Distribution of *Shewanella putrefaciens* and *Desulfovibrio vulgaris* in sulphidogenic biofilms of industrial cooling water systems determined by fluorescent *in situ* hybridisation

Elise S McLeod[1], Raynard MacDonald[2] and Volker S Brözel[2]*

[1] *Department of Microbiology, University of the Western Cape, P/Bag X17, Bellville 7535, South Africa*

[2] *Laboratory for Biofilm Physiology, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002, South Africa*[2]

Abstract

Limited research has been done on the distribution and role of sulphidogenic facultative anaerobes within biofilms in microbially influenced corrosion (MIC). Sulphate-reducing bacteria (SRB) cause MIC and occur in the anaerobic zone of multispecies biofilms. Laboratory-grown multispecies biofilms irrigated with sulphate or sulphite-containing synthetic cooling water, and biofilms from an open simulated cooling water system, were hybridised with a rhodamine-labeled probe SPN3 (*Shewanella putrefaciens*) and fluorescein-labeled probe SRB385 (*Desulfovibrio vulgaris*) and investigated using scanning confocal laser microscopy. The facultative anaerobe *S. putrefaciens* and the strict anaerobe *D. vulgaris* synergistically coexisted in multispecies biofilms, but as time progressed, *S. putrefaciens* flourished, displacing *D. vulgaris.* The results show that *S. putrefaciens* is capable of growing in sulphidogenic biofilms in aerated environments such as industrial cooling water systems, colonising sulphidogenic biofilms and out-competing the true sulphate-reducing bacteria.

Introduction

Microbially influenced corrosion (MIC), believed to be caused by sulphate-reducing bacteria (SRB) in multispecies biofilms, has been researched extensively using *Desulfovibrio desulfuricans* and *D. vulgaris* as models, but little research has been performed on the role of sulphidogenic facultative anaerobes such as *Shewanella putrefaciens* within such biofilms (Hamilton, 1985; Lee et al., 1995; McLeod et al., 1998). In regions where water resources are limited, water cooling systems are operated at up to 16 cycles of concentration in order to minimise water consumption. The make-up water added to compensate for evaporation and blow-down in cooling towers results in the continuous addition of dissolved and suspended solids and salts, including sulphate (Cloete et al., 1992). The concentration of SO_4^{2-} in such systems can be as high as 3 200 mg/l.

The SRB are the only bacteria known to reduce sulphate and therefore the sulphur cycle in natural systems can only be initiated by reduction of SO_4^{2-} to S^{2-} by the true SRB. Once sulphide production from sulphate has been initiated, SO_3^{2-} and/or $S_2O_3^{2-}$ will be generated at the sulphide-oxygen interface by chemical oxidation, generally in the upper layers of the biofilm (Lee et al., 1995). These oxidised sulphur compounds are then available to those bacteria capable of utilising them as terminal electron acceptors, i.e. SRB, *S. putrefaciens* and certain *Aeromonas* species (McLeod et al., 1998). The current view is that the true SRB are the most important catalysers of MIC, generating hydrogen sulphide (H_2S) from sulphate (Hamilton, 1985; Lee et al., 1995). The ability to grow in the anoxic deeper layers of biofilms and to generate hydrogen sulphide near the metal surface is, however, not unique to the SRB. Sulphidogenic *S. putrefaciens* forms profuse biofilms on metal surfaces (Bagge et al., 2001), and is also capable of sulphite and ferric iron reduction under anaerobic conditions as well as utilisation of cathodic hydrogen (Semple and Westlake, 1987; Arnold et al., 1990; De Bruyn and Cloete, 1995; Dawood and Brözel, 1998). *S. putrefaciens* has been isolated from various industrial cooling water systems (De Bruyn and Cloete, 1995, McLeod et al., 1998).

The activities of consortia in biofilms enable organisms to maximise their metabolic capabilities and maintain community integrity and stability (Wolfaardt et al., 1994). We questioned whether *S. putrefaciens* would compete with true SRB in a biofilm exposed to industrial cooling water high in sulphate, as a synergistic relationship could result in a greater turnover of potentially corrosive ferrous sulphides (Obuekwe et al., 1981). The objective of this study was to obtain information about the spatial distribution and occurrence of *S. putrefaciens* and *D. vulgaris* within multispecies biofilms cultured in industrial cooling water by making use of fluorescent oligonucleotide probes and confocal laser microscopy.

Experimental

Cultures used

Facultatively sulphidogenic bacteria previously isolated from industrial cooling waters and identified as *Shewanella putrefaciens* (McLeod et al., 1998) and the control organisms *S. putrefaciens* ATCC 8072 and *Pseudomonas aeruginosa* PAO1 were grown in Luria-Bertani broth (LB) (Sambrook et al., 1989) while continuously agitating at 160 r·min^{-1} at 30°C, harvested during mid-exponential phase, washed and re-suspended in phosphate-buffered saline (PBS) (130 mM NaCl, 10mM Na_2HPO_4 [pH 7.2]). *D. vulgaris* subspecies *vulgaris* LMG 7563 was grown in pre-reduced modified medium 104 (2 mM K_2HPO_4, 19 mM NH_4Cl, 7 mM Na_2SO_4, 1 mM $CaCl_2.2H_2O$, 17 mM $MgSO_4.7H_2O$, 14 mM lactic acid, 1 g yeast extract, 1 mg resazurin, 2 mM $FeSO_4.7H_2O$, 0.6 mM ascorbic acid [pH 7.8] per l) in Hungate tubes and incubated anaerobically at

* To whom all correspondence should be addressed.
☎(012) 420 3262; fax: (012) 420 3266; e-mail: vsbrozel@postino.up.ac.za
Received 25 July 2001; accepted in revised form 18 December 2001.

30°C for 48 h. Titanium(III)citrate (1.3 mM) was used as the reductant and resazurin (1 ml of a 0.1 % w/v solution) as the redox indicator (Zehnder and Wuhrman, 1976). The cells were washed and re-suspended in PBS.

Oligonucleotide probes used

Probe SPN3 (5'-ECCGGTCCTTCTTCTGTAGGTAACGTCACAG-3', target position 477 – 506 on the 16SrDNA genes in the *E. coli* numbering system) (Brosius et al., 1981; Amann et al., 1995) is complementary to a 30-nucleotide region that distinguishes *S. putrefaciens* and *S. alga* from other members of the genus *Shewanella* and all other eubacteria in the rRNA (ribosomal RNA) database (DiChristina and DeLong, 1993). Probe SPN-3 was covalently linked to tetramethylrhodamine-5-isothiocyanate (TRITC) at the 5' end (Genosys). Probe SRB385 (5'-FCGGCGTCGCTGCGTCAGG-3', target position 385 – 402 on the 16S rDNA genes) was covalently linked to fluorescein at the 5' end (Genosys). The region targeted by probe SRB385 is specific to most members of the delta subclass of Proteobacteria which includes the gram negative SRB and myxobacteria. Probe EUB 338 (5'- GCTGCCTCCCGTAGGAGT- 3', target position 338 – 355 on the 16S rDNA genes) was covalently linked to CY-5 (Genosys) at the 5' end, and detects all members of the Eubacterial domain (Amann et al., 1995). The specificity of probe SPN-3 for all *S. putrefaciens* isolates used was determined by hybridising ribosomal RNA of all strains used to DIG-labelled probe DNA according to the method of Manz et al. (1992).

Optimisation of probe specificity and target accessibility

Whole cell hybridisation was performed according to the method of DiChristina and DeLong (1993). Exponential phase cell suspensions of *S. putrefaciens* ATCC 8072, *D. vulgaris* subspecies *vulgaris* LMG 7563 and *P. aeruginosa* PAO1 respectively were fixed with 0.1 volume of 37% (v/v) formaldehyde for 3 h at room temperature. A volume of 3 µl of each fixed cell suspension was combined and spotted on a pre-cleaned gelatin-coated slide. The air-dried slides were washed for 3 min consecutively with 50%, 75% and 90% ethanol. Gene frames (Advanced Biotechnologies Ltd; catalogue # AB-0555) consisting of adhesive frames and polyester covers, encasing 100 mm^2 (20 µl) cell spot area on the slide, were used to eliminate evaporative loss of hybridisation solution during fluorescent *in situ* hybridisation (FISH). A pre-warmed mixture of 10 µl of hybridisation buffer (5 x SET buffer, 0.01%[m/v] SDS [pH 7.8]), approximately 300 ng of rhodamine-labelled probe SPN3 and approximately 150 ng of fluorescein-labelled probe SRB385 was pipetted onto the polyester cover (1 x SET buffer contained 0.15 M NaCl, 1.0 mM EDTA, and 20 mM Tris base [pH 7.8]). The slide with the adhesive frame facing down was pressed on top of the cover. The slide was incubated at 46°C for 18 h in a 50 ml polypropylene tube floating in a water bath. Un-hybridised and non-specifically bound probes were removed by washing the slides in pre-warmed washing buffer (0.2 x SET buffer [pH 7.8]) at 48°C for 30 min. The slides were stored at 4°C in the dark.

Continuous-flow laboratory-scale apparatus for simulation of cooling water system

A reticulating flow system, facilitating a continuous flow of artificial cooling water, was constructed from perspex. Two parallel staircases on which glass cover-slips and mild steel coupons were placed, were irrigated constantly by circulating cooling water from the sump over the staircases by a water pump. The system had two removable panels for easy access and a sump accommodating a volume of 5 l. A closed-circuit operating system was used in order to prevent excessive evaporation and subsequent cooling of the cooling water to below ambient (*ca*. 5 °C). Sterile artificial cooling water was fed by a peristaltic pump (Watson Marlow 505 S) at a flow rate of 225 $ml \cdot h^{-1}$ maintaining an average growth rate of $0.045 \cdot h^{-1}$. The system was loaded with 5 l artificial cooling water, based on the analysis of Howarth and McEwan (1989), containing per 5 l: 5.0 g starch, 1.67 g tryptone, 1.67 g yeast extract, 5 g glucose, 1.92 g $CaSO_4$, 0.1 g $FeSO_4$, 7.45 g NaCl, 3.45 g Na_2SO_4, 0.17 g $NaNO_3$, 1.0 g $NaSO_3$ and 0.1 g ferric citrate. The pH was set at 8.5 making use of 5 M NaOH. The cooling water was then inoculated with 1 ml of overnight cultures of *P. aeruginosa* PAO1, *D. vulgaris* and *S. putrefaciens* 10. Sodium sulphite was excluded in certain experiments in order to determine whether the presence of sulphate alone would affect the spatial distribution of *D. vulgaris* and *S. putrefaciens*10 in the biofilm.

Operating conditions of the simulated cooling water system

An open recirculating flow system feeding a modified Robbin's device (MRD) with synthetic cooling water at a flow rate of $1.5 m \cdot s^{-1}$ was operated as described previously (MacDonald et al., 2000). Mild steel coupons with a diameter of 25 mm were cleaned with 70% ethanol, air-dried and inserted into the system. The system was inoculated with 1 l of cooling water taken from the Kriel Power Station in Gauteng, South-Africa and operated for a 14 d period with daily replacement of 10% of the water with fresh synthetic cooling water, when 24 coupons were removed for analysis and processed as described previously (MacDonald and Brözel, 2000).

FISH of biofilms

Biofilms on the glass cover-slips were fixed with 0.1 volume of 37% (v/v) formaldehyde for 3 h at room temperature. After air-drying, the cover-slips were washed for 3 min consecutively with 50%, 75% and 90% ethanol. Rubber-O rings (2 mm in width encasing an area of 17 mm in diameter) embedded with petroleum jelly were placed on the cover-slips to restrict the hybridisation solution to a confined area of the biofilm during FISH.

Biofilms from the MRD were scraped from mild-steel and nylon studs and diluted in 10 ml 1x PBS. A 5 ml volume of the cell suspension was centrifuged for 5 min (10 000 x g) and the supernatant discarded. A 100 µl volume of 0.5% glucose and 0.5% yeast extract was added to the sample, and incubated for 2 h at 30°C. Then 20 µl of chloramphenicol ($5 g \cdot l^{-1}$) was added and incubated for a further hour. The pellet was washed twice in 1X PBS and cells were fixed by adding 0,1 volume 35% formaldehyde and incubating for 3 h at room temperature. Cells were harvested and resuspended in 50 µl 1X PBS and 50 µl 96% (v/v) EtOH, and stored -20°C. A 3 µl volume of fixed cells in suspension was spotted onto gelatin-coated slides and air dried for 2 h at 37°C. Slides were washed consecutively in 50%, 70% and 90% (v/v) ethanol for 3 min each. Gene frames were pasted onto slides to surround the fixed cell spots.

A pre-warmed mixture of 300 µl of hybridisation buffer (5 x SET buffer, 0.01%[m/v] SDS), 3 000 ng of rhodamine-labeled probe SPN3 and 1 000 ng of fluorescein-labeled probe SRB385 was added to each biofilm (1 x SET buffer contained 0.15 M NaCl, 1.0 mM EDTA, and 20 mM Tris base [pH 7.8]). A glass cover-slip was placed on the rubber ring to eliminate evaporative loss of

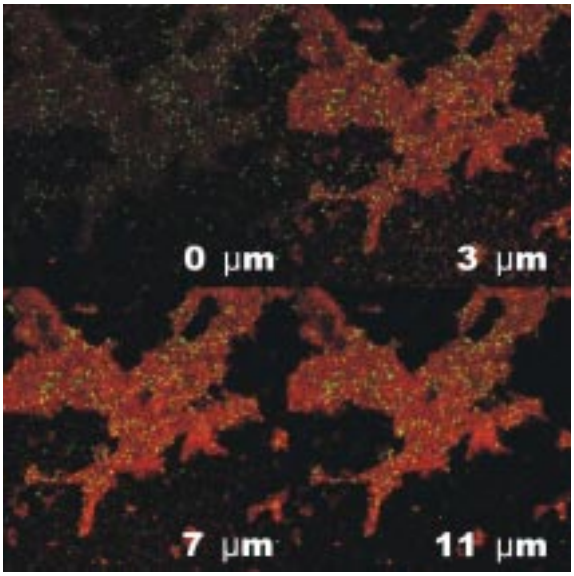

Figure 1
SCLM images (0 µm, 3 µm ,7 µm, 11 µm) of a 3 d-old biofilm formed in cooling water containing sulphite and sulphate and hybridised with rhodamine-labelled probe SPN3 specific for Shewanella putrefaciens *(red) and fluorescein-labelled probe SRB385 specific for δ-Proteobacteria (green)*

hybridisation solution during FISH. The samples were incubated at 46°C for 18 h in a 50 ml polypropylene tube floating in a water bath. Un-hybridised and non-specifically bound probes were removed by washing the cover-slips in pre-warmed washing buffer at 48°C for 1 h. The cover-slips were stored at 4°C in the dark before viewing by scanning confocal laser microscopy.

Determination of viability of cells on mild steel coupons

A volume of 5 ml of cell suspension was washed twice with PBS and centrifuged at 10 000 x g for 5 min at 4°C. The pellet was re-suspended in 1 000 ml PBS. A volume of 1 µl of the nucleic acid stains SYTO 9 and propidium iodide (live/dead Baclight™ bacterial viability kits, Molecular Probes) respectively was added to 10 µl of the cell suspension, and incubated for 10 min. The live bacteria with intact plasma membranes fluoresced green and the dead bacteria with compromised membranes fluoresced red.

Scanning confocal laser microscopy (SCLM)

A Carl Zeiss scanning confocal laser microscope (LSM 410; Carl Zeiss, Oberkochen, Germany equipped with a KrAr multiline laser [at 488, 568, 647 nm]) was used to obtain optical thin sections of the biofilms. The laser was operated at 1/30 maximum strength, and the pinhole was set to 20. Images were optimised by adjusting the brightness and contrast settings. A x63 Plan Apochromat objective (numerical aperture, 1.4; Carl Zeiss) was used in all experiments. Images were taken and processed using Zeiss LSM software (3D module). Fluorescein was detected using excitation 488 nm and a long-pass emission filter of 515 nm. Rhodamine was detected using excitation at 568 nm and a long-pass emission filter of 590 nm. CY5 was detected using excitation at 647 nm and an emission filter of 670 to 810 nm. Other microscope settings included laser attenuation using a 30 % ND filter and a 488/568 dichroic beam splitter to select the 488 and 568 excitation laser lines. For optimal imaging of the biofilm, the fluorophores were imaged sequentially producing grey

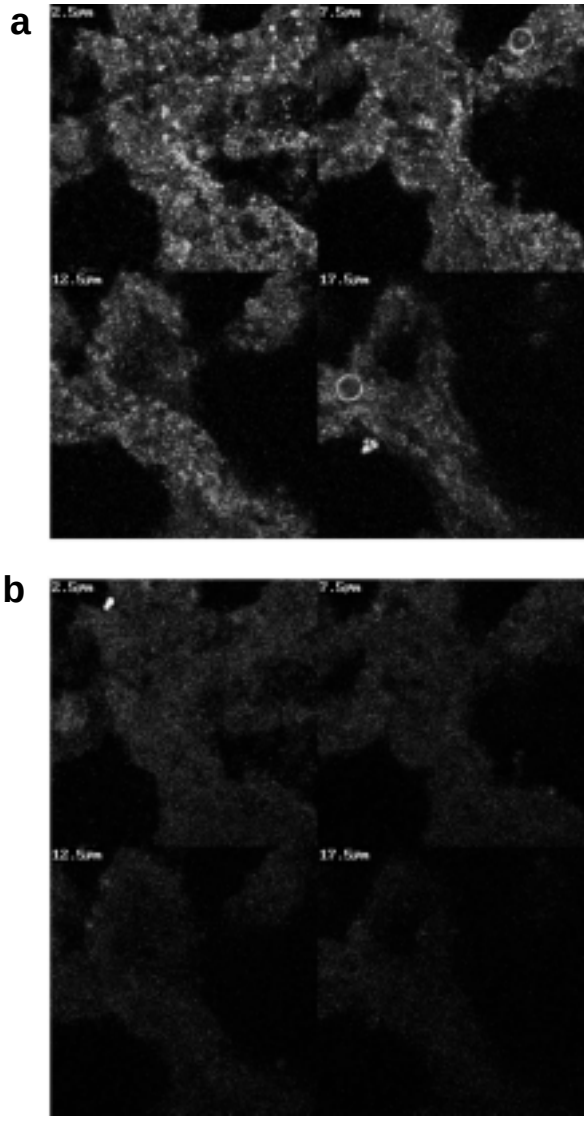

Figure 2
SCLM images (2.5 µm, 7.5 µm ,12.5 µm ,17.5 µm) of of a 7 d-old biofilm formed in cooling water containing sulphite and sulphate and hybridised with (a) rhodamine-labelled probe SPN3 specific for Shewanella putrefaciens *and (b) fluorescein-labelled probe SRB385 specific for δ-Proteobacteria*

scale 8-bit images, but the images were recombined into different color planes to produce color (RGB) images where necessary. Grey scale images were also converted to monochromatic color images using look-up tables, to improve the presentation.

Results

S. putrefaciens ATCC 8072 as well as various other *S. putrefaciens* isolates hybridised to probe SPN3, fluorescing red, whereas none of the sulphidogenic *Aeromonas*, SRB or *P. aeruginosa* PAO1 hybridised, confirming probe specificity and target accessibility (data not shown).

The glass cover-slips and mild steel coupons were left undisturbed in the continuous flow apparatus for 3 and 7 d respectively to allow biofilm development. After 1 d, attachment of bacteria was evident with the formation of discrete aggregates of densely packed cells and interstitial voids. As time progressed the microcolonies grew, and dense biofilm developed, covering most

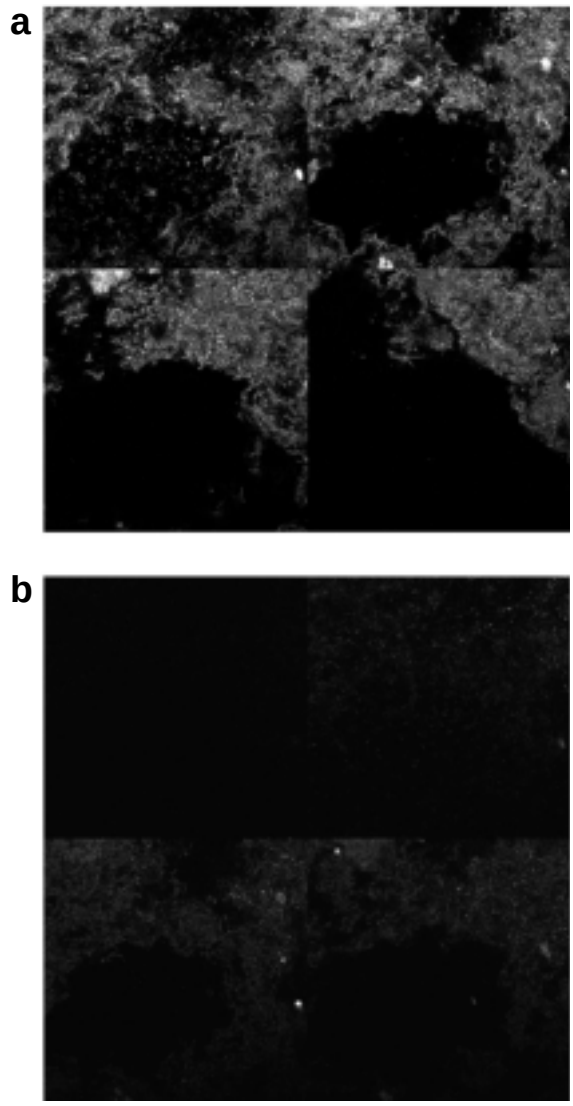

Figure 3
SCLM images (0 μm, 3 μm ,7 μm ,11 μm) of a 3 d-old biofilm formed in cooling water containing sulphate and hybridised with (a) rhodamine-labelled probe SPN3 specific for Shewanella putrefaciens *and (b) fluorescein-labelled probe SRB385 specific for δ-Proteobacteria*

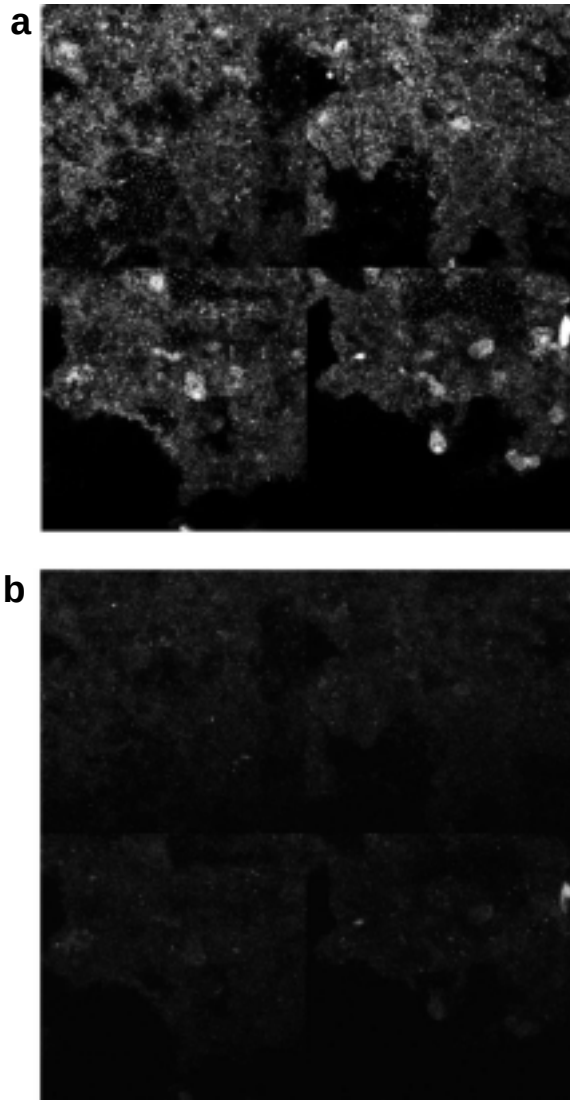

Figure 4
SCLM images (2 μm, 5 μm, 9 μm, 12 μm) of a 7 d-old biofilm formed in cooling water containing sulphate and hybridised with (a) rhodamine-labelled probe SPN3 specific for Shewanella putrefaciens *and (b) fluorescein-labelled probe SRB385 specific for δ-Proteobacteria*

of the surface. The biofilms on glass and on mild steel appeared black, indicating generation of S^{2-}. A 3d - old biofilm and a 7 d-old biofilm were removed from the system containing cooling water with (Figs. 1 and 2) or without SO_3^{2-} added (Figs. 3 and 4). The 3 d-old biofilms consisted of large microcolonies harbouring single cells of *D. vulgaris* dispersed between *S. putrefaciens* (Fig. 1). *D. vulgaris* appeared rod- or comma-shaped in pure culture and rod shaped to pleomorphic in biofilms. The *Desulfovibrio* cells were scattered and did not appear to be in clusters, indicating that they did not divide in the biofilm. This could be because they did not compete effectively with *S. putrefaciens* in the sulphidogenic/oxic environment. Although an equal number of both species were inoculated simultaneously, *S. putrefaciens* colonised the biofilm more rapidly. The optical sections through the biofilm show that *D. vulgaris* had a tendency to cluster with *S. putrefaciens* (Fig. 1), indicating a low degree of synergistic relationship between these two types. The 7 d-old biofilms also contained isolated cells of *D. vulgaris*, although fluorescence was weaker than in the 3 d-old biofilms (Fig. 2). The degree of fluorescence is known to be proportional to the ribosome count, which in turn is proportional to the growth rate (Møller et al., 1995). The artificial cooling water had been inoculated at day 0 with actively growing cultures, so that the *D. vulgaris* probably attached to the glass surface, but never multiplied in the system. Cells of *D. vulgaris* stopped growing after a while, and losing viability. *S. putrefaciens* was detected in microcolonies throughout the biofilm, increasing in dominance with biofilm age.

Biofilms from the system containing cooling water with no SO_3^{2-} added showed the same trend as biofilms growing with SO_3^{2-} (Figs. 3 and 4). The only difference between the biofilms was the higher concentration of *D. vulgaris* seen in the biofilms from the system containing SO_4^{2-} only (Figs. 3 and 4).

SCLM of biofilms on mild steel did not yield satisfactory fluorescent images due to the high proportion of debri, some of which autofluoresced. The direct viable count (DVC) using Baclight showed that most of the bacterial cells were viable. Biofilms were

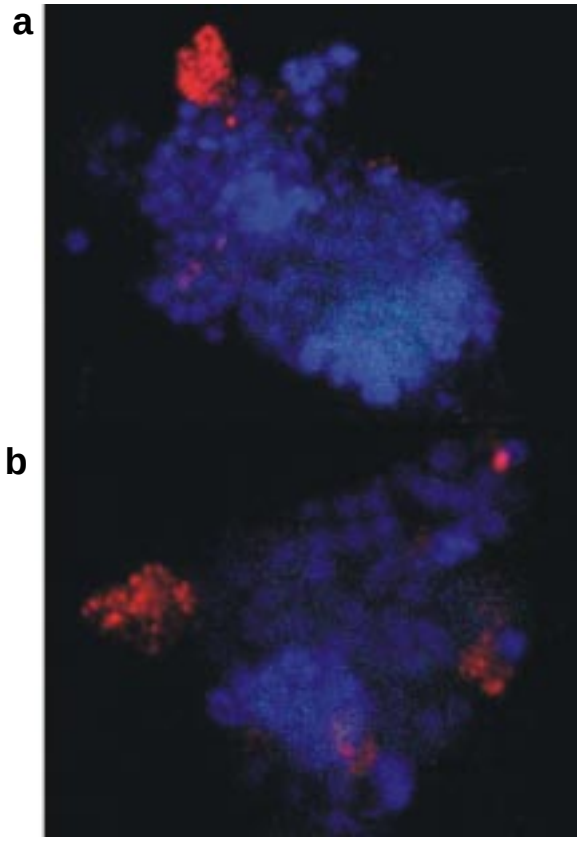

Figure 5
SCLM images of biofilm grown on mild steel in a modified Robbins device in cooling water and hybridised with rhodamine-labeled probe SPN3 specific for Shewanella putrefaciens *(red), fluorescein-labeled probe SRB385 specific for δ-Proteobacteria (green) and CY5-labelled probe EUB 338. CY5 was artificially allocated to the blue channel*

therefore scraped from mild steel coupons into PBS and allowed to increase in size using glucose and yeast extract while preventing cell division by addition of chloramphenicol, followed by hybridisation (Korber et al., 1999; MacDonald and Brözel, 2000).

Samples hybridised to the various probes following dispersal from the 24 mild steel stubs from the MRD were viewed by SCLM. *Shewanella putrefaciens* were detected in clumps originating from three of the coupons viewed (Fig. 5), and dispersed in one biofilm viewed (Fig. 6), but no SRB were detected. The detection limit of FISH is in the order of one in 100 cells (Amann et al., 1995) so that our observations indicate that true SRB, if present, did not predominate in the simulated cooling water system.

In an attempt to elucidate the predominance of *S. putrefaciens* over *D. vulgaris* in the biofilm fed only SO_4^{2-}, pure cultures of both were inoculated either separately or in combination into Hungate tubes containing only SO_4^{2-} and no SO_3^{2-}. *S. putrefaciens* was not capable of growing in pre-reduced modified medium 104 without SO_3^{2-}. However, in the presence of *D. vulgaris*, *S. putrefaciens* flourished as revealed by FISH, predominating over the *Desulfovibrio* (data not shown). It appears that SO_3^{2-}, the product of the first step in reduction of SO_4^{2-} by *D. vulgaris*, was released extra-cellularly and was therefore available for reduction to S^{2-} by *S. putrefaciens*. It is possible that a few cells of *D. vulgaris* can support numerous *S. putrefaciens*, resulting in a synergistic relationship. However, in the biofilm not fed with SO_3^{2-}, *S. putrefaciens* may also have used one of Fe^{3+} or NO_3^- during anaerobic respiration as terminal electron acceptor.

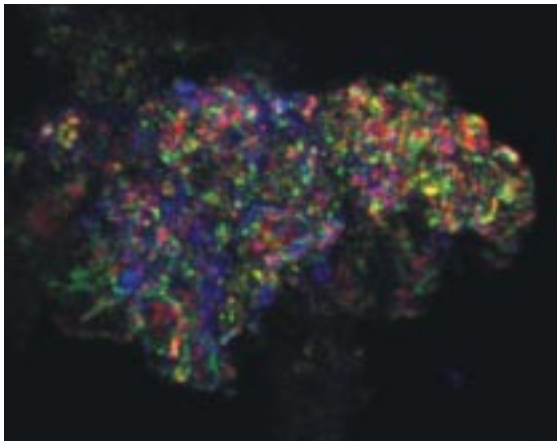

Figure 6
SCLM images of biofilm grown on mild steel in a modified Robbins device in cooling water and hybridised with rhodamine-labeled probe SPN3 specific for Shewanella putrefaciens *(red), fluorescein-labeled probe SRB385 specific for δ-Proteobacteria (green) and CY5-labelled probe EUB 338. CY5 was artificially allocated to the blue channel*

Discussion

It is shown here that *S. putrefaciens* is capable of growing in sulphidogenic biofilms in aerated environments such as industrial cooling water systems, colonising sulphidogenic biofilms on both glass and mild steel effectively and out-competing the true SRB, *D. vulgaris*. *S. putrefaciens* grew throughout the biofilm in the aerated simulated cooling water systems, from the oxygen-rich liquid interface to the anaerobic base. The corrosion-promoting potential of *S. putrefaciens* (Dawood and Brözel, 1998), together with its rapid growth rate and wide range of oxygen tolerance (Dawood et al., 1998), and extreme fitness in biofilms of industrial cooling water show that the current paradigm on MIC is too restrictive, and that facultative sulphidogenic bacteria are likely to play a significant role in this deleterious process.

Shewanella putrefaciens is a competitive biofilm-forming organism in contrast to *D. vulgaris*, the bacterium used as a model in studies of MIC. Bagge et al. (2001) showed that *Shewanella* forms well - developed stable biofilms on steel, and our results show that it grew well in cooling water biofilms. The predominance of *Shewanella* over *Desulfovibrio* in the laboratory system, and the presence of *S. putrefaciens* in the open simulated cooling water system, together with the absence of detectable levels of true SRB, indicates that the former has a greater degree of competitiveness in biofilms in the cooling water environment. *D. desulfuricans*, closely related to *D. vulgaris*, can grow at very low DO concentration, but become inhibited at 15 mmHg oxygen partial pressure (Abdollahi and Wimpenny, 1990), whereas *S. putrefaciens* is a true facultative bacterium, growing over a wide redox range (DiChristina and DeLong, 1993).

Facultative sulphidogenic bacteria such as *S. putrefaciens* cannot be argued to be the sole catalysts of MIC in industrial water systems as the sulphur cycle can only be initiated by the true SRB and selected Archaeabacteria. Biochemical SO_4^{2-} reduction is initiated by the transport of exogenous SO_4^{2-} across the bacterial membrane into the cell, followed by combination with ATP by adenosine tri-phosphate sulphurylase to produce the highly activated adenosine phosphosulphate (APS) (Lengeler et al., 1999). APS is then converted to SO_3^{2-} by the cytoplasmic enzyme APS reductase (Lengeler et al., 1999). SO_3^{2-} may then be reduced by sulphite

reductase via a variety of intermediates to form S^{2-} (Lengeler et al., 1999). The facultative sulphidogenic bacteria have been documented to reduce a variety of oxidised sulphur species other than SO_4^{2-} to S^{2-} (McLeod et al., 1998). Once the sulphur cycle has been initiated in a biofilm, chemical oxidation of S^{2-} at the sulphide-oxygen interface takes place (Lee et al., 1995), supplying the sulphidogenic community with an electron acceptor in the absence of molecular oxygen. In addition it appears that *D. vulgaris* supplies sufficient electron acceptor to *S. putrefaciens* to facilitate growth in the absence of alternate acceptors.

S. putrefaciens is likely to be a major catalyst of MIC in industrial water systems. It is capable of reducing both SO_3^{2-} and ferric iron using cathodic hydrogen as an electron donor, and is also capable of corroding steel in pure culture (Dawood and Brözel, 1998). The corrosion rate of steel in aerated, S^{2-}-containing water is higher than in anoxic water (Lee et al., 1995). *S. putrefaciens* begins reducing SO_3^{2-} at a dissolved oxygen concentration of 1.5 mg/l, producing S^{2-} (Dawood et al., 1998). The occurrence of *S. putrefaciens* in thin biofilms lacking true anaerobic deep zones, may therefore constitute a corrosive scenario, whereas the true SRB would only promote MIC in the anoxic zones of thicker biofilms (Cloete et al., 1992). The characteristic of being facultative would be the selective advantage for sulphite-reducing bacteria over true SRB growing in aerated waters harbouring sulphite-containing micro-zones.

Conclusions

Shewanella putrefaciens is capable of growing in sulphidogenic biofilms in aerated environments such as industrial cooling water systems, colonising sulphidogenic biofilms and out-competing the true sulphate-reducing bacteria.

The current paradigm on microbially influenced corrosion is too restrictive as the facultative sulphidogenic bacteria such as *S. putrefaciens* are likely to play a significant role in this deleterious process.

Acknowledgements

ESM and RM were supported by scholarships from the National Research Foundation. We would like to thank Dr IS Harper and Keith Williams at the Medical Research Council for their help with scanning confocal laser microscopy. This research was funded by a grant from the Water Research Commission of South Africa to VSB.

References

ABDOLLAHI H and WIMPENNY J (1990) Effects of oxygen on the growth of *Desulfovibrio desulfuricans. J. Gen. Microbiol*. **136** 1025-1030.

AMANN RI, LUDWIG W and SCHLEIFER K-H (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59** 143-169.

ARNOLD RG, HOFFMAN MR, DICHRISTINA TJ and PICARDAL FW (1990) Regulation of dissimilatory Fe (III) reduction activity in *Shewanella putrefaciens. Appl. Environ. Microbiol.* **6** 2811-2817.

BAGGE D, HJELM M, JOHANSEN C, HUBER I and GRAM L (2001) *Shewanella putrefaciens* adhesion and biofilm formation on food processing surfaces. *Appl. Environ. Microbiol*. **67** 2319-2325.

BROSIUS J, DULL TJ, SLEETER DD and NOLLER HF (1981) Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli. J. Mol. Biol.* **148** 107-127.

CLOETE TE, BRÖZEL VS and VON HOLY A (1992) Practical aspects of biofouling control in industrial water systems. *Int. Biodeterioration & Biodegradation* **29** 299-341.

DAWOOD Z and BROZEL VS (1998) Corrosion-enhancing potential of *Shewanella putrefaciens* isolated from industrial cooling waters. *J. Appl. Microbiol.* **84** 929-936.

DAWOOD Z, EHRENREICH L and BRÖZEL VS (1998) *Shewanella putrefaciens* reduces sulphite in the presence of molecular oxygen. *FEMS Microbiol. Letts.* **164** 383-387.

DE BRUYN EE and CLOETE TE (1995) The role of *Shewanella putrefaciens* in microbially induced corrosion. In: Bousher A, Chandra M and Edyvean R (eds.) *Biodeterioration and Biodegradation - 9*. Inst. of Chem. Eng., Warwickshire. 535-542.

DiCHRISTINA TJ and DeLONG EF (1993) Design and application of rRNA-targeted oligonucleotide probes for the dissimilatory iron- and manganese-reducing bacterium *S. putrefaciens*. *Appl. Environ. Microbiol.* **59** 4152-4160.

HAMILTON WA (1985) Sulphate-reducing bacteria and anaerobic corrosion. *Annu. Rev. Microbiol.* **39** 195-217**.**

HOWARTH DA and McEWAN JJ (1989) Monitoring and evaluation of water quality, corrosion, fouling and scaling in water reticulation systems in the gold mining industry. *Proc. S.A. Ind. Water Ass. Symp.* September 1989, Witkoppen, South Africa. 124-136.

KORBER DR, WOLFAARDT GM, BRÖZEL VS, MACDONALD R and NIEPEL T (1999) Reporter systems for microscopic analysis of microbial biofilms. *Meth. Enzymol*. **310** 3-20.

LEE W, LEWANDOWSKI Z, NIELSEN PH and HAMILTON WA (1995) Role of the sulphate-reducing bacteria in corrosion of mild steel: A review. *Biofouling* **8** 165-194.

LENGELER JW, DREWS G and SCHLEGEL HG (1999) *Biology of the Prokaryotes.* Blackwell Science, Stuttgart. 292-294.

MACDONALD R, SANTA M and BRÖZEL VS (2000) The response of a bacterial biofilm community in a simulated industrial cooling water system to treatment with an anionic dispersant. *J. Appl. Microbiol*. **89** 225-235.

MACDONALD R and BRÖZEL VS (2000) *In situ* analysis of bacterial biofilms in industrial cooling water systems, using fluorescently labeled rRNA-targeted oligonucleotide probes *Water Res.* **34** 2439-2446.

MANZ W, AMANN R, LUDWIG W, WAGNER M and SCHLEIFER K-H (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of Proteobacteria: problems and solutions. *System. Appl. Microbiol.* **15** 593-600.

McLEOD ES, DAWOOD Z, MACDONALD R, OOSTHUYZEN M, GRAF J, STEYN PL and BROZEL VS (1998) Isolation and identification of sulphite- and iron reducing, hydrogenase positive facultative anaerobes from cooling water systems. *System. Appl. Microbiol.* **21** 297-305.

MØLLER S, KRISTENSEN CS, POULSEN LK, CARSTENSEN JM and MOLIN S (1995) Bacterial growth on surfaces: Automated image analysis for quantification of growth rate-related parameters. *Appl. Environ. Microbiol.* **61** 741-748.

OBUEKWE CO, WESTLAKE DWS, COOK FD and COSTERTON JW (1981) Surface changes in mild steel coupons from the action of corrosion-causing bacteria. *Appl. Environ. Microbiol.* **41** 766-774.

SAMBROOK J, FRITSCH EF and MANIATIS T (1989) *Molecular Cloning. A Laboratory Manual* (2nd edn.) Book 1. Cold Spring Harbor Laboratory Press.

SEMPLE KM and WESTLAKE DWS (1987) Characterization of iron-reducing *Alteromonas putrefaciens* strains from oil field fluids. *Can. J. Microbiol.* **33** 366-371.

WOLFAARDT GM, LAWRENCE JR, ROBARTS RD, CALDWELL SJ and CALDWELL DE (1994) Multicellular organization in a degradative biofilm community. *Appl. Environ. Microbiol.* **60** 434-446.

ZEHNDER ABJ and WUHRMAN K (1976) Titanium (III) citrate as nontoxic oxidation-reduction buffering system for the culturing of obligate anaerobes. *Science* **194** 1165-1166.