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Early detection of oxidized surfaces using *Shewanella oneidensis* MR-1 as a tool

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Corrosion is a natural global problem of immense importance. Oxidation of iron and steel not only compromises the structural stability of a widely used and versatile material but it also creates an abrasive compound (iron oxide) that can score the surfaces of metals, rendering them useless for the purpose for which they were designed. Clearly, the identification of corrosion in its nascent stages is a high priority for reasons that range from aesthetics to economics. Many bacteria in the facultatively aerobic genus *Shewanella* have the capacity to respire some metal oxides, such as iron oxide, by way of a variety of oxide-binding proteins lodged in their outer membrane. In this study, a rapid, cost-effective system for the specific early detection of a variety of oxidized steel surfaces is described, taking advantage of bacteria with natural affinities for iron oxides, to identify the sites of nascent corrosion.

Keywords: oxidation; corrosion; *Shewanella*; biofilm; metal reducing; iron oxide

Introduction

Steel is one of the most common materials used in manufacturing (Gold et al. 1970). Its ability to be fashioned, hardened or machined to meet a very wide range of applications makes it an invaluable asset for structural and mechanical manufacturing of everything from precision instruments to vehicles to buildings. For steel to have such versatility, iron (the major component of all steels) is either specially treated or mixed with other components (eg chromium, manganese or molybdenum), leading to products that are both durable and structurally precise, two of the most industrially important qualities of steel (Beddoes and Parr 1999). One of the greatest foes of steel is oxidation (Bozack 1991; Koch et al. 2002; Farmer et al. 2003). As iron in steel is exposed to air, regardless of its stainless properties, it becomes increasingly susceptible to corrosion by oxidation (Asteman et al. 2000). Iron oxide particles that form on a steel surface are easily flaked off and can be harder than the steel itself, not only providing direct obstacles that decrease the ability of a machine to function but also creating an abrasive that can score the surface and indirectly impact optimum function (Lide 1997; Martin 1999). In addition, oxidative corrosion can convert structurally important material into structurally compromised forms such as iron oxides (rust), creating pitting, and inducing the formation of microcracks, all of which can significantly and permanently impact the material strength of the steel (Matlock and Grong 1986; Shakleford 2005a,b).

Corrosion is one of the most expensive natural phenomena to impact society on a global basis, both economically and environmentally (Rao 2001; Koch et al. 2002). Clearly, it would be advantageous to have methods for the early detection and removal of rust, but the predominant means currently available involve visual inspection or technologies with low spatial resolution that can only detect significant oxidative corrosion (Kirmeyer and Logsdon 1983; Lockheed Missiles and Space Company 1996; Fuhr and Huston 1998; Breen et al. 2002).

Many bacteria in the genus *Shewanella*, in the absence of oxygen as an electron acceptor, can respire manganese and iron oxides (Myers and Nealson 1988; Nealson and Saffarini 1994; Lower et al. 2001; Heidelberg et al. 2002; Tiedje 2002). Because these are solid, insoluble compounds incapable of traversing the outer membrane, this respiration process has to happen outside of the cell. Consequently, proteins that interact with these oxides are located in the outer membrane. Given that only a few reports describing bacterial attachment to iron oxides have been published (Brown 1992; Ohmura et al. 1993; Caccavo Jr. 1995; Lower et al. 2001, 2007; Shaprio et al. 2004; Parikh and Chorover 2006; Roberts et al. 2006),

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a study was carried out to investigate the potential of iron oxide attaching bacteria to detect naturally formed rusted steel *in situ*. This communication reports the use of the dissimilatory iron-reducing bacterium, *Shewanella oneidensis* MR-1, known for its ability to interact with a variety of different iron oxides (Lower et al. 2001, 2007; Heidelberg et al. 2002; Shapiro et al. 2004), to rapidly and irreversibly attach to rusted surfaces and indicate early stages of oxidative activity on surfaces.

Materials and methods

Bacterial growth and visualization

S. oneidensis MR-1 was grown to stationary phase in 5 ml of nutrient-rich Difco Luria–Bertani broth (LB) (Bertani 1951) from frozen glycerol stocks at room temperature (25°C) overnight shaking at 120 rpm, then inoculated 1:100 into 1 ml to 500 ml LB and grown at room temperature in a horizontal rotator, rotating 60 rpm overnight to obtain stationary phase cultures.

Levels of dissolved oxygen in cultures were determined by a YSI 5300A Biological Oxygen Monitor, standardized with air saturated LB and boiled nitrogen bubbled LB. LB saturated with air was used to standardize the instrument and measured as 98.0% $\pm$ 2.8% dissolved in LB with a dissolved oxygen concentration of 20.4% $\pm$ 0.5%. Dissolved oxygen in anaerobically prepared LB measured 0.1% $\pm$ 0.1%. Dissolved oxygen levels in stationary phase cultures of *Shewanella* strains used for attachment studies measured 0.9% $\pm$ 0.6%. Such low values are maintained because *Shewanella* uses oxygen as a preferred electron acceptor until it drives media into oxygen-limited conditions, at which time nitrate and/or metal oxides become the next most preferred electron acceptors.

S. oneidensis MR-1 containing plasmid p519ngfp (Stretton et al. 1998), constitutively expressing green fluorescent protein (GFP) by way of the npt-2 promoter was grown in 50 μg ml^{-1} kanamycin antibiotic at all times. Cultures were then observed for attachment to oxidized or clean surface steel. Bacteria and surfaces were visualized by standard or epifluorescent compound microscopy (Zeiss) either by superficial white light illumination or blue light excitation (Zeiss filter set 9, excitation 450–490 nm, emission 520 nm) or a combination thereof.

Generation of oxidized steels

Before oxidizing, surfaces were cleaned with sandpaper where appropriate, to remove any apparent non-steel components, then cleaned with ethanol and autoclaved at 121°C, 15 psi (1 bar) for 20 min. A variety of steel surfaces were exposed to 1% NaCl and monitored for oxide development under a compound microscope, at 5-min intervals for a minimum of 4 h. An array of steels were used in oxidation experiments, including mild steel (wire and bars), spring steel and 301 stainless steel (SS), where the maximum time allowed for oxidation was up to $\sim$300 h. The process of corrosion under these conditions will be referred to from this point as natural oxidation, meaning that the only controlled component is the addition of the NaCl electrolyte. All other components key to oxidation, such as pH, hydration, temperature and oxygen concentration were not tightly controlled. Rust growth was monitored for each type of steel. Time-lapse videos were used to document rust growth over time for both mild and 301 SS. In every case, rust growth behavior and appearance was consistent. Non-oxidized states were verified by visual inspection for changes in structural formation or discoloration to indicate signs of oxidation over time under a microscope at up to 1000 $\times$ magnification. 304 SS was exposed to up to 5% NaCl solution for 300 h, with no signs of oxidation.

There are many other components used in varying concentrations in steels to alter their mechanical properties and reactivity to the environment (Table 1; Beddoes and Parr 1999). Although Cr and Ni ions are added to create a protective barrier to iron oxidation in the steel, all stainless steels are still subject to some oxidative corrosion resulting in the generation of iron oxides. In addition, for each steel type, a variety of iron oxide compounds can be formed under various conditions as documented in many independent studies (Tomaszewicz and Wallwork 1983; Vertes et al. 1990;

Table 1. Elemental composition (%), other than Fe.

Steel type	Elements								
	C	P	S	Mn	Si	Cr	Ni	Cu	Mo
ASTM A510 mild steel wires	0.08	0.04	0.05	0.40	–	–	–	–	–
ASTM A36 mild steel bars	0.29	0.04	0.05	1.03	0.28	–	–	0.20	–
301 stainless steel bolts	0.15	0.045	0.03	2.00	1.00	16.00	6.00	–	–
304 stainless steel bars	0.08	0.045	0.03	2.00	1.00	18.00	8.00	0.3	2.0

Bozack 1991; Asteman et al. 2000). Magnetite, an iron (II/III) oxide powder, was purchased from Sigma–Aldrich.

X-ray diffraction (XRD) analyses of the oxidized steel surfaces generated in this study indicated that the major form of corrosion byproduct formed was lepidocrocite (γFeO(OH)). Below is the stoichiometry for how lepidocrocite is formed, where green rust, an iron (II/III) oxide, is formed as an intermediate.

$$6Fe^{2+}NaCl + 12H_2O + 1.5O_2 \rightarrow [(4Fe^{2+})(2Fe^{3+})](OH)_{12}Cl^{-}2H_2O + Na^{+} + 8H^{+} + \tfrac{1}{2}O_2 \quad (1)$$

$$[(4Fe^{2+})(2Fe^{3+})](OH)_{12}Cl^{-}2H_2O + Na^{+} + 8H^{+} + \tfrac{1}{2}O_2 \rightarrow \gamma\text{-}6FeO(OH) + 3H_2O + 12H^{+} + NaCl \quad (2)$$

Preparation of hydrous ferric oxide

Poorly crystalline hydrous ferric oxide (HFO) was prepared as described by Schwertmann and Cornell (Childs 1992; Cornell and Schwertmann 1996). Briefly, a solution of $FeCl_2.6H_2O$ was neutralized with NaOH to a pH of 7. This solution was repeatedly washed with deionized water to remove the chloride and sodium ions. The product yielded XRD patterns consistent with two-line ferrihydrite. The HFO solution was stored at 4°C until use.

Attachment assays

Oxidized and non-oxidized surfaces were exposed to bacterial cells from anaerobic stationary phase cultures for times ranging from ~1 s (immersed in culture and removed) to 3 days. Bacteria that remained after a thorough rinsing under a strong stream (~50 psi) of sterile (autoclaved at 121°C, 15 psi (1 bar) for 20 min), MilliQ filtered 18Ω water were deemed to be irreversibly attached. All surfaces were then assessed for co-localization of oxidized steel and bacteria by microscopic examination under white light and blue light (450 nm), respectively. The presence of bacteria was determined by visual detection of light-induced fluorescence of intracellularly expressed GFP.

Detailed analyses of signal-to-noise ratios indicated that the GFP signals from bacteria were 3 to 9 times as intense as the non-specific fluorescence of the bacteria-free background. Using this GFP signal, bacterial presence could be spatially localized across a surface at low (100×) magnification. Counting individual bacteria under higher (400× and 1000×) magnification validated bacterial presence ascertained via GFP fluorescence. Additionally, samples were assessed for autofluorescence with green light (546 nm), under which GFP does not fluoresce. Images of GFP signal, acquired under blue light, were filtered to eliminate background and autofluorescence, then overlayed with white-light images of rusted substrata to more clearly delineate the location of bacterial binding. The process of overlaying an image requires isolation of the relevant portion of the color spectrum, which slightly enhances the color and intensity of the bacterial cells. However, the results remain accurate.

Attachment was demonstrated across oxidized surfaces from 541 samples of mild steel wires and bars (from Anchor Wire and Industrial Metal Supply Co., respectively), 18 spring steel paper clips (from 3M Co.), four 301 SS bolts (from McMaster–Carr) and four 304 SS bars. Surface roughness was taken into account in these studies by measuring surfaces with VSI and monitoring rust growth with microscopic time-lapse photography. The roughest surfaces evaluated had a peak frequency 60.1 ± 1.6 peaks/100 μm^{-2} with an average height of 151.0 μm ± 69.5 μm. The smoothest surfaces evaluated had a peak frequency of 121.0 ± 5.7 peaks/100 μm^{-2} peaks and an average height of 5.7 μm ± 1.0 μm. The analyses did not reveal any correlation to bacterial binding and changes in iron oxide roughness. In Figure 5B, for instance, oxide roughness is similar in regions i and iii, however, bacterial binding is substantially different. In Figure 5C, the two oxide forms represented there have significantly different roughness, with similar bacterial binding abilities.

Results

Detection of naturally formed rust on mild steel by S. oneidensis *MR-1*

Previous studies have shown that some dissimilatory iron-reducing bacteria, including strain MR-1, are capable of binding to various forms of oxidized iron (Lower et al. 2001, 2007; Shapiro et al. 2004), and that this ability is enhanced by as much as 50 fold when the cells have been grown anaerobically. The anaerobic state of MR-1 was confirmed in all experiments by electrode measurement of oxygen content (Figure 1A, Materials and methods section).

As an initial investigation into the ability of *S. oneidensis* MR-1 to detect naturally formed iron oxides, the authors examined mild steel wire that had been rusted overnight and incubated in stationary phase bacterial cultures that constitutively express GFP for 2 h, and determined the length of time it took for MR-1 to colonize a surface as a monolayer (single layer of bacterial cells in direct contact with the surface). Monolayer biofilm formation was observed on both oxidized and fresh (non-oxidized) surfaces under both conditions, as expected, concurrent with the initial stage of biofilm formation. After a mild rinsing with

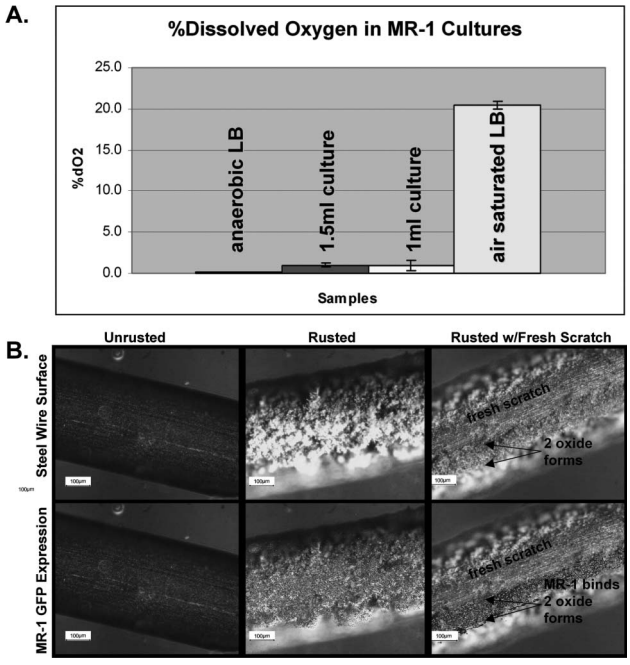

Figure 1. Specific detection of oxidized mild steel by GFP expressing MR-1. (A) Cultures were determined to be anaerobic and (B) did not attach to unrusted steel (left) or a freshly generated surface (right), but attached strongly to any location where rust was present, regardless of form. In the upper frames, the yellow areas are the oxidized product of steel in the images of the surface. The generation of a fresh scratch revealed both yellow iron oxide and a green iron oxide. In the lower frames, each green point is GFP expressing MR-1 from an overlayed fluorescent picture. Note in the lower panels of B that GFP fluorescence at the edges of the round wires cannot be seen in these images as those regions are too out of focus.

flowing 18 Ω MilliQ water, however, it was observed that the bacterial cells were readily removed from the fresh surfaces, but remained attached to and abundant on the oxidized surfaces (Figure 1B). In an attempt to confirm that bacteria on the oxidized surface were indeed attached and not merely the result of altered topography of the steel, attempts were made to remove the bacteria from the oxidized surface by rinsing vigorously. However, no visible change in attached bacterial density was observed (418 ± 99 cells mm^{-2}).

As further confirmation that bacterial cells were attaching directly and specifically to the oxidized surface, a scratch was placed in the middle of the oxide generating a pristine surface and exposing the various levels of oxidized surface that had developed during the corrosion process (see Materials and methods section for characterization and stoichiometry). Attachment analyses repeatedly showed very specific strong attachment to all levels of the oxidized surface only and not to the fresh surface (Figure 1B). Attachment was so specific and abundant that bacterial fluorescence perfectly mirrored the location of the oxidized steel surface. The sharp line of the oxide/steel interface defined by the fluorescent bacteria prompted an interest into the limits of the bacterial cells to detect naturally formed oxide.

In a series of time course attachment assays to determine variations in detection over time, oxidized steel was subjected to incubation from 0 to 120 min in anaerobic stationary phase culture. In every case, regardless of incubation time, MR-1 readily attached specifically to the rusted surface, with no apparent difference in the level of attachment. To determine the minimum sufficient time for detection, the rusted steel was dipped in the MR-1 culture (for ~2–5 s) followed by rinsing (for ~2–5 s). The levels of bacteria binding to iron oxide were very strong and could not be differentiated from those that were incubated extensively.

Rapid recognition of early rust development by MR-1

Until this point, bacterial detection had only been tested with heavily rusted surfaces. To define the minimum level of rapid detection, surfaces were monitored for oxide growth under the microscope (Figure 2). The first appearance of oxide growth occurred in ~10 min on mild steel, and was only visible under the microscope. Under oxidizing conditions, oxide growth was visible to the eye as mild discoloration at about 20 min (Figure 3).

Viewing the topographic, amorphous surface of naturally oxidized steel under the microscope requires a large depth of view, which can only be attained under lower magnification. To more accurately define the lower limit of MR-1 to detect naturally formed iron oxide, the rusted surface was scraped off of oxidized steel and subjected to the stationary phase, anaerobic, GFP expressing bacterial cultures. Immediately upon exposure, robust attachment was observed to the dislodged rust particles (Figure 4A).

Preferential binding of MR-1 to individual iron oxides below the resolution of the human eye

Naturally formed iron rust can be a complex entity, which is seldom comprised of only one type of oxide. According to Cornell and Schwertmann (1996), there are 16 different types of iron oxides, with a wide range in properties. In all the experiments conducted, rust formation was never completely uniform across any one surface (Figure 5A), commonly creating multiple types of oxide, the great majority of which were bound to MR-1 when exposed (Figures 1B,5C). To reinforce this observation, purified iron III oxides were challenged with MR-1 cultures. Attachment was observed to be rapid and strong in the presence of both HFO,

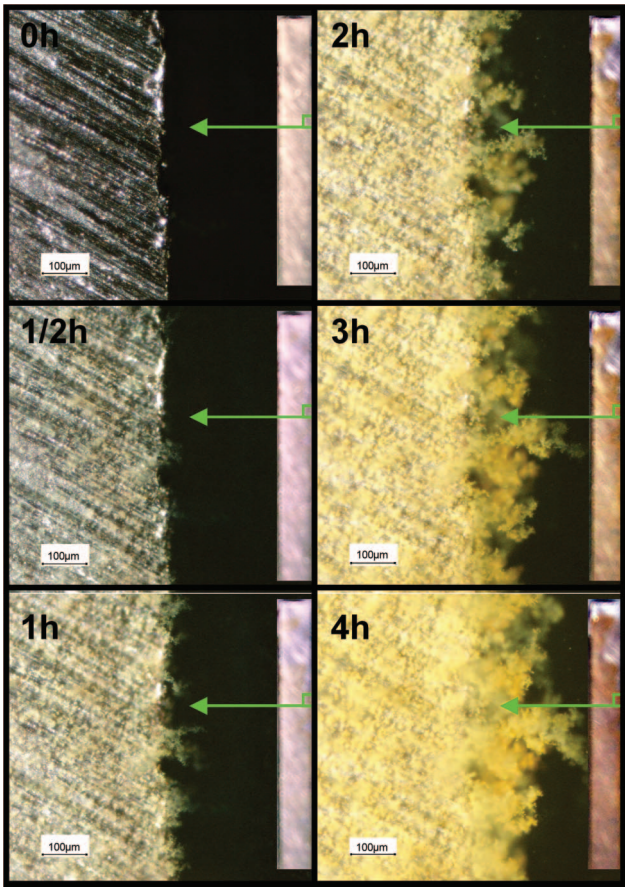

Figure 2. Time course comparison of rust detectability by light microscopy (left) and the unaided eye (right). The green square and arrow in the visible section denotes the area that corresponds to the microscopic image.

the most common and abundant oxide formed in the early stages of steel oxidation in aqueous conditions (Evans and Taylor 1972; Stratmann 1990; Balasubramaniam et al. 2003), and hematite, another common mineral form of iron oxide (Schwertmann 1991; Balasubramaniam et al. 2003) (Figure 4B,C). HFO, which had the smallest particles, defined the limit of detection for MR-1 of oxides to < 0.5 μm (Figure 4C) where resolution of the microscope became the limiting factor. When tested against magnetite, a mixed valence iron II/III oxide, MR-1 attached with significantly lower affinity (Figure 4D).

Recognition of iron oxides generated in the presence of other metals

Not all oxidized steel surfaces were equally recognized by MR-1 in the present experiments. Oxidation of the steel surface was never a completely even process, and while some areas became oxidized, others appeared untarnished in the same period of time (Figure 5A).

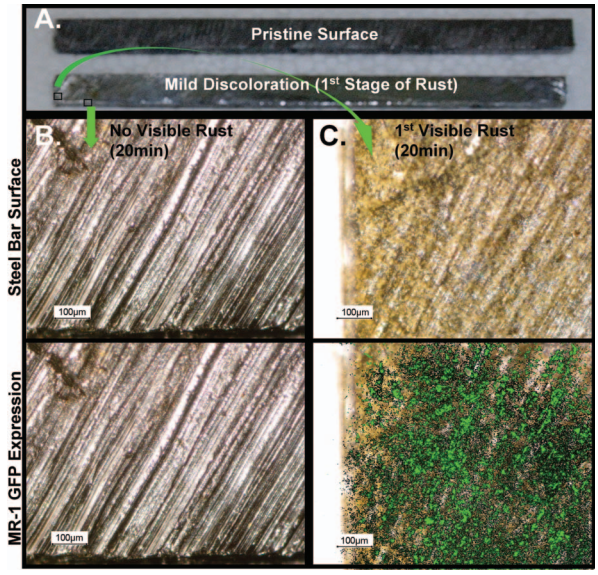

Figure 3. Detection of oxidized mild steel by GFP expressing MR-1 prior to becoming visible to the unaided eye. (A) An unoxidized bar (top) and a bar exposed to oxidizing conditions for 20 min (bottom) are compared with the naked eye. Note that the oxidized bar shows only signs of mild discoloration. The boxes on the left side of the bar correspond to B, and C as indicated by the green arrows. (B) This represents an area where no visible signs of rust can be detected by eye or under the microscope (top) and an overlay of a corresponding fluorescent image (bottom). (C) On the same bar, what appears as mild discoloration to the eye is easily visible under the microscope (top) and readily detected by GFP expressing MR-1 (bottom). Each green point is GFP expressing MR-1 from an overlayed fluorescent picture.

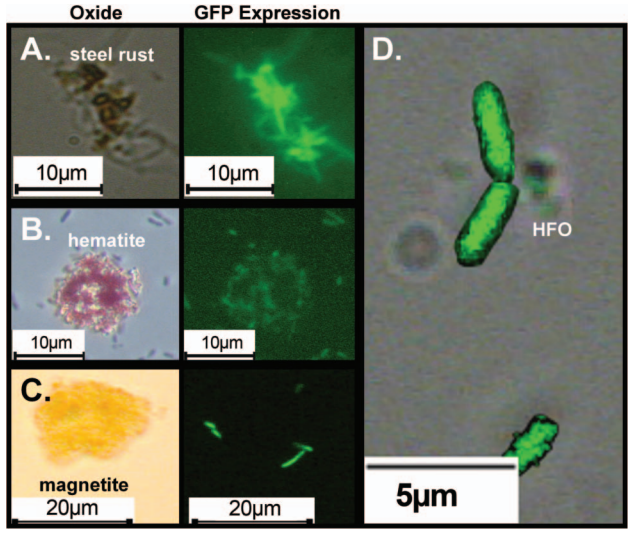

Figure 4. Recognition of microscopic iron oxide particles by MR-1. (A) Rust scraping from mild steel overnight (top) and bound MR-1 (bottom). (B) A hematite particle with (top) MR-1 attached (bottom). (C) A magnetite particle (top) displaying representative weak attachment to MR-1 (bottom). (D) An overlay of the fluorescent and phase contrast images of two GFP expressing MR-1 attached to a particle of HFO measuring < 0.5 μm in diameter.

However, if the steel was left to sit over a longer time frame, the remaining surface would oxidize and a barrier would form between the two oxides. The two oxidized surfaces were topographically similar and appeared to be similar in color; however, MR-1 would not always rapidly recognize all of the surfaces (Figure 5B). Although the rusted surfaces not rapidly bound by MR-1 appeared to be in the minority, these visual observations highlight the important point that MR-1, when grown under these conditions, did not bind rapidly to all forms of natural rust (Roberts et al. 2006).

Detection of oxidation in stainless steel

The experiments reported above dealt with the abilities of MR-1 to bind to oxidized mild steel or purified iron oxides. The experiments were then extended to test the ability of MR-1 to detect natural oxides created by the corrosion of stainless steel. The first experiments were performed with 301 SS, which, even though it contains chromium and nickel, is nevertheless susceptible to oxidation. When 301 SS oxide was exposed to MR-1, rapid attachment was observed (Figure 6A).

In contrast, efforts to oxidize 304 SS (comprised of the same components as 301 SS but with higher concentrations of chromium and nickel) for up to 2 weeks in up to 5% NaCl were unsuccessful, as judged by the lack of any signs of oxidation, either via MR-1 binding or visual inspection of the metal.

Another method of steel protection is achieved by coating the steel with other metals, resistant to corrosion. To investigate the potential of MR-1 to detect rust from coated steel, binder paper clips (spring steel coated with two other proprietary metals) were bent beyond the point of elasticity to crack the coat and expose the steel, then inspected for rust after exposure to 1% NaCl and rinsed in the bacterial culture. Of the visible layers of oxide, MR-1 displayed variable binding affinity between layers and in some cases no binding at all, again reinforcing the point that MR-1 will bind rapidly to many, but not all, oxidized surfaces where iron is a principle component (Figure 6B).

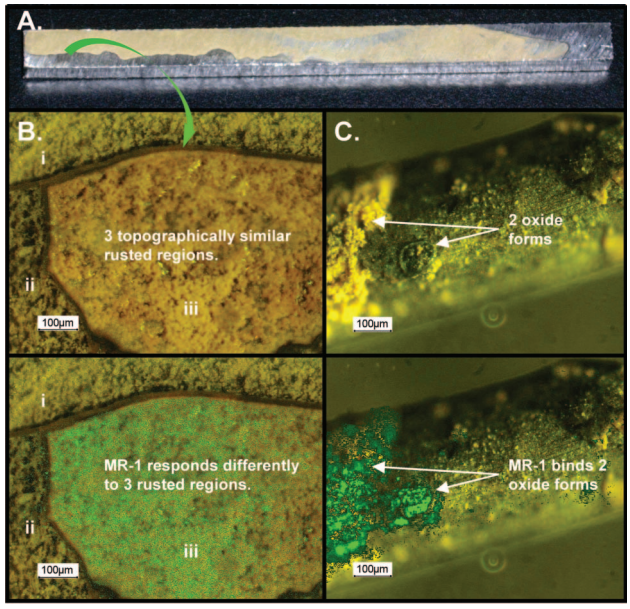

Figure 5. Specific detection of different oxides from naturally rusted mild steel. (A) A mild steel bar allowed to oxidize for 4 h displays an uneven oxidation, where a clear barrier is present between the oxidized and untarnished surface. The green arrow points to a representative region indicative of the typical barrier that develops with continued oxidation. (B) Oxidation for 8 h shows a clear barrier between three topographically similar oxidized regions (top), two of which did not instantly bind/attract MR-1 (bottom). (C) An area of a mild steel wire allowed to rust overnight showed at least two different types of oxide development (top) both of which were instantly detected by GFP expressing MR-1 (bottom). Each green point is GFP expressing MR-1 from an overlayed fluorescent picture.

Steel rust recognition by several bacterial strains

Throughout the series of experiments above detailing the ability of *S. oneidensis* MR-1 to rapidly recognize a

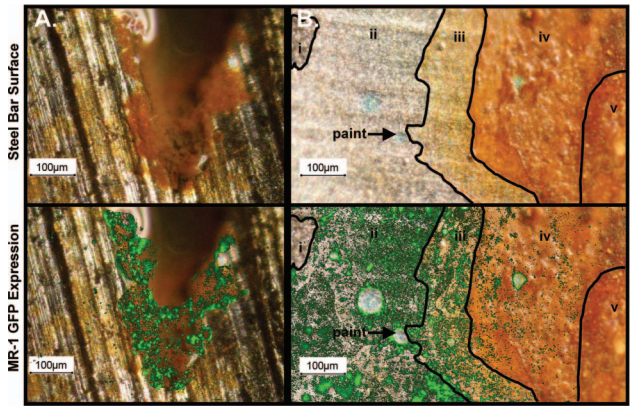

Figure 6. MR-1 detection of rust from complex commercial metals. (A) Cross-section of the thread of a 301 SS bolt, exposed to oxidizing conditions for 8 h (top) and a corresponding fluorescent image (bottom) showing MR-1 binding specifically to the visibly rusted areas. (B) Regions i–v (top) represent the visibly distinguishable regions of the variety of oxide formations seen when a steel binder paperclip plated with other metals was allowed to oxidize, and the representative binding to each region by GFP expressing MR-1 (bottom). Each green point is GFP expressing MR-1 from an overlayed fluorescent picture. The large blank spots, surrounded by a halo, are filtered out autofluorescent particles not relevant to this experiment such as acrylic paint deposited during production of the product B.

range of iron oxides, a particularly high affinity to iron III oxides was displayed. This phenomenon may easily be explained in part by the expression of numerous proteins known to bind to iron oxides, such as the c-type decaheme cytochromes OmcA, MtrA and MtrC, and perhaps others, which play a part in the ability of the bacterium to utilize iron oxides as electron acceptors in anaerobic respiration (Myers and Nealson 1988; Nealson and Saffarini 1994; Lower et al 2001; Heidelberg et al. 2002; Tiedje 2002). As expected, because there are several other bacteria capable of respiring iron oxides, MR-1 is not the only environmentally safe bacterium that possesses the capacity to bind well to naturally formed iron oxides. Upon testing five other *Shewanella* species, all were found to be able to bind to some degree to some forms of iron oxide (Table 2) (Nealson et al. 1991; Fredrickson et al. 1998; Ziemke et al. 1998; Murray et al. 2001; Brettar et al. 2002).

Discussion

To the best of the authors' knowledge, this report is the first description of rapid detection of iron oxide at submicron levels by bacteria. There are many known genera (Brown 1992; Wightman and Fein 2005; Parikh and Chorover 2006; Roberts et al. 2006) of metal binding and reducing microbes. *S. oneidensis* MR-1 was chosen as a model organism for the following properties: (1) MR-1 is a facultative aerobe and an effective and versatile respiratory microbe that uses a wide variety of electron acceptors (including iron) (Myers and Nealson 1988; Nealson and Saffarini 1994; Heidelberg et al. 2002; Tiedje 2002), therefore, once aerobically grown cultures reach a high density, they are rapidly driven to an anaerobic state (Figure 1). (2) Under anaerobic conditions, MR-1 has been shown to bind with high affinity to iron oxide (Lower et al. 2001) and even protect steel from further corrosion (Dubiel 2002).

It is concluded from the results that: (1) *S. oneidensis* MR-1 cultures displayed the ability to attach specifically to naturally rusted steel, exclusively in the areas of oxidative corrosion (Figure 1). (2) The earliest stages of oxidative corrosion are invisible to the naked eye (Figure 2). (3) However, by microscopic examination, it was determined that *S. oneidensis* MR-1 is capable of detecting not only the early stages of rust but also several different forms of naturally occurring iron oxide well below the resolution limits of the human eye ($\sim$85 μm) (Figures 3 and 4). (4) The ability to detect naturally formed rust on steel is not confined to a single strain of the bacterium. Repetition of these experiments with several different species of *Shewanella* confirmed that all had the capacity to specifically detect natural rust (Table 2), including *S. denitrificans* OS217, which lacks iron-reducing proteins and is incapable of reducing iron (Brettar et al. 2002). The initial bacteria iron-binding experiments were conducted based on the hypothesis that iron-reducing proteins, such as OmcA and MtrC, were primarily responsible for binding attachment phenotypes (Lower et al. 2007), as they are known to be ubiquitous and abundant in the outer membrane of the *Shewanella* genus. However, because these and other genes are absent in *S. denitrificans* OS217, and the strain still retains the ability, albeit reduced, to bind to iron oxides, it is suggested here that other factors may play a role in bacterial attachment/detection of oxides.

The complexity of iron oxide formation increases when the oxidation process is conducted in the presence of other metals (Tomaszewicz and Wallwork 1983; Vertes et al. 1990; Bozack 1991; Asteman et al. 2000). To examine the range of oxidative corrosion byproducts rapidly detected by specific bacterial attachment, the *Shewanella* strains were exposed to a variety of oxidized amalgam and plated steels. It was discovered that none of the strains, individually, were capable of attaching to all types of rust generated. Furthermore, the levels of specificity to different rust

Table 2. Levels of rapid bacterial attachment to steel rust.

Bacteria	Culture collection	Reference*	Instant rust attachment[†]
S. oneidensis MR-1	ATCC 700550 TS	Myers and Nealson 1988	+++
S. baltica OS155	ATCC BAA-1091	Ziemke et al. 1998	+
S. putrefaciens CN32	ATCC BAA-453	Fredrickson et al. 1998	++
S. oneidensis MR-4	N/A[‡]	Nealson et al. 1991	++
S. sp. W3-18-1	N/A[‡]	Murray et al. 2001	++
S. denitrificans OS217	ATCC BAA-1090	Brettar et al. 2002	+

*All strains used were cultural environmental isolates.

[†]Attachment was ascertained on rusted mild steel surfaces by comparing with attachment rate to MR-1. Those that bound to steel rust at a rate significantly less than MR-1 were then exposed to MR-1 for comparison.

[‡]N/A = not applicable (strains MR-4 and W3-18 are not yet available in the ATCC).

types varied within an individual strain and between species (Figures 5 and 6, Table 2). Although, all of these strains (except *S. denitrificans* OS217) possess iron reduction genes, the relative expression levels have never been determined in comparative studies. Variable expression levels of proteins capable of binding to iron oxide, may, in part, account for differences observed in rust detectability between strains and even species. Interestingly, the strongest levels of attachment were observed where exclusively iron (III) oxides were present, which are the form of iron oxide capable of being electron donors. In addition, iron oxide-specific attachment always appeared to be a monolayer, indicating that direct contact to iron oxides is important in this detection process. These observations support the theory that proteins involved in respiratory iron reduction are the reason for the specificity of *Shewanella* strains to iron oxides.

With such a wealth of metal-binding and -reducing microbes available, perhaps they will offer a plethora of alternative methods and advantages for more specific and/or broad range applications. So far *Shewanella* is the only known facultative aerobe that is a good iron reducer (Myers and Nealson 1988; Nealson and Saffarini 1994; Heidelberg et al. 2002; Tiedje 2002), and thus might be one of the few that might easily be used for applications in the field, where anaerobiosis would be very difficult to maintain.

The ability of bacteria to detect oxidized surfaces has great potential to be intensified and adapted to a wider range of substrata, or even focussed to be used as a diagnostic tool to differentiate between different types of oxidation (Brown 1992; Carlsson et al. 2006; Lower et al. 2007).The ability of *Shewanella* to interact rapidly and irreversibly with iron oxides offers great potential to identify and highlight early corrosion at spatial scales below those visible to the naked eye via GFP or a DNA stain (Le Pecq and Paoletti 1966; Hiem et al. 1994; Dickinson 1999; Hansen et al. 2000; Pan et al. 2000; Augustine et al. 2004). It should be noted that GFP and most other fluorescent proteins require a minimum of 0.1 μM oxygen for activation and may not function in completely anaerobic systems (Hansen et al. 2001). However, this requirement is significantly lower than the dissolved oxygen concentration in most of the ocean (Karl 1979; Castro-Gonzalez et al. 2005). Utilizing fluorescent bacteria to detect oxidative corrosion facilitates the development of technology that could complement that currently employed to prevent or remove oxidation from the surface of steel (Lemmer 1972; Meisel et al. 1983; Lockheed Missiles and Space Company 1996; Partridge 1998; Rao 2001; Shakleford 2005c).

In addition to many different strains of *Shewanella*, there are a large number of mutants of strain MR-1 that are altered with regard to their ability to reduce iron oxides, including strains that are incapable of transporting proteins to the outer membrane. Studies are now under way to analyze these mutants and other critical genera with regard to their abilities to bind to iron oxides. In this way, it will be possible to begin to unravel the components and mechanism of oxide recognition.

Probiotic technological approaches offer a unique opportunity to take advantage of the highly discriminating sensory systems of microbes, opening the possibility of using this as an approach for following corrosion, or other structurally damaging events, such as micro-cracks. Using such environmental bacteria may offer methods for rapid, cheap and practical detection of material defects in steel products of a wide variety, detection that could help ameliorate a very large economic problem, numbering hundreds of billions of dollars per year.

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