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

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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

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Received 25 July 2001; accepted in revised form 18 December 2001.