MICROBially INDUCED IRON OXIDATION:
WHAT, WHERE, HOW

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INTRODUCTION

The study of Geomicrobiology is not new and applies to many aspects of our lives. Since the "study of microbial processes currently taking place in the modern sediments of various bodies of water, in ground water circulating through sedimentary and igneous rocks, and in weathered earth crust . . . [and also] the physiology of specific microorganisms taking part in presently occurring geochemical processes" (Ehrlich, 1996), geomicrobiology impacts our water supply, rock formations, and the corrosion of metals. Among the first contributors to the field of geomicrobiology was Ehrenberg with his discovery of the association between *Gallionella ferruginea* and iron (Ehrlich, 1996). Another important contribution was made by Winogradsky who realized that *Leptothrix ochracea* could oxidize FeCO₃ to ferric oxide (Ehrlich, 1996). These and other studies led to the investigation of microbial iron oxidation and precipitation (Ehrlich, 1996).

Both *Gallionella* and *Leptothrix* are neutrophilic iron-oxidizing bacteria. They come in many varieties and can occupy many different niches of the environment. These bacteria thrive in conditions with a pH range of 6.0-8.0 (Ehrlich, 1996; Mulder & Deinema, 1992) although they can survive in a more acidic or basic environment. They can also be autotrophic or mixotrophic. Among the most common types of neutrophilic iron-oxidizing bacteria are *Gallionella, Leptothrix, Sphaerotilus, Crenothrix, and Siderococcus*. They can bind and precipitate ferric iron onto their cell surfaces or sheaths (Ehrlich, 1996).

*Gallionella ferruginea* is the most common species in the genus *Gallionella*. It derives its energy from enzymatic iron oxidation (Ehrlich, 1996) of preformed organic chemicals (ferrous iron) and its carbon from CO₂ (Boyd, 1988). This bacteria is a gradient organism that develops at the anoxic-oxic interface where conditions are neither strongly reducing nor highly oxidizing (Hanert, 1992). It occurs in environments where water is moving from an anoxic into an oxic region (Emerson & Moyer, 1997), and ferrous ion content is exposed to sufficient oxygen to satisfy growth requirements (Kucera & Wolfe, 1957). *Gallionella* is usually found in iron-bearing waters in cool climates, such as cool springs and brooks, wells, and pipe lines (Breede et al., 1957; Skerman, 1967). Its cells are usually kidney or bean-shaped but they may also be rod-shaped. It secretes a ribbon-like stalk which grows from the cell in a twisted manner to give *Gallionella* its differentiating characteristic (Breede et al., 1957; Emerson & Moyer, 1997; Kucera & Wolfe, 1957; Skerman, 1967). These stalks may have a multitude of strands twisting together at nodes resulting in a "string of pearls" like appearance.

The genus *Leptothrix* is characterized by trichomes of cylindrical or rod-shaped, colorless cells within a sheath encrusted with iron or manganese oxide (Breede et al., 1957; Skerman, 1967). This bacteria is unicellular but usually occurs in chains (Skerman, 1967) and is mostly found in fresh water (Breede et al., 1957). *Leptothrix* is often confused with the genus *Sphaerotilus* as they are very similar in morphology. *Sphaerotilus* cells can be either free-floating or attached in chains or trichomes (Breede et al., 1957). They are colorless rods or ellipsoids, are surrounded by a firm sheath, and are found in stagnant or running water (Breede et al., 1957). While these two genera are very similar, there are some differences between them. First, some *Leptothrix* strains have the ability to oxidize manganese, *Sphaerotilus* does not
(Mulder & Deinema, 1992). Next, there is a difference in the take-up of organic substrates. *Sphaerotilus natans* is able to process these and thus can exist in sewage and waste waters. On the other hand, *Leptothrix* cannot and can survive only in fresh water (Mulder & Deinema, 1992). There are also differences in the nutritional requirements between these two genera and in the morphologies of their cells (Mulder & Deinema, 1992).

Because iron is the fourth most abundant element in the Earth's crust (Ehrlich, 1996), the oxidation of ferrous iron (Fe$^{2+}$) to ferric iron (Fe$^{3+}$) by bacteria has proven to be very important in the area of biocorrosion. Since iron-oxidizing bacteria are abundant worldwide, microbial corrosion has become a significant and widespread problem in the water and aqueous waste systems (Tatnall, 1981), also affecting the petrochemical industry (Kobrin, 1976). The iron-oxidizing bacteria indicate their presence with large reddish-brown deposits or rust-colored streaks most often occurring on stainless steel (Kobrin, 1976; Tatnall, 1981). These iron oxidizers can concentrate chlorides into ferric and manganic chloride-rich deposits. This acts like dilute HCl and causes general corrosion of steel (Tatnall, 1981). They usually form thick, bulky deposits (Kobrin, 1976) which can lead to highly localized damage, such as subsurface pit cavities and stress corrosion cracking (Tatnall, 1981). Also, with the ferric hydroxide they produce, they can form extensive deposits called tubercles on the inside of iron water pipes (Miller, 1970).

It is often quite difficult to control these bacteria-induced corrosions. Most attempts are unsuccessful when trying new materials or applying a film over the metal. The only metals resistant to the damage of the iron-oxidizing bacterium *Gallionella* are titanium and other noble metals (Tatnall, 1981). Thin film coatings do not work because instead of protecting the metal, they invite bacterial colonies to grow (Tatnall, 1981). The only method of controlling biocorrosion may be through the use of chemicals which are used to break up the deposits (Tatnall, 1981). While it may be the best option available, it is often the most expensive one and requires continuous monitoring and testing of the metals.

In order to develop methods to grow and study iron-oxidizing bacteria, bacteria were collected from iron-stained fresh water springs and introduced into "sloppy" gel test tubes containing iron bearing metal.

**MATERIALS AND METHODS**

*Preparation of the Medium.* A Manganese Gradient Tube Medium was prepared using a basal medium, carpet tacks, and a co-factor solution. The basal medium was created with 0.25g NaCl, 0.25g CaCO$_3$, 0.25g MgSO$_4$$\cdot$7H$_2$O, 0.375g K$_2$HPO$_4$, 0.125g NaH$_2$PO$_4$, 0.05g KNO$_3$, and 2.0g of agar. 500 mL deionized water was added and the entire solution was heated to boiling. One carpet tack was put in each of 125 test tubes to provide a source of iron for the iron-oxidizing bacteria. The carpet tack at the bottom of the tube released iron upwards while oxygen diffused down into the tube. This allowed the bacteria to grow at the levels of iron and oxygen concentrations suitable for them. Four mL of the basal medium was pipetted into each of the test tubes (over the tack) and autoclaved for 15 minutes. The co-factor solution was
prepared by first making a yeast solution by combining 0.67g of yeast nitrogen base with 10 mL of deionized water. The yeast solution was then filter-sterilized. 0.1g of Casamino Acids, Vitamin free was added to 100 mL of deionized water and filter-sterilized. 2.6 mL (enough for 40 µL of solution to be added to half of the tubes) of both the yeast and casamino acid solutions were combined to form a master mix of co-factor solution. 80 µL of the co-factor solution was added to half of the tubes and vortexed.

A Hyphomicrobium Enrichment Medium was prepared by a modification from the media given in Atlas and Parks (1997). It was created with 2.66g Na2HPO4•12H2O, 0.5g (NH4)2•SO4, 0.4g KNO3, 4.8mg MgSO4•7H2O, 2.34mg FeCl3•6H2O, 2.5mg MnCl2•4H2O, 2.5mg NaMoO4•2H2O. Enough deionized water was added to create one liter of the solution. Five grams of rock flour from sites was added. 1 Normal HCl was added until the pH was almost neutral. 10g FeSO4•7H2O and 10g (NH4)2SO4 were then added to the solution. The solution was pipetted into the test tubes and autoclaved.

**Source of Bacteria.** Red-orange sediments were obtained from the Tierra Amarilla Springs, iron-bearing cold springs, north of Albuquerque, New Mexico. The bacteria were collected from three different spring sites and at three depths at each site: deep inside the hole of the spring, at the surface of the spring, and more than 30 cm away from the spring. First, the Manganese Gradient Tube Media culture and the the Hyphomicrobium Enrichment Medium was made. At the first two springs, four different samples were collected at each depth. A pipette was used to inoculate the bacteria into two different Manganese Gradient Tube Media tubes: one with co-factor solution and one without. A loop was used to inoculate bacteria from the same depth into two more Manganese Gradient Tube Media tubes (one with co-factor solution and one without). This method provided four cultures from a single location and was performed at six specific locations. At the third spring, three different tools were used to collect bacteria at only two depths. The two depths taken at the third spring were deep in the hole of the spring and at the surface of the spring. A pipette, loop, and syringe were each used to collect bacteria from a single location and inoculate the bacteria into media with and without co-factor solution. Since only two depths were cultured at the third spring, 12 cultures were made. There were 36 total cultures made from the Manganese Gradient Tube Media.

More cultures were also made with the Hyphomicrobium Enrichment Medium. However, before arriving at the site, most of the tubes had settled into two layers with the top layer being clear and the bottom layer orange and full of sediment. The same collecting process that was used for the Manganese Gradient Tube Medium was also used for the Hyphomicrobium Enrichment Medium. Six cultures were made from each spring using a combination of tools and locations for a total of 18 cultures. Because there was no co-factor difference in this media, there were less cultures to be made.

Eight controls were made. Three controls were made with the pipette. One of these was a Hyphomicrobium Enrichment Medium control and two were Manganese Gradient Tube Media controls, with one having the co-factor solution and the other
without the solution. Three controls were made with the loop in the exact way the controls with the pipette were created. Two controls were made with the syringe. These were Manganese Gradient Tube Media control, one having co-factor solution and the other without.

**Observing Growth of Cultures.** The cultures were incubated together at 64°F and were observed for growth and changes weekly for five weeks. Growth was noted in some of the Manganese Gradient Tube Media tubes by the second week. This was marked by a change from white, orange, and rust-colored particles to bright, orange particles. Growth was also noted by white, cloudy growth bands that formed inside the tube. Most of the particles and bands grew from the middle to the top of the tube, concentrating mostly at the top of the media. As the cultures continued to grow, their color brightened and the particles increased in size. There was no growth observed in the Hyphomicrobium Enrichment Media tubes. Upon reading the pH after 2.5 weeks, it was determined that the media had oxidized too fast for anything to grow and no further methods were applied to these cultures.

**Staining and Optical Microscopy.** At the end of the fifth and seventh weeks, samples were made, stained with acridine orange, and observed with an epifluorescent microscope in the laboratory facility at the University of New Mexico Biology Annex. Bacteria was first pipetted from one of the tubes onto a clean, glass slide. The slides were then dried in a fume hood at room temperature. When the slides were dry, they were stained with 1-2 drops of 0.1% weight by volume, filter-sterilized acridine orange. The acridine orange stayed on the slides for 30 seconds and then they were rinsed with deionized water two to three times. Next, the slides were put back into the fume hood to dry. Once dry, 1-2 drops of filter-sterilized deionized water was placed on top of the stained bacteria. A cover slip was placed on top of the sample and excess water was wiped away from the edges of the cover slip. Then, the cover slip was sealed in place with clear, top coat nail polish.

The stained slides were observed through an epifluorescent microscope at 100x, 450x, and through an oil immersion lens at 1000x. The epifluorescent microscope uses a mercury vapor lamp to produce energetic waves which strike certain molecules and impart energy that is released in the form of greater wavelengths (Boyd, 1988). The acridine orange stain is a nucleic acid-binding fluorescent dye which makes hard to see microbes visible under an epifluorescent microscope (Emerson & Moyer, 1997).

Two Gram stains were also performed using the traditional stains of crystal violet, iodine, and safranin.

**TEM and Sample Preparation.** Three different methods were employed in an attempt to prepare cultured samples for observation in the transmission electron microscope (TEM). The first method used formalin-acetic acid-alcohol or FAA. The FAA was made by combining 18 mL of ethyl alcohol (50%), 1 mL of glacial acetic acid, and 1 mL of formaldehyde (40%) and adding the mixture to a well that already contained bacteria and a holey carbon grid suitable for TEM as described in Postek et al.’s *Scanning Electron Microscopy* (1980). The well sat untouched for about 36 hours in a loosely
sealed jar for slow evaporation. After this time period, all liquids had evaporated. Next, deionized water was added and allowed to partly evaporate. The sample was then moved to a dry well. A small amount of acetone was placed in the well and evaporated slowly in a bell jar.

The second method used gluteraldehyde and ethanol. An approximately 3% concentration of gluteraldehyde was created and put in a vial with bacteria and a holey carbon grid. This mixture sat overnight. Each vial was poured into a high-grade ceramic well and covered with water and ethanol. Ethanol was added three more times to concentrate the ethanol to at least 80% and then completely evaporated.

All samples from these first two methods were then coated with gold palladium (60:40) by evaporation. This was done for additional contrast in the TEM.

The third method only used deionized water. Deionized water, bacteria, and a holey carbon grid were placed in a ceramic vial. A small amount of water was allowed to evaporate from the vial. The grid was pulled out of the vial and placed on filter paper to remove any additional water. The grid was then submersed in liquid nitrogen and immediately placed in a cryogenic liquid nitrogen TEM holder.

All TEM, including the cryogenic microscopy, was done on the JEOL 1200 at Sandia National Laboratories.

RESULTS

Epifluorescence Microscopy and Gram Stain. The cells stained with acridine orange appeared orange and green and revealed themselves to be a population of diverse morphology. There were cocci, rods, curved rods, and s-shaped cells present. Several long filaments were also observed. These filaments contained cells of mostly a rod shape, although some were elliptical or round, see figure 1. The third type of bacteria most often noted consisted of three to four cocci strung together which could either be like pancake stacks, as sausage links, or as a string of pearls. The features seen with epifluorescence microscopy were also seen with the TEM which, we believe, adds to the validity of our findings.

The two slides that underwent the Gram stain produced gram negative results. The cells kept the color of the secondary stain, safranin, and appeared pink.

TEM. The cryogenic samples were looked at first. We found all of the types of cells seen with epifluorescence except for the long filaments. A curved rod was seen along with the “string of pearls” or three to four cocci joined together. We also noted a branching stalk characteristic of that of Gallionella, see figure 2. This had not been seen before with the epifluorescent microscope.

The FAA-prepared samples were the best. They had the most contrast and least amount of gel present. We again saw the curved rods, strings of pearls, and rod-shaped bacteria. However, the FAA solution caused the outer edges of the bacteria to be jagged.

The gluteraldehyde-prepared samples had the least predictable results. There were some areas of the grid that had well-defined bacterial cells and others that were contorted from their original shape. But, once again, there were rods, curved rods, and twisted stalks.
FIGURE 1. a: String of pearls and many different cell types. b: A long filament with cells in the middle. Both taken with epifluorescent microscope.

FIGURE 2. a: A curved rod. b: A twisted stalk characteristic of Gallionella. Both taken by TEM.
DISCUSSION

From the results of the different bacterial cells seen, it is fairly certain that Gallionella is present because of the bean-shaped cells and twisted stalks found with the TEM. We cannot confirm, though, what other iron-oxidizing genera exist in our tubes, since our media was only preferential and not one that isolated a specific genus of bacteria. Based on the environment in which they live and the source of the water, we believe our cultures contain Gallionella, Leptothrix, and possibly Crenotrich and Sphaerotilus. We believe the genus Leptothrix rather than Sphaerotilus exist in our tubes because the water source was fresh, unlike the polluted water in which Sphaerotilus are usually found.

The TEM preparations worked well. The cryogenic method rapidly froze the cells in place and allowed us to view their morphology. The FAA method, as stated previously, was the best of the three methods because it gave the best contrast. The gluteraldehyde samples did not come out as well. It is possible that the gluteraldehyde we prepared was still too concentrated and did not mix well.

Although these bacteria were collected from springs and then cultured in an environment containing a presumably pure iron-bearing metal, it seems the tube already containing Manganese Gradient Medium could be used with a piece of metal containing these bacteria. A small piece of corroding metal could then be inserted into the test tube and cultured to study the bacteria.

CONCLUSION

Bacteria from a known source were inoculated into test tubes containing an iron-rich media which supported the growth of different genera of bacteria. And, while complete identifications were not made, methods of growing and characterizing iron oxidizing bacteria were developed. So that isolation and identification of these bacteria can be done from many different settings.

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