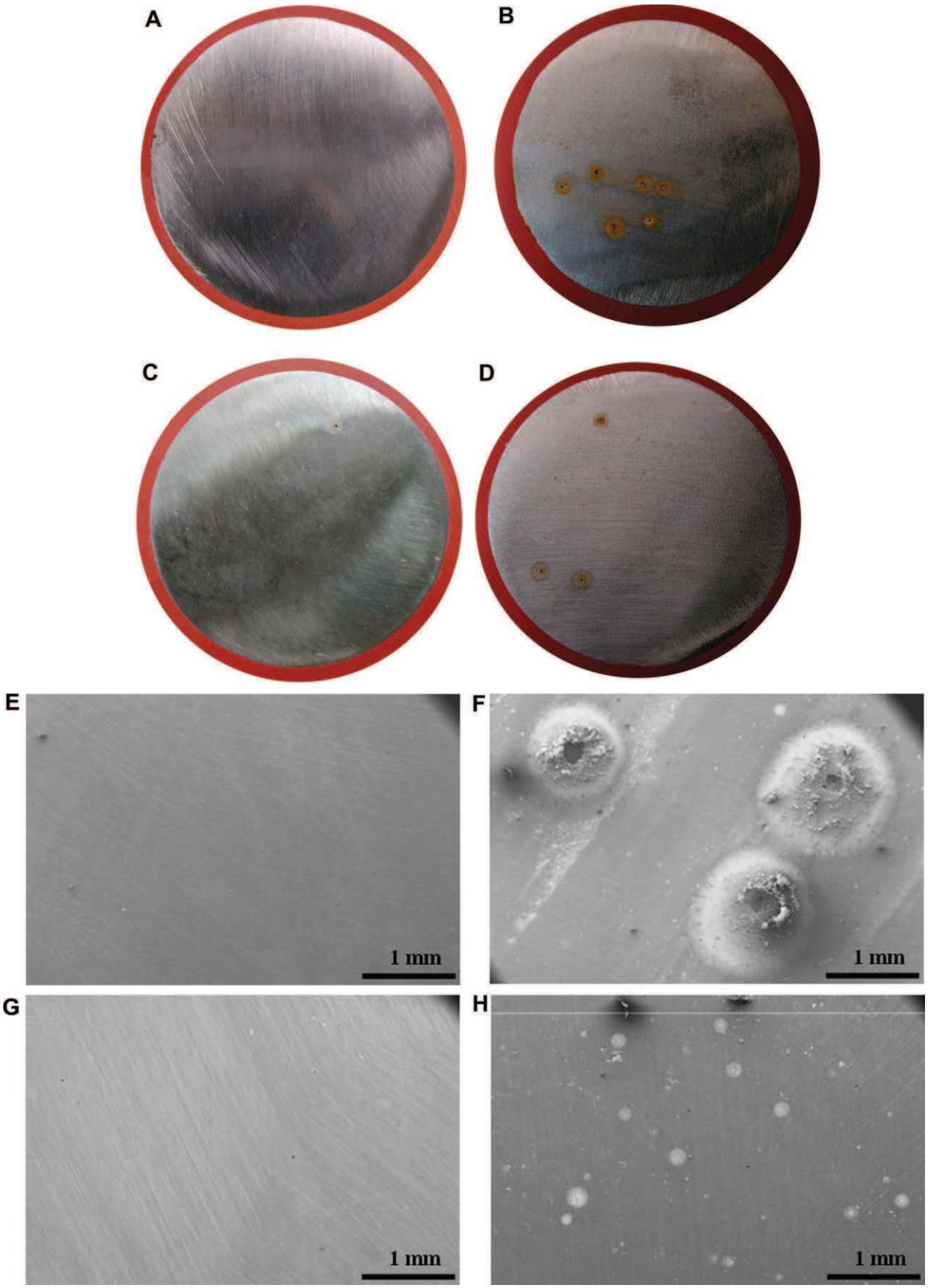

Fig. 2. Pictures and SEM micrographs of coupons after 20 days' immersion in an anaerobic medium in the absence of bacteria (first column: A, C, E, G) or inoculated with 142,000 CFU mL^{-1} *G. sulfurreducens* (second column: B, D, F, H). (A, B, E, F): acetate 10 mM. (C, D, G, H): acetate 0 mM.

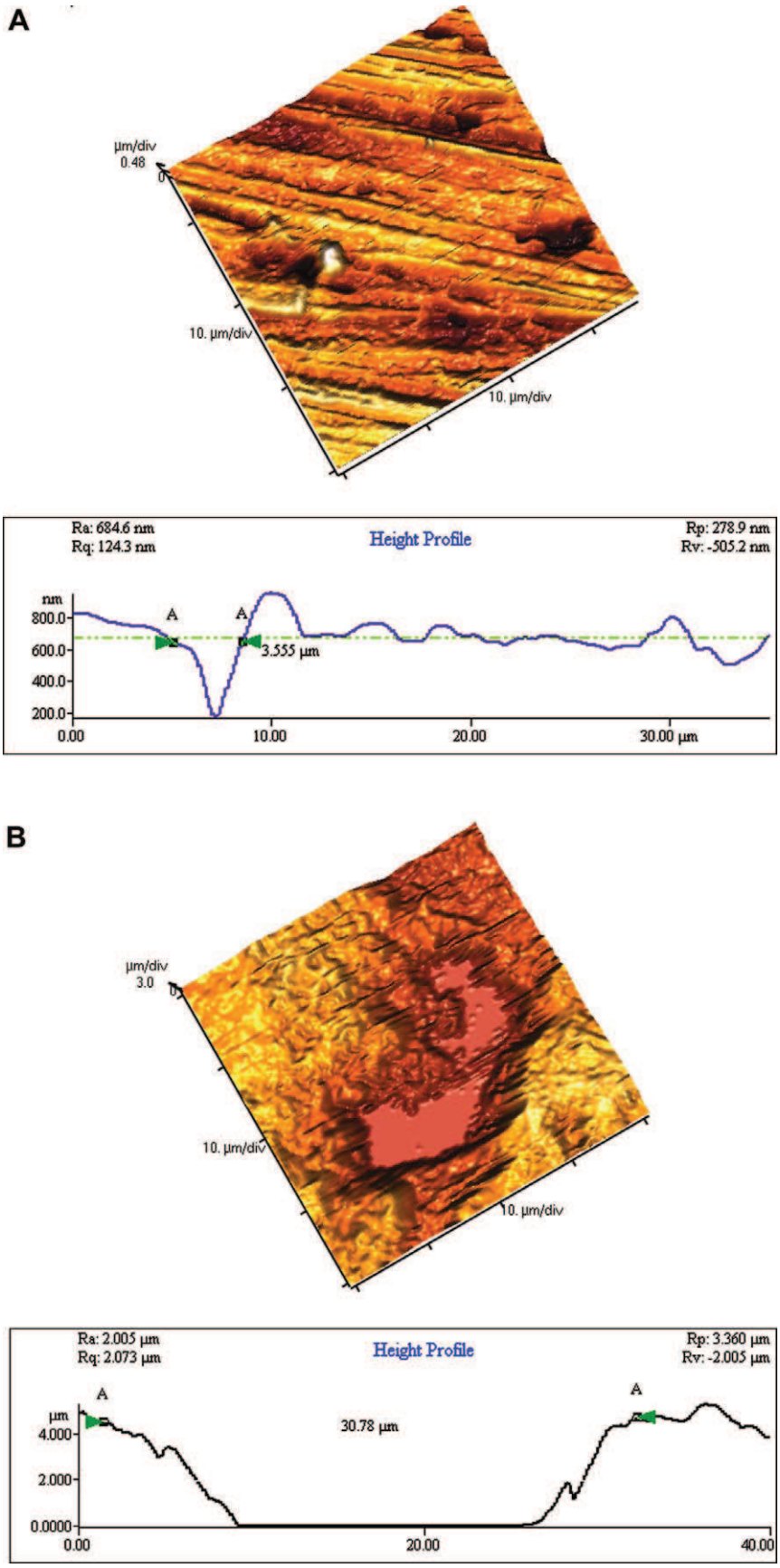

Fig. 3. AFM image and topographic representation of pits on a 304L SS from the experiments reported in Fig. 1B (acetate 0 M): (A) and (A′) in the absence of bacteria, showing small pits (width $w \approx 3$ µm, depth $d \approx 0.3$ µm); (B) and (B′) in the presence of 142,000 CFU mL^{-1} *G. sulfurreducens*, showing a large pit (width $w \approx 31$ µm, depth $d > 3$ µm); (C) and (C′) in the presence of 284,000 CFU mL^{-1} *G. sulfurreducens* showing a large pit (width $w \approx 27$ µm, depth $d > 3$ µm).

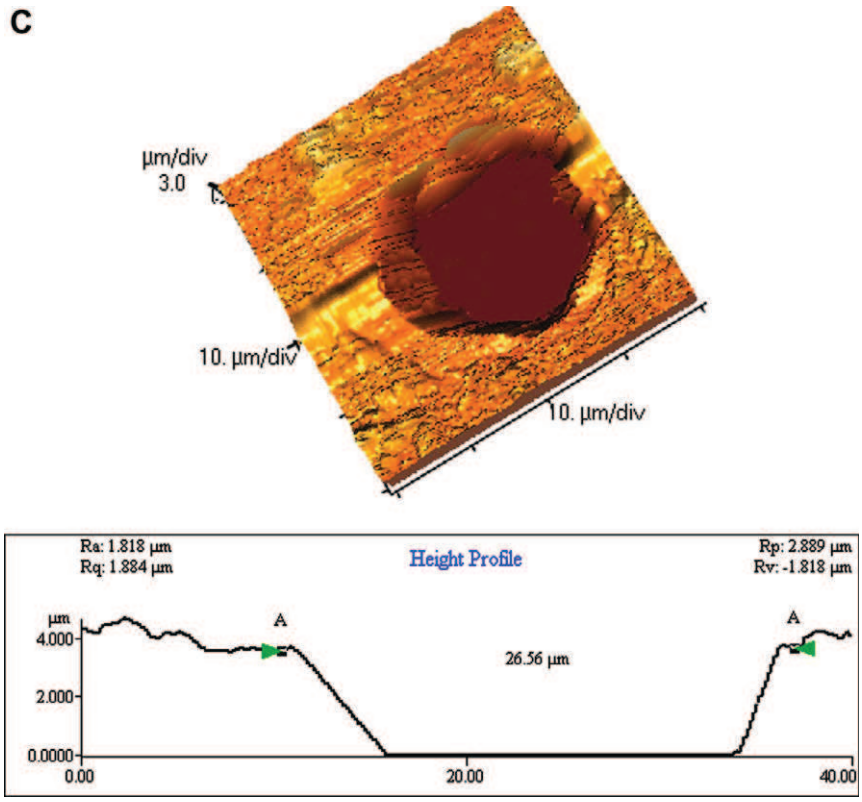

Fig. 3 (*continued*)

320 mV from the initial values. The E_{oc} value then remained stable for 10 more days. Microbial cells that came into contact with the material surface created a cathodic reaction that shifts the open circuit potential in the positive direction. This cathodic reaction has been attributed to direct electron transfer between the material surface and the redox system of the cell [11].

After 20 days on open circuit in these media, pitting curves were recorded by scanning the potential at 0.5 mV s^{-1} from the E_{oc} value (Fig. 1, Table 1). The presence or absence of acetate did not have a significant effect on the 10 control experiments performed without bacteria. The presence of bacteria significantly shifted E_{pit} towards positive values. The results were well reproducible in the eight experiments performed with 5 mM and 10 mM acetate. They were less regular in the three experiments performed with 1 mM acetate, because it is more difficult for the bacteria to grow with so low a concentration of electron donor, as shown by the lower concentration of planktonic cells (Table 1). *G. sulfurreducens* forms biofilms that catalyse the oxidation of acetate on steel surfaces and transfer the electrons produced directly to the material [7]. This external source of electrons is suspected to modify the behaviour of the passive layer and to delay the occurrence of pitting. Nevertheless, when the passive layer finally disrupted at very high potential values, pitting occurred at higher energy levels than in the control experiment and resulted in larger pits that required more charge for repassivation.

Epifluorescent images exhibited a heterogeneous coverage of the material surface by the biofilm (data not shown). Small surface areas were covered by dense biofilm, while large areas supported only scattered bacteria. In most cases, pits were observed close to or beneath biofilm settlement. With no acetate in the medium, E_{pit} values in the presence or the absence of bacteria were the same (Fig. 1B). The absence of electron donor in the medium was stressful for the cells, which developed only thanks to the small amount of acetate that remained in the inoculum. Epifluorescent microscopy with acridine orange staining allowed active cells (red fluorescence linked to ARN) to be distinguished from dead or inactive cells (green fluorescence linked to DNA). The weakness of red fluorescence observed on biofilms formed in acetate-free medium indicated a low living activity for these biofilms.

In acetate-free medium, the biofilm was no longer able to affect the E_{pit} value and pitting occurred in the same range of potentials with and without bacteria. Nevertheless, the propagation currents were higher in the presence of bacteria, suggesting the presence of larger pits. This was confirmed by macroscopic and microscopic images (Fig. 2). In the absence of bacteria, only one or two small corroded spots were visible and the pits initiated were very small and difficult to identify, scattered over the electrode surface. In contrast, several large corroded areas were visible in the presence of *G. sulfurreducens* and SEM detected a number of wide, deep pits, most often in zones of dense microbial settlement. AFM analysis of the surface topography (Fig. 3) showed only very small pits of 3 µm width and 0.3 µm depth on average for control experiments in the absence of bacteria. In the presence of 5% *G. sulfurreducens*, the pits were around 30 µm wide and their depth, greater than 3 µm, could not be evaluated because the size of the tip. The presence of the cells resulted in pits up to 10 times as wide and more than 10 times as deep. This conclusion resulted from SEM or AFM examination of 20 different plots for each analysed electrode. In the absence of acetate, pitting beneath biofilms and in the control experiments occurred in the same range of potentials; in such acetate-lacking condition, the marked differences can consequently not be attributed to differences in potential values, the high increase in the propagation rate was consequently due to the biofilm only. It can be concluded that *G. sulfurreducens* biofilms in the absence of acetate increased the width and depth of pits. This effect can be explained by two mechanisms, either *G. sulfurreducens* biofilm promotes pit propagation or it inhibits pit initiation resulting in a smaller number of pits with larger size. This last assumption is

most likely, as the number of pits observed was lower in the presence of bacteria.

Moreover, it must be noted that the repassivation potentials were significantly decreased by the presence of biofilms (Fig. 1). The repassivation potential was lowered of at least 260 mV on the 14 pitting curves recorded in the presence or in the absence of acetate, with respect to the 10 control experiments performed without bacteria. The presence of mature *G. sulfurreducens* biofilm led to larger pits but it has a clear beneficial effect on repassivation. The beneficial effect on repassivation is in accordance with the hypothesis assuming inhibition of pit initiation.

4. Conclusion

The catalysis of acetate oxidation that occurs with well-established biofilm of *G. sulfurreducens* delays the occurrence of pitting. In the absence of acetate, biofilms were still able to form thanks to the residual acetate contained in inoculum, but were less active and did not affect the pitting potential. In this case, pitting occurred with and without bacteria in the same range of potential values and it was thus demonstrated that *G. sulfurreducens* biofilms drastically enhanced pit width and depth. Actually, in the presence of acetate, the catalysis of the oxidation of acetate masked the local effect of the biofilm on pit formation. In the absence of acetate, this masking effect disappeared. It was suggested here that *G. sulfurreducens* biofilms act through inhibition of pit initiation and they promote repassivation; nevertheless, they also favour disruption of the passive layer as the large pits were systematically observed close to or beneath the densest microbial settlements. Because of the ubiquitous presence of *Geobacter* strains in soils and sediments, this genus must now be considered as a possible contributor when sources of microbial corrosion are investigated.

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References

[1] I.B. Beech, Int. Biodeter. Biodegr. 53 (2004) 177.
[2] M.A. Lopez, F.J.Z. Diaz de la Serna, J. Jan-Roblero, J.M. Romero, C. Hernandez-Rodriguez, FEMS Microbiol. Ecol. 58 (2006) 45.
[3] D.R. Bond, D.R. Lovley, Appl. Environ. Microbiol. 69 (2003) 1548.
[4] K. Rabaey, W. Verstraete, Trends Biotechnol. 23 (2005) 291.
[5] D.R. Lovley, Nature Rev. Microbiol. 7 (2006) 497.
[6] B.E. Logan, J.M. Regan, Trends Microbiol. 14 (2006) 512.
[7] C. Dumas, R. Basseguy, A. Bergel, Electrochim. Acta 53 (2008) 5235.
[8] Y.-H.R. Ding, K.H. Hixson, C.S. Giometti, A. Stanley, A. Estev-Núñez, T. Khare, S.L. Tollaksen, W. Zhu, J.N. Adkins, M.S. Lipton, R.D. Smith, T. Mester, D.R. Lovley, Biochim. Biophys. Acta 1764 (2006) 1198.
[9] K.B. Gregory, D.R. Bond, D.R. Lovley, Environ. Microbiol. 6 (2004) 596.
[10] C. Dumas, R. Basseguy, A. Bergel, Electrochim. Acta 53 (2008) 2494.
[11] M. Mehanna, R. Basseguy, M.-L. Délia, A. Bergel, Electrochem. Commun. 11 (2009) 568.
[12] C. Dumas, R. Basseguy, A. Bergel, Electrochim. Acta 53 (2008) 3200.