View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Open Archive Toulouse Archive Ouverte

Possible role of *Geobacter sulfurreducens* in anaerobic corrosion of steels

MEHANNA Maha; BASSEGUY Régine; DELIA Marie-Line; BERGEL Alain

Laboratoire de Génie Chimique CNRS-INPT
5 rue Paulin Talabot BP 1301, 31106 Toulouse, France
E-mail : Maha.Mehanna@ensiacet.fr

Geobacteraceae are the most widespread microorganisms in soils and sediments in which microbial reduction of Fe(III) is an important process, either in the natural degradation of organic compounds or in their bioremediation. Geobacter species have been shown to be predominant microorganisms on electrodes harvesting electricity from the sediments. They have the capability to oxidize organic electron donor to carbon dioxide transferring the electron directly to electrodes [1]. On the other side, the ability of *Geobacter sulfurreducens* to reduce nitrate to nitrite or fumarate to succinate with a graphite electrode serving as electron donor has also been demonstrated [2]. Direct electron transfer to solid electrodes is achieved through periplasmic and outer membrane c-type cytochromes [3]. Outer membranes proteins and even some kind of conductive pili that serve as biological nanowires are also involved in the electron transfer chains, mainly to Fe(III) and Mn(IV) oxides [4]. The aim of this study was to assess the possible influence of *G. sulfurreducens* on the occurrence of corrosion of steels. Experiments were performed with pure cultures of *G. sulfurreducens* on mild steel (XC45) and three different kinds of stainless steels (ferritic steel, 304L, 316L). In each case the free potential increased by 200 to 300 mV after the injection of the bacteria. On the contrary, control experiments performed with the injection of the sterile medium or the bacteria suspension after filtration on a 0.2 µm filter did not induce any variation in the free potential. The presence of the cell was consequently directly responsible for the potential increase of the coupons. The occurrence or not of corrosion was discussed with respect to this potential increase and the nature of the medium. Besides, preliminary results allow assessing the possibility to use G. sulfurreducens to design protective biofilms.

Keywords
Biocorrosion; biofilm; mild steel; stainless steel.

Introduction

Microbial fuel cells (MFC) are devices that use bacteria as the catalysts to oxidize organic and inorganic matter and generate current. This can be achieved when bacteria switch from the natural electron acceptor such as oxygen or nitrite to an insoluble acceptor such as MFC anode. Electrons produced by the bacteria are transferred to the anode and flow to the cathode through an external resistance (or a load). **(Logan review , Rabaey 2005)**. The advantages of MFC compared to other types of fuel cells is that it can operate at room temperature, it's self sustaining and renewing as it uses microorganisms that conserve energy from electron transfer to electrodes and it offers the possibility of extracting over 90% of the electrons from organic compounds. **2006** Recent development of a MFC that can harvest electricity from the organic matter stored in marine sediments has demonstrated the feasibility of producing useful amounts of electricity in remote environments. Further study of these systems has led to the discovery of microorganisms that conserve energy to support their growth by completely oxidizing organic compounds to CO_2 with direct electron transfer to electrodes. Molecular analysis of the microbial community on anodes surfaces revealed that microorganisms in the

family *Geobacteraceae* accounted for about half of the microorganisms, but there was no similar enrichment of *Geobacteraceae* on graphite buried in the sediment that was not connected to a cathode **(lovley current. Op in biotec 2006)**
Geobacteraceae offer a useful strategy for bioremediation of metal-contaminated subsurface environments as micro-organisms growing via Fe(III) reduction in subsurface environments can simultaneously reduce toxic metals such as U(VI), Tc(VII), Co (III) and V(V). **Mehta 2006** ,+**Butler** In addition, some *Geobacter* species can oxidize aromatic contaminants coupled to Fe(III) reduction allowing thus the removal of aromatic compounds from polluted aquifers. **Esteve-Núñez et al., 2005**Moreover *Geobacteraceae* can remove chlorinated solvents from contaminated ground water through reductive dechlorination. **Nature review Lovley**

Geobacter sulfurreducens can completely oxidize organic electron donors to carbon dioxide by using only an electrode as the electron acceptor **(Bond App. Env. Microbiol. 2003+ Esteve-Núñez et al., 2005).** On the other side, the ability of *G. sulfurreducens* to reduce nitrate to nitrite or fumarate to succinate with a graphite electrode serving as an electron donor has also been demonstrated (Gregory et al., 2004). Direct electron transfer to solid electrodes, without the need for electron shuttles or mediator, is achieved through periplasmic and outer membrane c-type cytochromes. In fact, the genome of *G. sulfurreducens* encodes 111 c-type cytochromes, which is significantly higher number of cytrochrome genes than reported in any other organism whose sequence is available. **Ding 2006** OmcS (Outer membrane cytochrome S) and to a lesser extend OmcE are important in electron transfer to electrodes. OmcB which is required for optimal electron transfer, not only to Fe(III) oxide but also to Fe(III) citrate, was not required for electricity production, this demonstrates that even though Fe(III) oxide and electrodes both represent extracellular electron acceptor, there may be significant difference between the pathways involved in electron transfer to Fe(III) and electrodes. (**Holmes 2ENVi006**) Outer membranes proteins such as OmpJ **Afkar 2005** and even some kind of conductive pili that serve as biological nanowires are also involved in the electron transfer chains, mainly to Fe(III) and Mn(IV) oxides **(Holmes, 2006).** Pili aren't absolutely required for electron transfer to the anode but are necessary for maximum power. They are needed in order to reduce Fe(III) oxides because pili are the electrical connection between the cell and the surface of the Fe(III) **(reguera nature 2005 +reguera 2006 app envi microbiol).**

The aim of this study was to assess the possible influence of *G. sulfurreducens* on the occurrence of corrosion of different kind of steels. Many facts have led us to emit this hypothesis: it was shown that four [Ni-Fe]-hydrogenases are encoded in the *G. sulfurreducens* genome: two periplasmic oriented, membrane bound hydrogenases, Hya and Hyb and two cytoplasmic hydrogenases, Mvh and Hox. It is the presence of Hyb that permits *G. sulfurreducens* to exploit hydrogen as an electron donor. (**Maddalena Coppi 2005+** Rabaey 2005). Yet hydrogenase implication in biocorrosion was proven **(publis da silva)**; for example hydrogenase sensors for quantifying hydrogenase activity and thus controlling corrosion are commercialised. (**boivin et al 1990).** Besides *G. sulfurreducens*, previously classified as a strict anaerobe, tolerated exposure to atmospheric oxygen for at least 24h and grew with oxygen as the sole electron acceptor at concentrations of 10% or less in the headspace. These results help explain how *Geobacter* species may survive in oxic subsurface environment, being poised to rapidly take advantage of the development of anoxic conditions. These findings are important for biocorrosion where the presence of both oxic zones and anoxic zones favors appearance of both anodic and cathodic zones on the same electrode. **(LIN 2003)**

2. Experimental

2.1. Metal sample preparation

Working electrodes were 2 cm diameter cylinders. They were made of either mild steel XC 45 or ferritic steel (Z6 C13) or stainless steels (316L or 304L) and embedded in resin (Resipoly Chrysor). The elemental composition by weight percentage of the steels is given in Table 1. Electrical connection was done through titanium wire protected with resin. Coupons were polished successively with SiC papers of P120, P180, P400, P800 grit (Lam Plan) and rinsed thoroughly with distilled water. The reference electrode was an Ag/AgCl electrode. A platinum grid served as a counter electrode. Experiments were carried out in 0.5 L corrosion cells, with continuous 80% N_2/20% CO_2 bubbling, at 30°C for optimum bacteria growth. Open circuit measurements of E_{corr} were done using a multipotentiostat Ec-lab.
The electrodes, after being removed from the cells were cleaned in a solution containing 50% in volume of HCl (36%) and 5 g/L of corrosion inhibitor hexamethylentetramine $C_6H_{12}N_4$.
They were analysed afterwards using an objective (10x) on a Carl Zeiss Axiotech 100 microscope equipped for epifluorescence with an HBO 50/ac mercury light source and the Zeiss 09 filter (excitor HP450-490, reflector FT 10, barrier filter LP520). Images were acquired with a monochrome digital camera (Evolution VF) interfaced to a computer with the Image-Pro Plus 5.0 software.

Table 1: Chemical composition of the steels (wt %)

Alloy	Ni	C	Mn	Cu	Si	S	P	Mo	Cr
XC45	0.1	0.46	0.65	0.11	0.31	0.032	0.01	0.02	0.1
Z6 C13		≤ 0.08	≤ 1		≤ 1	0.03	0.04		11.5/13.5
304L	9.68	0.02	1.43	0.35	0.35	0.03	0.03	0.40	18.26
316L	10.69	0.03	1.41	0.33	0.33	0.02	0.04	2.10	17.09

2.2. Microbiological cultivation and inoculation

G. sulfurreducens culture was grown under anaerobic conditions in ATCC medium (g/L): 1.5 NH_4Cl, 0.6 NaH_2PO_4, 0.1 KCl, 2.5 $NaHCO_3$, 0.82 $NaCOO^-$, 8 fumarate, 10 mL of ATCC vitamins and 10 mL of ATCC minerals (pH 7.2) **(Gregory et al. 2004)**. Bacteria were then inoculated at 5% (v/v) in the same culture medium but with a lower concentration of acetate (1 mM instead of 10 mM), previous studies having demonstrated that *Geobacter sulfurreducens* could be cultured under acetate-limiting conditions with fumarate or Fe(III)-citrate as the electron acceptor at growth rates between 0.04 and 0.09h^{-1} **(A Estrev nunez Envi micobiol 2005**).
Also, when the mild steel was employed, bacteria were inoculated at 5% (v/v) in the similar culture medium as described above but with lower chloride levels and lower acetate (ATCC medium (g/L): 0.2 NH_4Cl, 0.2 NaH_2PO_4, 0.2 KH_2PO_4, 2 $NaHCO_3$, 0.082 $NaCOO^-$, 8 fumarate, 10 mL of ATCC vitamins and 10 mL of ATCC minerals (pH 7.2)).

3. Results and discussion

Electrodes were immersed in the solution under continuous N_2/CO_2 flow. The microbial inoculum was injected after 24 hours. Fig. 1 shows the variation of the open circuit potentiel (E_{oc}) during 30 hours. Injection of 0.5% or 5% (v/v) *G. sulfurreducens* increased E_{oc} by +0.25 V and +0.30 V respectively. Control experiments performed in the same conditions by injecting only the initial medium or the bacteria suspension after filtration on a 0.2 µm filter after a 5-day culture did not induce any significant changes in E_{oc}. Sudden jumps in the potential were previously observed by Javaherdashti et ***al.*** in mild steel immersed in pure

sulphate reducing bacteria (SRB) culture during 40 days: E_{oc} decreased from -0.2 V versus Ag/AgCl reference down to -0.45 V, remained stable at this value for three days and then raised to potentials around + 0.2 V. This fluctuating pattern showing decreasing and increasing potentials was repeated after 21 days. The presence of (SRB) promoted corrosion cracking after 45 days exposure in Postgate Medium B a containing 35g/L of sodium chloride.**(Javaherdashti et al., in press**).

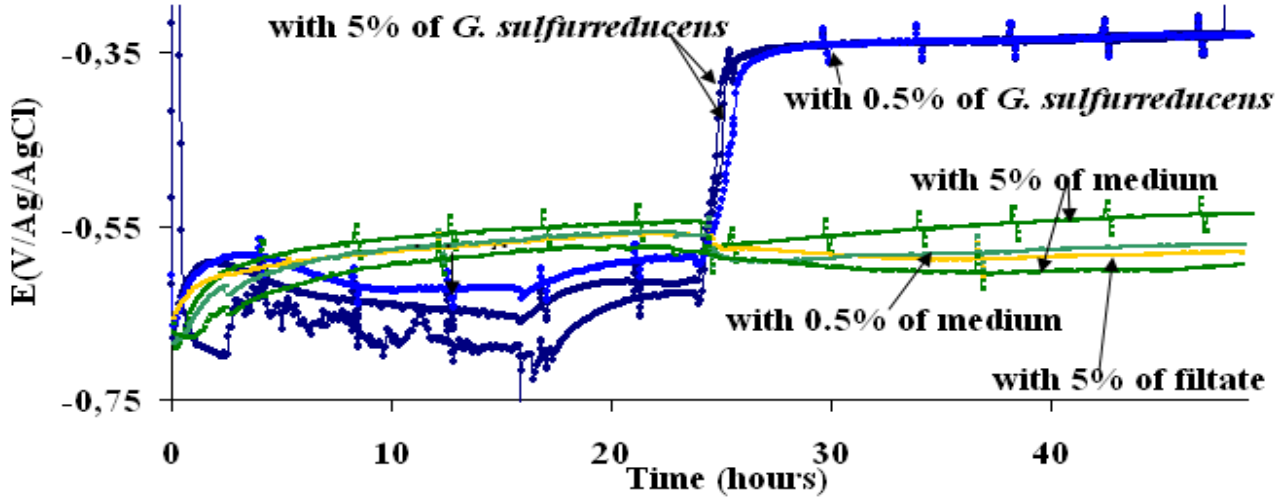


Fig. 1: Evolution of the free potential as a function of time for XC45 mild steel in the absence and presence (5% and 0.5%) of G. sulfurreducens; 1mM acetate. Fluctuations of +/-10 mV that appear on the graph each four hours are due to polarization resistance measurements.

Electrodes in the absence or the presence of bacteria were both covered with a black deposit, certainly because mild steel XC45 corroded in the culture medium, due to high chloride levels (30 mM). It was consequently difficult to assess the different levels in corrosion.
Therefore experiments were repeated with the same medium but with less chloride (4 mM) (Fig. 2). The scale of time is zoomed between 23 h to 30 h potential in order to show that the potential increase was gradual, it did not happen in just a few seconds but it took at least one hour indicating that the potential increase is not a phenomena only related to the double layer capacity. The potential increase is not due to some increases neither in the potential drop in the passive film nor to a variation in the double layer potential difference because such phenomena happen very fast in just a couple of seconds. So the potential jump is indeed due to the bacteria. **(L. Boulangé+Bellon-Fontaine)** Addition of 5% *G. sulfurreducens* at t = 24 h induced an increase in the free potential by + 0,33 V (from -0.64 V to -0.32 V/Ag/AgCl). When 0.5% *G. sulfurreducens* were injected at t = 24 h, the free potential increased slower than when more bacteria were added. There was no sudden jump but the free potential augmented gradually from -0.59 V to stabilize at -0.37 V after 28 h. E_{oc} remained constant after adding the sterile medium or the bacteria suspension after filtration on a 0.2 µm filter.

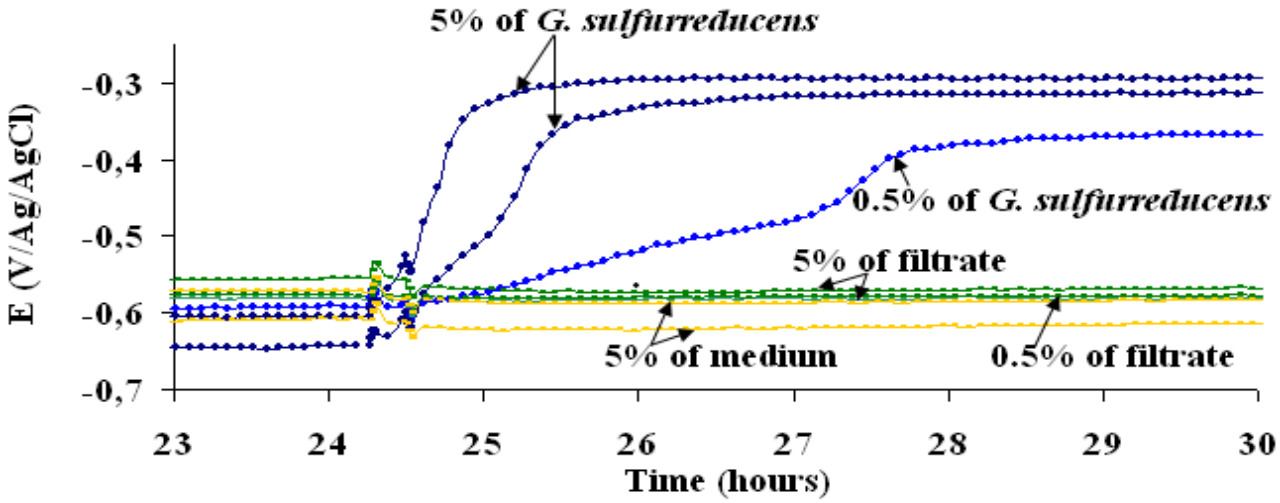


Fig. 2: Evolution of the free potential as a function of time for XC45 mild steel in the absence and presence (5% and 0.5%) of G. sulfurreducens;1mM acetate, with less chloride (4 mM).

The steels after removal from the different cells showed significant signs of corrosion whether they were immersed into a medium containing *G. sulfurreducens* 5% or filtrate eventhough more corrosion was observed in the presence of the bacteria (Fig. 3). Nevertheless, SEM photomicrography showed some pits on mild steel XC45 after 48 h exposure in a sterile medium containing 5% of filtrate (Fig. 4).

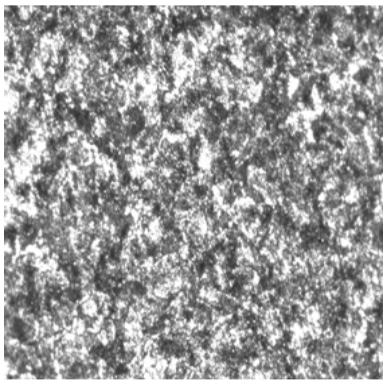
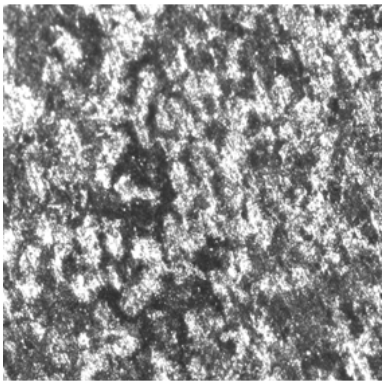
(a)  (b) 

Fig. 3: Micrographs of mild steel XC45 after 48 h exposure (a) in a sterile medium containing 5% of filtrate,(b) with presence of 5% of G. sulfurreducens. Je sais je n'ai pas mis l'échelle, je vais la mettre.

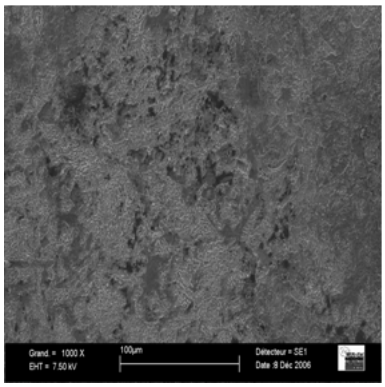


Fig. 4: SEM photomicrograph showing corrosion on mild steel XC45 after 48 h exposure in a sterile medium containing 5% of filtrate.

As mild steel did not resist well in the sterile medium even with lesser chloride, ferritic steel Z6 C13, containing 13% of chromium was chosen.

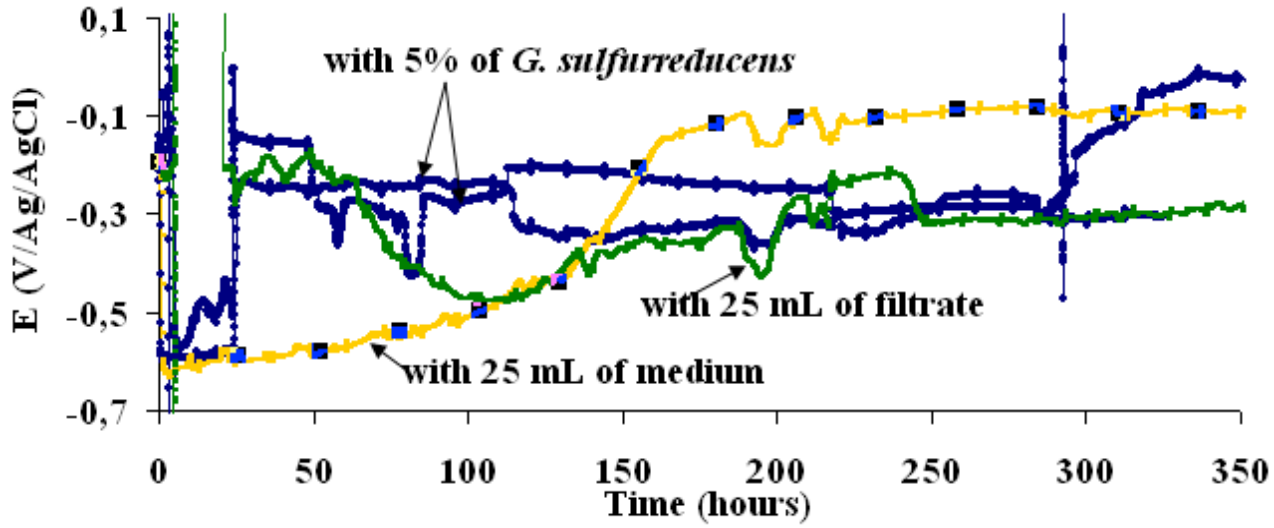


Fig. 5: Evolution of the free potential as a function of time for 304L SS in the absence and presence (5% and 0.5%) of G. sulfurreducens;1mM acetate.

NO COMMENT, j'ajouterais les photos SEM

SEM micrographs showed large and deep pits on the electrode immersed in the medium that contained 5% *G. sulfurreducens*: pits of 5.64 µm diameter and 4.71 µm depth were observed. Whereas, much smaller pits were shown on the electrode that was immersed in a medium containing 5% of filtrate: 2.94 µm diameter and 3.38 µm depth. The amount of pits was globally the same in the presence of both the bacteria or the filtrate but the size and the distribution of the pits was different in the presence of *G. sulfurreducens*: the bacteria induced the appearance of bigger and deeper pits that were grouped by zone whereas in the absence of the bacteria, pits were randomly distributed and they were smaller.
As small pits were observed on the ferritic steel even in the absence of *G. sulfurreducens*, stainless steel 316 L was chosen because it's known to better resist to corrosion.

Injection of 5% *G. sulfurreducens* into a medium lacking with acetate ennobled E_{oc} of 316L stainless steel up to +0.20 V. All of the four experiences that were done in these conditions were perfectly reproducible. The potential of the control electrodes that were immersed into a medium that contained neither acetate nor bacteria did not oscillate as it did in the presence of the bacteria, nevertheless E_{oc} increased slowly and after 14 days, all the steels had almost the same free potential nearby 0 V (Fig. 6). After removing the steel from the cells, the surfaces of 316L did not show any significant changes and the colour of the medium was almost limpid. Microscopic observation of this medium after marking the cells with Syto 9 and iodized propidium showed that the amount of dead cells was higher than that of the viable cells, so it can be assumed that 0% of acetate was not an ideal concentration for the viability of the bacteria, therefore the same experiment was repeated but with a medium that contained 10% of acetate (1 mM). When 5% *G. sulfurreducens* were added in a medium containing 1 mM acetate, the free potential increased by +0.16 V (from -0.20 V to +0.04 V) after 330 hours. Observation of the electrode after the experiment at a macrometer scale did not show any significant sign of corrosion. Surprisingly, when the medium didn't contain any acetate, the ennoblement of the free potential was higher than when 1 mM of acetate were added, this could be explained by the fact that when the medium lacks with the electron donor (acetate),

G. sulfurreducens uses more electron from the electrode thus E_{oc} increases more. Large evolutions in E_{oc} have also been previously reported by Xu et *al.* in pure cultures of SRB bacteria**:** in the presence of SRB, the free potential was reduced by about -0.37 V (from -0.06 to -0.43 V vs. SCE). Micrometer-scale pitting was observed on the 316L SS surface in the presence of SRB after 1000 h exposure. (**Xu et al., 2007, + in press).**

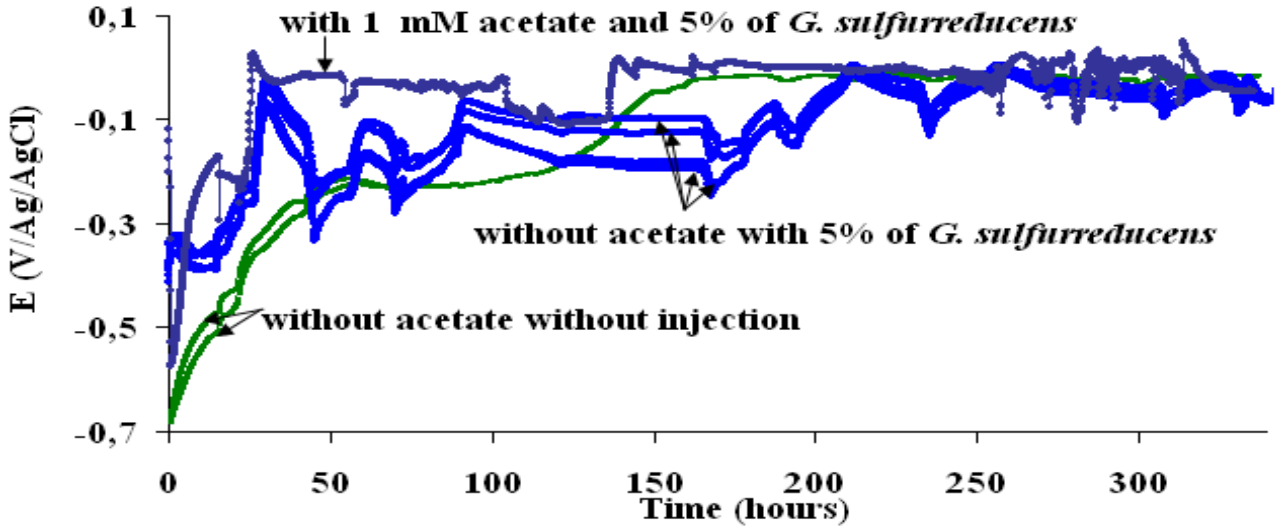


Fig. 6: Evolution of the free potential as a function of time for 316L SS in the absence and presence (5% and 0.5%) of G. sulfurreducens;without acetate or with 1 mM acetate.

316L stainless steel appeared to be highly resistant to corrosion therefore 304L was chosen which is an intermediate steel between mild steel, ferritic steel and 316L SS regarding its resistance to corrosion. Evolution of the free potential as a function of time was also observed for 304L SS (Fig. 7). Addition of 5% *G. sulfurreducens* ennobled E_{oc} of 304L stainless steel up to +0.32 V (from -0.34 V to -0.02 V), this was reproducible in all of the three cases. Injection of 0.5% *G. sulfurreducens* induced in increase of E_{oc} by also +0.32 V (from -0.33 V to +0.01 V). No significant jumps of the free potential were observed in the absence of bacteria. After 300 hours, the highest values of E_{oc} were obtained in the presence of bacteria independently on the quantity of bacteria that was added (5% or 0.5%), in fact the E_{oc} of the medium that contained 0.5% of *G. sulfurreducens* reached the same value as the medium containing 5% of *G. sulfurreducens* after 150 hours.

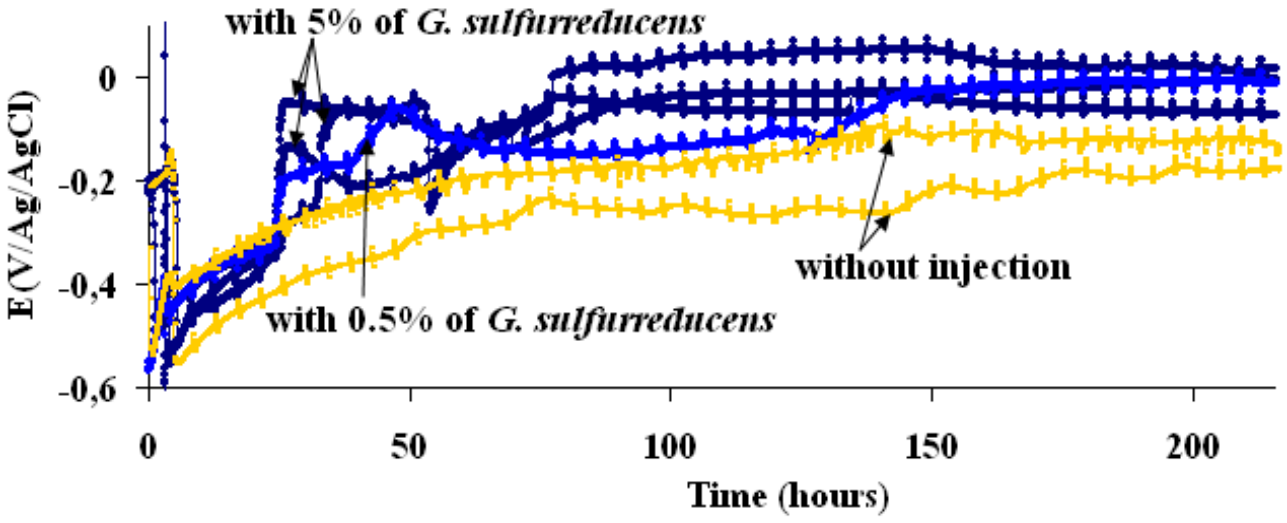


Fig.7: Evolution of the free potential as a function of time for 304L SS in the absence and presence (5% and 0.5%) of G. sulfurreducens;1mM acetate. Fluctuations of +/-10 mV that appear on the graph each four hours are due to polarization resistance measurements.

4. Conclusion

In conclusion, *G. sulfurreducens* induced an increase in the free potential up to +0.35 V on mild steel and stainless steels, which is controlled by the initial amount of bacteria inoculated and the concentration of electron donor in solution. Experiments performed with different initial concentrations of acetate indicated that the potential evolution was monitored by the depletion of the electron donor (acetate) in the medium, which forces *G. sulfurreducens* to use the material as electron source. Evolution in potential may thus be explained here by the capacity of this strain to implement direct electron transfer with conductive materials. These preliminary results may introduce for the first time into the framework of biocorrosion the new mechanism of microbial direct electron transfer. Additional results for anodic polarization curves, polarization resistances and SEM micrography would be presented in a further paper.
Besides, preliminary results allow assessing the possibility to use *G. sulfurreducens* to design protective biofilms. But this hypothesis should be considered carefully as other experiments performed using *G. sulfurreducens* in reduction have demonstrated that the reduction potential was not able to rise above -0.3V vs Ag/AgCl **(publi Claire, Busalmen)** nevertheless if some *G. sulfurreducens* strains were able to grow at higher potentials, then promising results would be next to come.

Acknowledgements
This work was supported by grants from CNRS-DRI. It was a part of network ERG+ "Surfaces of materials in living environments (SMILE)". We would like to thank Luc Etcheverry (LGC) for his technical support and Marie-Line De Solan (LGC) for SEM facilities.

References

1. B. E. Logan, B. Hamelers, R. Rozendal, U. Shröder, J. Keller, S. Freguia, P. Aelterman, W. Verstraete and K. Rabaey, *Environmental Science & Technology*, Review, **2006**, 40(17), 5181.
2. K. Rabaey and W. Verstaete, *Trends in Biotechnology*, Review, **2005**, 23(6), 291.
3. D. R. Lovley, *Nature reviews Microbiology*, Review, **2006**, 4, 327.
4. J. E. Butler, R. H. Glaven, A. Esteve-Núñez, C. Núñez, E. S. Shelobolina, D. Bond. And D. R. Lovley, *Journal of Bacteriology*, **2006**, 188(2), 450.
5. M. V. Coppi, *Microbiology*, **2005**, 151, 1239.
6. D. R. Bond and D. R. Lovley, *Applied and Environmental Microbiology*, **2003**, 69(3), 1548.
7. D. R. Lovley, *Current opinion in Biotechnology*, **2006**, 17, 327.
8. A. Esteve-Núñez, M. Rothermich, M. Sharma and D. R. Lovley, *Environmental Microbiology*, **2005**, 7(5), 641.
9. K. B. Gregory, R. D. Bond and D. R. Lovley, *Environmental Microbiology*, **2004**, 6(6), 596.
10. E. Afkar, G. Reguera, M. Schiffer and D. Lovley, *BioMed Central Microbiology*, **2005**, 5(41), 1471.

11. W. C. Lin, M. V. Coppi and D. R. Lovley, *Applied and Environmental Microbiology*, **2004**, 70(4), 2525.
12. G. Reguera, K. D. McCarthy, T. Mehta, J. S. Nicoll, M. T. Tuominen and D. R. Lovley, *Nature*, **2005**, 435(23), 1098.
13. G. Reguera, K. P. Nevin, J. S. Nicoll, S. F. covalla, T. L. Woodard and D. R. Lovley, *Applied and Environmental Microbiology*, **2006**, 72(11), 7345.
14. Y-H. R. Ding, K. K. Hixson, C. S. Giometti, A. Stanley, A. Esteve-Núñez, T. Khare, S. L. Tollaksen, W. Zhu, J. N. Adkins. M. S. Lipton, R. D. Smith, T. Mester and D. R. Lovley, *Biochemica et Biophysica Acta*, **2006**, in press.
15. D. E. Holmes, S. K. Chaudhury, K. P. Nevin, T. Mehta, B.A. Methé, A. Liu, J. E. Ward, T. L. Woodard, J. Webster and D. R. Lovley, *Environmental Microbiology*, **2006**, 8(10), 1805.
16. R. Javaherdashti, R. K. S. Raman, C. Panter and E. V. Pereloma, *Journal of International Biodeterioration & Biodegradation*, **in press**.
17. C. Xu, Y. Zhang, G. Cheng and W. Zhu, *Material Science Engineering*, **2007**, A 443, 235.
18. C. Xu, Y. Zhang, G. Cheng and W. Zhu, *Materials Characterization*, **in press.**
19. L.Boulangé-Petermann, M-N. Bellon-Fontaine and B. Baroux, *Journal of Microbiological methods*, **1995**, 21, 83.
20.