Bacterial colonization and modification of grain boundaries on 316L stainless steel
by Richard John Gillis

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University

Abstract:
Localized pitting corrosion has become a significant problem with 316L stainless steel used as a piping material for service water systems in nuclear power facilities. Biofilms and the activities of the microorganisms associated with them are frequently implicated. Although much is known about abiotic corrosion, comparatively little is known about microbially influenced corrosion of stainless steel. Current research focused on using surface sensitive techniques including scanning confocal laser microscopy (SCLM), image analysis, energy dispersive X-ray spectroscopy (EDX), Auger electron spectroscopy (AES), and atomic force microscopy (AFM).

A dual species biofilm containing Pseudomonas aeruginosa and Desulfovibrio gigas was grown on 316L hand polished stainless steel under aerobic conditions. Microcolonies of D. gigas were detected using an SRB polyclonal antibody, however stainless steel surface preparation changes discontinued these experiments. A dual species biofilm containing a facultative anaerobic bacterium, Citrobacter freundii, and a sulfate reducing bacterium, Desulfovibrio gigas, was grown on a 316L 2B finish stainless steel under anaerobic conditions, After 16 days, direct enumeration revealed densities of C. freundii on the surface to be approximately 1x10^7/cm^2, whereas Desulfovibrio gigas was found to be in lower average densities and growing in areas containing C. freundii. Using SCLM and image analysis techniques, C. freundii was shown to preferentially colonize grain boundaries on the stainless steel surface. AFM showed the grain boundaries were deep and wide enough to harbor bacteria. Subsequent analysis by small spot AES showed a depletion in chromium relative to nickel in the grain boundaries of the coupons exposed to C. freundii with or without D. gigas and a depletion in iron relative to nickel in the coupons exposed to both C. freundii and D. gigas. AES also revealed sulfur and phosphorus nutrient deposition within the grain boundaries and may provide rationale for preferential bacterial colonization. The present study demonstrates that bacterial colonization of grain boundaries on 316L SS results in depletion of Cr and Fe relative to Ni. Chromium depletion is thought to promote localized attack on this alloy. Selective removal of Cr and Fe may represent a mechanism of localized microbially influenced corrosion of 316L stainless steel.

BACTERIAL COLONIZATION AND MODIFICATION OF GRAIN BOUNDARIES ON 316L STAINLESS STEEL

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Richard John Gillis

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Dec 1, 1993
Date

Chairperson, Graduate Committee

Approved for the Major Department

12/3/93
Date

Head, Major Department

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ABSTRACT

Localized pitting corrosion has become a significant problem with 316L stainless steel used as a piping material for service water systems in nuclear power facilities. Biofilms and the activities of the microorganisms associated with them are frequently implicated. Although much is known about abiotic corrosion, comparatively little is known about microbially influenced corrosion of stainless steel. Current research focused on using surface sensitive techniques including scanning confocal laser microscopy (SCLM), image analysis, energy dispersive X-ray spectroscopy (EDX), Auger electron spectroscopy (AES), and atomic force microscopy (AFM).

A dual species biofilm containing *Pseudomonas aeruginosa* and *Desulfovibrio gigas* was grown on 316L hand polished stainless steel under aerobic conditions. Microcolonies of *D. gigas* were detected using an SRB polyclonal antibody, however stainless steel surface preparation changes discontinued these experiments. A dual species biofilm containing a facultative anaerobic bacterium, *Citrobacter freundii*, and a sulfate reducing bacterium, *Desulfovibrio gigas*, was grown on a 316L 2B finish stainless steel under anaerobic conditions. After 16 days, direct enumeration revealed densities of *C. freundii* on the surface to be approximately $1x10^7/cm^2$, whereas *Desulfovibrio gigas* was found to be in lower average densities and growing in areas containing *C. freundii*. Using SCLM and image analysis techniques, *C. freundii* was shown to preferentially colonize grain boundaries on the stainless steel surface. AFM showed the grain boundaries were deep and wide enough to harbor bacteria. Subsequent analysis by small spot AES showed a depletion in chromium relative to nickel in the grain boundaries of the coupons exposed to *C. freundii* with or without *D. gigas* and a depletion in iron relative to nickel in the coupons exposed to both *C. freundii* and *D. gigas*. AES also revealed sulfur and phosphorus nutrient deposition within the grain boundaries and may provide rationale for preferential bacterial colonization. The present study demonstrates that bacterial colonization of grain boundaries on 316L SS results in depletion of Cr and Fe relative to Ni. Chromium depletion is thought to promote localized attack on this alloy. Selective removal of Cr and Fe may represent a mechanism of localized microbially influenced corrosion of 316L stainless steel.

INTRODUCTION

Stainless Steel

Stainless steel has been used as a piping material for many years and its metallurgical properties are well characterized (80). A variety of different types of stainless steel alloys are available, however austenitic stainless steels are most commonly used for piping materials. Austenitic stainless steels, by definition, are of face-centered cubic lattice. This allows them to have high ductility and tensile strength making them ideal candidates for tubing and piping fabrication. Austenitic steels consist of the American Iron and Steel Institute (AISI) 300 series stainless steels, most commonly 304, 304L, 316, and 316L. These stainless steels contain nickel, iron, chromium, molybdenum, manganese, silicon, and less than 1% carbon, phosphorus, and sulfur depending on the particular alloy. These particular stainless steels are designed to have increased corrosion resistance due to their alloy composition (43). The high chromium content (as high as 20%) reduces corrosion by forming a passive film of chromium oxide across the surface (58). Low carbon content reduces intergranular corrosion caused by the migration of carbides to the grain boundaries. Molybdenum enhances the corrosion resistance to pitting and crevice corrosion by allowing formation of a more uniform passive layer (47). Interestingly, Scott, *et al*, report that there may be some type of maximum concentration of molybdenum that enhances corrosion resistance and

that 316L may contain the optimal concentration for resistance to microbially influenced corrosion (MIC) (61). Because molybdenum is a potent ferritizer, nickel must be present in fairly high concentrations (10%) to prevent delta ferrite formation, which may be structurally detrimental to the stainless steel, as will be discussed later. A passive chromium oxide layer forms on the surface of these alloys under aerobic conditions. This passive layer forms an electronic insulating barrier decreasing the electric field strength and cationic transport into solution that occurs during the corrosion process (22). If this oxide layer is breached, either biotically by the activity of microorganisms or abiotically by welding and heating, corrosion can occur at an accelerated rate (69).

Stainless Steel Microstructure

The fabrication of stainless steel causes it to be metallurgically separated into areas of grains and grain boundaries. During the annealing process, cations nucleate crystal growth. As adjacent crystals (grains) converge, boundaries are formed between them, termed grain boundaries. These grain boundaries tend to be higher in carbon and other impurities due to carbide precipitation (6,8,39) which causes localized breakdown in the oxide film (1,69) and have a thinner chromium oxide surface layer as well as a lower general amount of chromium (up to 40% less) than the adjacent grains in instances where extensive carbide precipitation occurs (7). The metallurgy of the grains and grain boundaries in stainless steel has been extensively studied using a multitude of surface analytical instruments (ie. scanning Auger spectroscopy, energy dispersive x-ray spectroscopy, x-ray

photoelectron spectroscopy, scanning tunneling microscopy, transmission and scanning electron microscopy, etc.) (10,23,36,44,47,49,50,58). Most of the current research has analyzed metallurgical stresses that stainless steel undergoes in certain situations, such as high temperatures and pressures, and how grain boundary composition and structure are affected. . Many studies have been published using the aforementioned surface techniques to investigate corrosion of stainless steel. McIntyre (1983) reviewed the most frequently used instruments, Auger electron and X-ray photoelectron spectroscopies, to study corrosion. Quantification still seems to be a challenge for these analytical techniques (47).

Surface Analysis of Corrosion

More recently, new applications have been reported using other types of surface analytical techniques. Fan and Bard (1989) (23) investigated corrosion of 304L stainless steel in aqueous chloride media using scanning tunneling microscopy, however results reported were more of a qualitative than quantitative nature. The majority of the past studies have focussed on abiotic corrosion, however some scientists have used these surface analytical instruments to investigate MIC. Chen, *et al* (1988) (12) used scanning Auger electron spectroscopy to investigate biological corrosion of copper, titanium, aluminum brass, and stainless steel suspended in the open ocean for 56 and 92 days. Unfortunately, no chemical characterization of the stainless steel surface was reported, other than to note that the surface "appeared" visually corroded. Because of the difficulties of combining microbiological and surface chemical

analysis, not much research has been done in this area (Gustavo Cragnolino, Jeff Kearnes, personal communication).

MIC of stainless steel has been studied on a larger scale using electrochemical techniques. Mansfield, *et al*, (1992) (46) studied 304 and 316 stainless steels exposed to flowing seawater. After 124 days they reported no corrosion either visually, microscopically, or electrochemically, measured by open circuit potential. Studies are ongoing in this area, but laboratory study of microbially influenced corrosion has proved challenging and difficult to reproduce under defined conditions therefore warranting further investigation.

Microbially Influenced Corrosion

The corrosion process from an abiotic standpoint has been well characterized but, in many ways, the processes of MIC remain elusive and somewhat speculative (27,33,77). A growing appreciation of the significance of MIC in corrosion processes has led to a resurgence in investigation. Recent studies have primarily focussed on mild steel (17,18,32,34,48) due to its extensive use in industrial water systems. Stainless steels were generally thought to be corrosion "resistant" and thus were the material of choice in applications where corrosion resistant materials were needed (33). These situations include power generating industries, chemical plants, paper mills, waste water systems, etc (31,79). Localized corrosion of stainless steel piping, usually in the form of pitting, has been documented, especially in areas of stagnant or low to intermittent flow (31,37,42,68,78,79). It has been particularly troublesome in the nuclear power

industry where service water systems may be filled with untreated water and left stagnant for months prior to placing into service, resulting in rapid extensive corrosion and subsequent system failure (78). Some temporary remedies included welding new stainless steel sleeves over corroding stainless steel pipes, however this is usually temporary, with system failure occurring as soon as 2 years (68). Once MIC occurs, it is difficult to halt and until a better understanding of the mechanisms and means of initiation are known, the problem will continue.

Mechanisms of MIC

Cathodic Depolarization

MIC is a phenomenon that was hypothesized by von Wolzogen Kühr and van der Vlugt in 1934 (74). They speculated that anaerobic corrosion of buried iron pipes was caused by the presence of a sulfate reducing bacterium, *Desulfovibrio desulfuricans*. Based on the research of Stephenson and Strickland (66), who had shown that this bacterium was able to use molecular hydrogen for the reduction of sulfate to hydrogen sulfide, von Wolzogen Kühr and van der Vlugt proposed the theory of cathodic depolarization, being applicable to all materials containing iron. In this theory, the iron surface is negatively polarized in water by losing metal cations (anodic reaction). The electrons left on the surface reduce protons from the water to atomic hydrogen (cathodic reaction). The bacterial hydrogenase scavenges the atomic hydrogen to reduce sulfate, thus acting as a depolarizing agent (cathodic depolarization reaction). This action eliminates the build up of hydrogen on the surface, which is thought to polarize the cathodic

reaction, although hydrogen accumulation may cause other problems such as stress corrosion cracking (26). From the inception of cathodic depolarization, researchers have studied MIC and appended the theory as more became known (32,57). A number of reviews (14,26,27,33,60,73) have been written on the subject as the theory is updated, but much remains unknown and speculative. Consequently, other localized corrosion mechanisms have also been proposed.

Biofilms and MIC

The idea of biofilm formation on a surface and its association with MIC is a relatively new idea when compared to the previously discussed cathodic depolarization theory. Nonetheless, this area of study has spawned a number of new theories about MIC (73). On stainless steel, breaching of the passive layer, generally considered to be chromium oxide is one means by which biofilms can contribute to MIC (27). As previously mentioned, the chromium oxide layer is one of the major characteristics of stainless steel that enhances corrosion resistance (43). Biofilm detachment, which may occur more frequently under differential flow conditions (73), may expose a breached passive layer, whereby the protective chromium oxide layer is no longer intact. This breaching may be caused by the formation of crevices, differential aeration zones, or a more aggressive environment beneath the biofilm (31). Once this passive layer is breached, corrosion can occur at an accelerated rate if no repassivation occurs as might be the case under anaerobic conditions.

With the chromium oxide layer being more unstable at the grain boundaries,

a corrosive biofilm may be able to breach the passive oxide layer in this area more rapidly than at the grains. Once the oxide layer is breached, bacteria can then assist in dissolution of the metal (31). Bacteria may contribute to certain anodic effects by production of organic acids such as short chain fatty acids, thereby increasing the proton concentration (17,27,33). The production of sulfides such as FeS and H_2S by SRB may have an anodic or cathodic effect depending on the specific conditions of that particular surface area. SRB are generally confidered to be the major culprits in MIC of stainless steel. It has been reported that SRB are responsible for over 77% of corrosion that occurred in a specific oilfield (78). Others have speculated that annual monetary loss due to MIC caused by SRB is in the billions of dollars per year (27,33). Consequently, most laboratory studies on MIC have used SRB to mimic corrosive field conditions.

Patchy biofilms containing aerobic organisms may also accelerate corrosion by creating concentration cells along the substratum. Exopolymeric substances (EPS) produced by many attached bacteria may have this effect (4,53). The EPS can contain certain acids (ie uronic acids) that may have a corrosive nature. Costerton and Geesey (14) have proposed the formation of a corrosion cell where there are localized anodic and cathodic sites due to microbial activity on the surface. This phenomenon may be due to aerobic bacteria producing EPS, but it may also be due to SRB as well as iron-oxidizing bacteria. If the SRB are metabolically active, they will be producing sulfides which have been shown to be very corrosive (33). Iron-oxidizing bacteria such as *Gallionella* have been

implicated in corrosion of stainless steel (42). These bacteria have the ability to produce sulfuric acid, which is very corrosive to stainless steel (33).

Localized Pitting Corrosion

The most common type of MIC to occur on stainless steel is that of localized pitting corrosion associated with biofilms (22,31,33,61). Pits generally begin as small "pinholes" that can expand into large cavities within the bulk metal. Pit formation is thought to be caused by a hydrolysis reaction which both concentrates chlorides and acidifies the immediate environment within the pit (22). Microbial activity, particularly of SRB, is thought to propagate the phenomenon (27). The formation of oxygen concentration cells caused by the presence of biofilms, as aforementioned, can also cause pit enhancement (22). Microbial activity can also be responsible for initial pit formation by causing the adsorption of aggressive ionic species, as discussed below.

Chloride Effects and Stress Corrosion Cracking

Once a biofilm has been established on a stainless steel substratum, it has the ability to trap and entrain certain chemical species that may be particularly aggressive toward stainless steel (73). Chloride is one such chemical that has been shown to be of particular importance (43,60). Chloride concentrations have been found to be surprisingly high within an established tubercle or corrosion deposit, even when the chloride concentration in the surrounding medium is quite low (5,68). Certain species of iron-oxidizing bacteria, ie *Gallionella*, have the ability

to concentrate chlorides, usually complexed as ferric and manganic chlorides within the tubercle (70). Chloride ions have the ability to permeate the passive film thus initiating or hastening the electrochemical reactions that are responsible for corrosion. The effect can be two-fold. Chloride can cause small pits to form beneath the tubercle that can penetrate stainless steel piping walls in as short a time as a few weeks (37). In addition, chloride initiates the phenomenon known as stress corrosion cracking that affects all alloys and has been well characterized in stainless steel (43). Stress corrosion cracking begins at the anodic site and causes metal dissolution to occur at the crack front. A brittle fracture results which is transgranular in austenitic stainless steels. Once this fracture has occurred, it will enlarge to the point of material failure.

Hydrogen Embrittlement

Hydrogen embrittlement is another means by which MIC can cause material failure of stainless steel (25). Hydrogen embrittlement involves atomic hydrogen entering the stainless steel lattice causing loss of ductility, tensile strength, and crack propagation. Any bacterium that produces an acid byproduct may be a problem, as well as SRB which produce sulfides that prevent transformation of atomic hydrogen to molecular hydrogen. Some SRB can consume hydrogen, thus cathodically depolarizing the stainless steel. The overall outcome of hydrogen embrittlement may be determined by the competition between the metal, hydrogen producing bacteria, and hydrogen consuming bacteria. Since the metal surface characteristics will determine the number of adsorption sites for the atomic

hydrogen, biofilm composition remains the controlling variable for hydrogen adsorption.

The microstructure of the stainless steel surface, primarily the grain boundaries, could be ideal regions for hydrogen embrittlement. As was aforementioned, these areas are inherent "weak spots" on the stainless steel surface. If there was preferential bacterial attachment to these areas, hydrogen embrittlement could occur.

MIC and Weldments

For reasons not entirely understood, MIC is more frequently reported at or near a weld than any other area on a stainless steel surface (5,31,44). Welding of stainless steel leads to a biphasic condition, where some of the austenite (face-centered cubic) is converted to delta ferrite (body-centered cubic). The delta ferrite phase is generally more susceptible to corrosion than the austenitic phase, however, the austenitic matrix may be preferentially attacked under oxidizing acidic conditions (35). Reducing, acidic conditions tend to favor attack at the delta ferrite regions (31). The biphasic condition seems to be more susceptible to MIC. This condition is known to occur in heat affected zones (HAZ) and has been shown to be susceptible to MIC (31,42). This may be due to the fact that the HAZ is sensitized during welding, causing chromium carbide precipitation at the austenite-ferrite interphase. As a result, chromium depleted zones are created (7) which will be subject to MIC (31). The weld metal is also more susceptible to MIC in comparison to the base metal (5,31). Although a variety of different weld metals

have been tried, so far all have shown susceptibility to MIC. Little investigation has been done into why this may occur (5).

Bacterial Coexistence and MIC

MIC of metals is frequently reported to occur to greater extent in aerobic systems than anaerobic systems (33). As aforementioned, SRB are considered to be the most problematic in association with MIC and are commonly isolated from corrosive biofilms (27,47). Because SRB require anoxic conditions for growth, many reviews on MIC have speculated that biofilms provide these conditions even in systems where the water contains oxygen (14,27,73). Hamilton (1985) reports highest sulfate reduction rates occur near the aerobic/anaerobic interface (27). This implies that the occurrence of SRB in aerobic environments may not be improbable, provided there are small anaerobic microzones for sulfate reduction to occur. Microorganisms undergoing aerobic metabolic processes could create such zones (27,73). Other metabolic "cross-feeding" may also be involved. For SRB to be growing in these "microenvironments", an intricate symbiosis has evolved. In an attempt to study these phenomena, researchers have tried to recreate conditions that may initiate MIC.

Undefined Consortia to Study MIC

One approach to studying MIC has been to use undefined consortia to gain information about how these events may occur. Scotto, *et al.*, (1985) investigated the corrosive behavior of an undefined marine aerobic biofilm on 304 and 316

stainless steel (63). Although their results were qualitative, they did report the "microbiota" had corroded the stainless steel, evidenced by crevice corrosion on loops fed with natural seawater. They speculated that this was due to the breakdown of the passive film followed by nucleation of localized attack, although they provided no support for these findings. Zambon, *et al*, (1984) investigated marine fouling films on mild steel. They used specific polyclonal antibodies to identify various microbial species they suspected were growing in the films produced from an undefined sea water inoculum. Unfortunately, because an undefined seawater inoculum was used to grow the films, it was impossible to prove that there was no cross reactivity of the antibodies. A multitude of studies have been carried out using undefined consortia (27,73), but traditionally, results are virtually impossible to reproduce. It is for this reason that others have attempted to reproduce corrosion using defined systems.

Defined Consortia to Study MIC

Many researchers have attempted to study MIC by using organisms reported to cause MIC, primarily SRB, in an attempt to mimic corrosive biofilms. Jack (1990) constructed a three member bacterial consortium consisting of an obligate aerobe, a facultative anaerobe, and an SRB (34). The corrosion rate of mild steel, measured by open circuit potential, was reported to be highest in the presence of the SRB. Dowling, *et al.,* (1992) created a consortium of a *Eubacterium* sp. and two sulfate reducing bacteria on a carbon steel surface. They used scanning electron microscopy coupled with electrochemical impedance

spectroscopy to measure the corrosion rate. They speculated that both the fermentative and sulfate reducing bacteria caused an increase in the corrosion rate. Beech, *et al.*, (1991) measured the amount of exopolysaccharide (EPS) produced by *Desulfovibrio desulfuricans* and *Pseudomonas fluorescens* in the presence of mild and stainless steel (British Standard 02134) (4). They reported that the amount of biofilm present on the stainless steel was much less than that on the mild steel and suggested that some EPS may be aggressive to metal dissolution due to high acid concentration at the surface. Dowling, *et al.*, (1988) used a mixed culture of *Vibrio natriegens*, *Desulfovibrio* sp., and *Desulfobacter* sp. to investigate corrosion of mild steel (18). Measuring corrosion via electrochemical impedance, they reported highest corrosion rates when both SRB and *Vibrio natriegens* were present. Other researchers have also shown defined mixed cultures containing SRB to be corrosive to stainless steel. Deshmukh, *et al*, (1992) showed that cultures of *Comamonas testosteronii* and *Desulfovibrio vulgaris* caused more extensive localized pitting corrosion, as measured by pit size and number, on 304 stainless steel than either culture alone (16). Research and field observation have given some clues as to what types of bacteria may cause or initiate MIC. Certainly in natural environments, more than one or two organisms work together to carry out this process. As a result, other types of bacteria have been implicated as well.

Other Potentially Corrosive Bacteria

Numerous researchers have proposed other bacteria that may also be responsible for MIC of various iron and iron-based alloys, including stainless steel. In 1949, Olsen and Szybalski (55) reported metal oxidizing bacteria that could accelerate the corrosion of cast iron. Iron oxidizing bacteria, i.e. *Gallionella sp.* have been reported to cause corrosion in a variety of industrial systems (19,27,33,42,73). Iron oxidizing bacteria produce sulfuric acid, which is very corrosive to most metals (42). Others have shown additional types of bacteria may also be responsible. Nivens, *et al.*, (1986) reported a 13 fold increase in corrosion current density on 304 stainless steel using the marine bacterium *Vibrio natriegens* grown in seawater (53). Obuekwe, *et al.*, (1986) concluded that energy was a limiting factor in the corrosion of mild steel by a *Pseudomonas* sp. (54). Others have speculated that bacteria capable of growing facultatively could produce organic acids that could be corrosive in a localized manner (27,73). Manganese-oxidizing bacteria have also been reported in causing MIC of 304 stainless steel in a service water system at a nuclear power facility (68). A multitude of metabolically different organisms have been implicated in causing or initiating MIC. For bacteria to cause MIC, they must first be attached to the substratum. As prevention of MIC is easier than control of these corrosion processes, other researchers have investigated bacterial colonization in hopes of discouraging attachment.

Bacterial Colonization of Stainless Steel

Zobell (81) was one of the first to propose bacterial attachment to surfaces when describing attachment of marine bacteria to sampling bottle walls. He speculated that bacteria were attached due to nutrient availability at the interface. Since that time, a multitude of studies have been undertaken to determine why bacteria attach to surfaces and how attachment may be discouraged in order to prevent bacterial fouling and corrosion of surfaces (21). It is widely accepted that once a bacterium attaches, if conditions are conducive to growth, a biofilm can develop over time. It is also accepted that bacteria can attach to virtually any surface, however attachment to some surfaces may occur much more readily than to others.

Relationships between surface free energies, surface charge, and bacterial attachment have been described to further understanding of the attachment process (2,9,21,24,71,72). Due to its ease in investigation, most work has been done on plastic or glass, however some information is available on stainless steel (21). One of the more defined studies was reported by Vanhaeke, *et al*, (1990) who investigated attachment of *Pseudomonas aeruginosa* to 304 and 316L stainless steel electropolished and 120 grit mechanically polished surfaces in static systems (71). They reported that bacterial cell surface charge (bacterial cell surface hydrophobicity measured by the bacterial adherence to hydrocarbons test and the contact angle measurement test) may be of minor importance in adhesion to stainless steel as bacterial attachment was very similar and independent of the

stainless steel type or surface. Mueller, *et al.* (1992) described initial attachment of *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* to hand polished 316 stainless steel, copper, silicon, and glass in a laminar flow system (51). They found that the adsorption rate was positively correlated with surface free energy, surface roughness of the substratum, and the hydrophobicity of the cells.

General "attachment" of specific species of bacteria to stainless steel surfaces has been investigated. Traditionally, little relevance has been given to either the type or surface treatment of stainless steel. Some work has been done for the milk industry using pure cultures of bacteria (28,38,45,64,67,82). Although details were given to the type and culturing of the bacteria used in these studies, little effort was made to define surface features of the substratum before, during, or after bacterial colonization. Bacterial attachment to stainless steel in drinking water distribution systems has also been investigated. Pedersen (1990) (56) examined biofilm development of an undefined mixed culture on a bright annealed or electropolished stainless steel compared to polyvinyl chloride (PVC) pipe in an attempt to compare surface roughness and wettability properties. Although the surface finish of the stainless steel was reported, no mention was made as to the type of stainless steel that was used.

The effects of environmental conditions on bacterial attachment to stainless steel have also been investigated. Stanley (1983) investigated irreversible attachment of *Pseudomonas aeruginosa* to 304 stainless steel in static conditions and reported that growth phase, pH, and motility may influence attachment (64).

Duddridge, *et al*, (1982) examined the effects of surface shear stress on attachment of *Pseudomonas fluorescens* to 316 stainless steel and reported that high shear stress may not prevent microbial attachment (20). Some research has been conducted on bacterial colonization of stainless steel, however surface preparation and characterization are often neglected and no information is currently available on bacterial attachment to stainless steel in relation to stainless steel microstructure.

Significance of Initial Colonization Events

The study of initial colonization events may be integral to understanding MIC of stainless steel. The stainless steel microstructure and initial colonization events by bacteria may be intimately responsible for MIC (44). Primary colonizing bacteria attach at grain boundaries and become metabolically active to form microcolonies. An ideal primary colonizing bacterium would be an organism that can grow well under low nutrient environments and is very "sticky" (52). Many aerobic bacteria and some facultative bacteria, which could be corrosive under the proper conditions and have been shown to readily attach to stainless steel surfaces in low nutrient environments, could be envisioned to fit this scenario (51,52,65). If environmental conditions are conducive to growth, these bacteria will begin to multiply, forming small microcolonies on the surface. Within these microcolonies, microniches form that may be conducive to growth of other types of bacteria, ie sulfate reducing bacteria (SRB). One can envision this consortium becoming a very complex and dynamic ecological setting with small microenvironments

throughout (27). Depending on the type of organisms and the environment, this biofilm could be corrosive to the stainless steel surface, as aforementioned.

Summary

For MIC to occur on stainless steel, bacteria must first attach to the surface. Stainless steel microstructure contributes to bacterial spatial distribution during early biofilm formation as will be presented in this study. Some of these microstructure regions, such as grain boundaries, may be inherent weaknesses in the corrosion barrier of the stainless steel. If bacteria attach to these potentially sensitive areas localized pitting corrosion may occur. As was previously mentioned, abiotic corrosion of stainless steel has been extensively studied, although biotic corrosion of stainless steel in a localized manner has not. In order to more thoroughly understand this problem, further characterization of bacterial attachment to stainless steel and correlation to surface chemistry is needed. Traditionally, little credence has been given to either the surface, surface preparation, or types of bacteria used. The objectives of this study are to use a specific surface, a specific surface preparation, and defined types of bacteria to more clearly define the role of microbes in the corrosion of stainless steel. This study investigates this unique endeavor using a novel combination of surface analytical (Auger electron spectroscopy, energy dispersive X-ray spectroscopy, atomic force microscopy), microscopic (scanning confocal laser microscopy), and computerized image processing techniques to address this problem.

Hypothesis

The hypothesis of this study was that bacterial attachment to stainless steel occurs in a non-random manner and that stainless steel microstructure (grain boundaries) contributes to this process.

STATEMENT OF RESEARCH PROBLEM

The purpose of this research was to conclusively prove whether or not bacteria could selectively attach to grain boundaries on the surface of 316L stainless steel. The second objective was to prove whether or not bacteria could change the surface chemistry of the stainless steel in such a manner that could prove detrimental to material life.

METHODS AND MATERIALS

Chemicals

The following were obtained from Sigma Chemical Co., St. Louis, MO: acridine orange; 4,6 diamidino-2-phenylindole (DAPI); sodium lactate; sodium succinate; sodium sulfate.

The following chemicals were obtained from J. T. Baker Chemical Co., Phillipsburg, NJ: ammonium nitrate; dibasic potassium phosphate; monobasic potassium phosphate.

MacConkey agar and yeast extract were obtained from Difco Laboratories, Detroit, MI.

Ferric chloride and L(+) ascorbic acid were obtained from Matheson, Coleman, and Bell, Norwood, OH. Sodium sulfite was obtained from General Chemical Company, New York, NY.

Resazurin was obtained from Manufacturing Chemists, Norwood, OH.

Equipment and Supplies

The following supplies were obtained from Fisher Laboratory Supplies, Santa Clara, CA: sterile cell scrapers; multi-well cover slides; frosted cover slides; cover slips.

The following supplies were obtained from Gelman Sciences, Ann Arbor, MI:

0.2 μm pore size Acrodiscs.

The polycarbonate 0.22 μm filters came from Poretics Corporation, Livermore, CA. The 316L 2B finish stainless steel was obtained from Metal Goods Services, Seattle, WA.

The following supplies were obtained from Cole-Parmer, Niles, IL: silicone tubing. The tubing (Manosil silicone tubing 3/32" ID, 1/32" wall) clamped into the pump cassettes was obtained from Manostat Corp., New York, NY.

The following items of equipment were used in this study: Peristaltic Pump (Manostat model 72-500-000, Manostat Corp., New York, NY); Four peristaltic pump cassettes (Manostat model 72-550-000, Manostat Corp., New York, NY); Ten liter polypropylene carboy (Nalgene, location); Anaerobic chamber (model #12356, Coy Laboratory Products, Ann Arbor, MI); scanning electron microscope (JEOL model JSM 6100, Peabody, MA) equipped with an energy dispersive X-ray spectrometer and software (Noran Instruments, Middleton, WI); scanning Auger electron spectrometer (Perkin-Elmer, Physical Electronics Division, Phi Model 595, Eden Prairie, MN) equipped with a scanning Auger microprobe (Physical Electronics Instruments, Inc., Eden Prairie, MN); membrane filtration towers (Millipore Corp., Cambridge, MA), Gast Vacuum pump (Model#DOA-P104-AA, Gast Manufacturing Corp., Benton Harbor, MI).

The following items of equipment were used for the microscopy: Olympus BH2 microscope (Olympus Optical Co., Ltd., Japan) with an Olympus BH2-RFL-T2 mercury lamp as the light source. An Olympus model UG1 filter (excitation 340

nm, barrier filter 420 nm) was used for viewing DAPI stained preparations, an Olympus model BP490 filter (excitation 490nm, barrier 515nm) was used for viewing acridine orange stained preparations, and an Olympus model BP545 filter (excitation 545 nm, barrier 590 nm) was used for viewing TRITC stained preparations. An Olympus D Plan Apo UV series 160/1.30 oil immersion objective was used for epifluorescent microscopy. Photomicrographs were taken using Kodak Ektar 1000 film (Eastman Kodak Co., Rochester, N.Y.) using a Olympus camera (Model C-35AD-2).

Reactor Design

The reactor system was designed to allow multiple parallel tubing to originate from a common medium container (See Figure 1). Each line of tubing was connected to an experimental chamber containing a stainless steel coupon. Each of three chambers was inoculated with a different matrix of bacteria while the fourth chamber was maintained as a sterile control as will be described. Each chamber contained one 316L 20 gauge 2B finish nitric acid passivated stainless steel coupon (20 cm x 2 cm). The flow rate of medium through the chamber was approximately 20 ml/hour. The flow rate was set to mimic low flow conditions where corrosion problems on stainless steel are generally experienced in industrial systems (Susan Borenstein, personal communication). The volume of the chamber containing the stainless steel coupon was approximately 60 ml, consequently the residence time was approximately 3 hours.

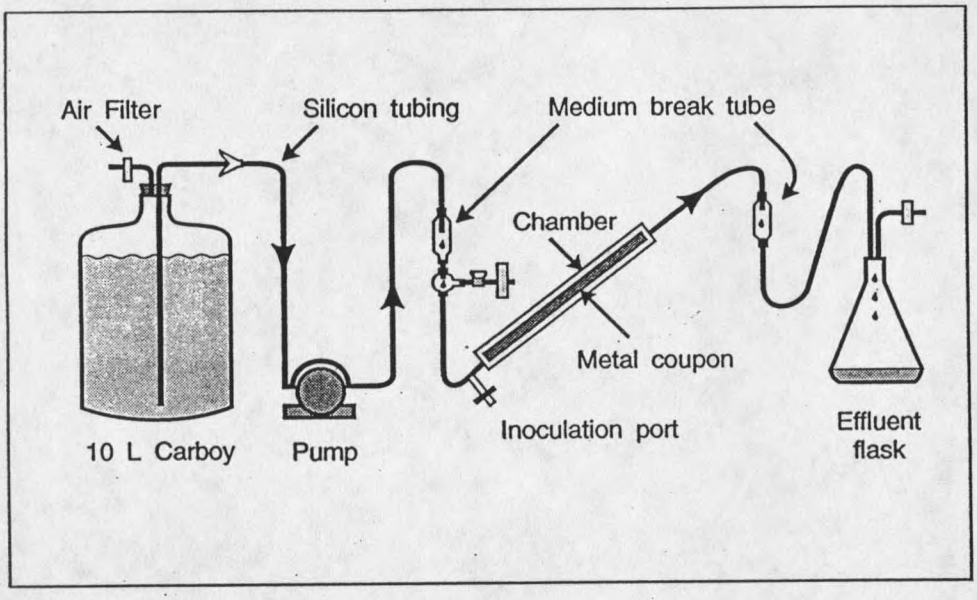

Figure 1. Diagram of reactor system used for biofilm studies.

Coupon Selection and Surface Preparation

A stainless steel material (316L) was selected because of its relevance to the nuclear power industry. This stainless steel is frequently used in the nuclear power industry due to its corrosion resistant properties (Jeff Kearnes, personal communication). The coupon surface was bright annealed classified as 2B finish by AISI. The 2B surface was passivated as is done in most industrial piping

systems using a standard industrial procedure according to the American Society for Testing and Materials procedure #A-380-78 (see #1 in Appendix). This procedure is performed to remove slag and grit which may accumulate on the surface during manufacture and piping installation.

The 316L stainless steel coupons used for the antibody experiments were hand polished using a 0.3 μm grit alumina polish (Buehler, Lake Bluff, Il) for 5 minutes, followed by a 0.05 μm grit alumina polish (Buehler, Lake Bluff, Il) for 5 minutes.

Assembly of the Reactor System

A ten liter polypropylene carboy sealed with a #16 rubber stopper was used to hold the sterile culture medium. Four borosilicate glass tubes were used to draw the medium from the carboy. A stoppered test tube embedded within the rubber stopper was used to aseptically transfer concentrated media into the carboy. All tubing used for the reactor system was either borosilicate glass or silicone. Two medium break tubes were placed between the peristaltic pump and the silicone tube chamber containing the stainless steel coupon to minimize back growth and contamination of the carboy. Another media break tube was located downstream from the chamber to minimize potential contamination from the effluent flask. The entire reactor system was assembled, connected to the carboy containing 9 liters reverse-osmosis (RO)-treated water, leak-tested, the carboy refilled with 9 liters RO water, then steam sterilized for 4 hours.

Bacteria

The facultative anaerobic bacterium, *Citrobacter freundii* a sulfate reducing bacterium, *Desulfovibrio gigas*, and an aerobic bacterium, *Pseudomonas aeruginosa* were used in the studies. *P. aeruginosa* (strain 8830), which was only used in the antibody studies, was obtained from Ananda Chakrabarty, University of Illinois at Chicago. Strain 8830 was selected due to its ability to produce alginate. The alginate matrix has been shown to impede oxygen transfer. *C. freundii* was obtained from M.W. Mittelman, University of Tennessee at Knoxville and was an environmental isolate from an active tubercle from a Tennessee Valley Authority pipeline that was identified by an Analytical Profile Index (API) 20E assay (Analytical Products Division, Sherwood Medical, Plainview, NY). *C. freundii* was a good candidate to study MIC on metal surfaces as it belongs to the Enterobacteriaceae family (Bergey's Manual of Systematic Bacteriology). Some members of this family have been implicated in MIC processes (25) and have been proposed to coexist with sulfate reducing bacteria (27,73,77). *D. gigas* (ATCC #19364) was obtained from the American *Type Culture* Collection (ATCC) Rockville, MD. It was used because of its unusually large size, and characteristic morphology allowing identification based on these features within the thin films. Sulfate reducing bacteria have been reported to cause or initiate MIC on stainless steel (42).

Suspended Cell Growth Studies

A growth curve was performed using *D. gigas* in the presence or absence of *C. freundii*. Replicate stopper-sealed flasks were filled with 10 strength EPRI medium as described below, amended with resazurin (0.1 mg/ml) and ascorbic acid (1 g/l) as a reducing agent, flushed with nitrogen, then autoclaved for 15 minutes. Modified Hutner salts and iron chloride were added after autoclaving. An inoculum of approximately $1x10^5$ cells of *D. gigas*, verified via AODC, from a culture grown anaerobically for 14 days at 22°C was used. An inoculum of approximately $1x10^8$ cells of *C. freundii* was used from a 24 hour batch culture of *C. freundii* grown in EPRI medium at 22°C. Samples of suspended cells were enumerated daily for 27 days via AODC on membrane filters as will be described. *C. freundii* was enumerated at 1000X magnification, while *D. gigas* was enumerated at 400X and differentiated from *C. freundii* on the basis of morphology (Figure 2).