Characterization of microbes implicated in microbially-influenced corrosion from the proposed Yucca Mountain Repository

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CHARACTERIZATION OF MICROBES IMPLICATED IN MICROBIALYL-
INFLUENCED CORROSION FROM THE PROPOSED
YUCCA MOUNTAIN REPOSITORY

by

Beth J. Pitonzo

A dissertation submitted in partial fulfillment of the
requirements for the degree of

Doctor of Philosophy

in

Biology

Department of Biological Sciences
University of Nevada, Las Vegas
August 1996
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ABSTRACT

This project addressed the recovery and characterization of the microbial community present in the densely welded volcanic tuff of Yucca Mountain. Yucca Mountain is located in the Topapah Spring Range on the Nevada Test Site and is the site of the proposed high-level nuclear waste repository. Emphasis of study was placed on the populations of microorganisms commonly implicated in microbially-influenced corrosion (MIC). Historically, these bacterial groups include: 1) sulfate-reducing bacteria; 2) iron-oxidizing bacteria; 3) acid-producing bacteria and 4) exopolysaccharide-producing (EPS) bacteria.

Three deep subsurface locations on the Nevada Test Site (Rainier Mesa, Yucca Mountain and Yucca Flat) were characterized microbiologically and physically to assess the potential for MIC. Various parameters were compared to determine whether Rainier Mesa or Yucca Flat might serve as a structural analog to Yucca Mountain for microbiological investigations. While some similarities existed between the sites, each had unique microbiological and physical
characteristics. MIC-implicated microbes were isolated from each of the three locations in varying numbers, i.e., sulfate-reducers, iron-oxidizers and exopolysaccharide-producing strains.

Since exopolysaccharide (EPS)-producing microorganisms provide the backbone for biofilm formation and subsequently MIC, it is important to understand what factors might control polymer production. All heterotrophic bacterial isolates obtained from Yucca Mountain were tested on different culture media to determine their ability to produce EPS under variable nutrient conditions. To further characterize specific isolates, MIDI-FAME (fatty acid methyl ester) analysis for bacterial identification was performed on twelve distinct aerobic EPS-producing heterotrophs. This analysis failed to identify nine isolates which indicated that these microorganisms were unique or had not been previously described. A principal components analysis clustered all of the isolates into four groups with known organisms. Exopolysaccharide material was isolated and purified from eight of the isolates after growth on R2A medium and compositional analysis was done by GC-mass spectrometry. The results indicated a diverse group of

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exopolymers whose carbohydrate compositions varied greatly.

As MIC-related microbes were determined to be present within Yucca Mountain, it became important to determine what effects the environmental conditions of the repository might have on their survival. Thus, a timecourse experiment was conducted to evaluate the effects of gamma-radiation on the microbial community. To simulate in situ conditions, a microcosm approach was used. After a 233.1 Krad dose of gamma radiation, administered at a low dose rate (163 rads/min), radiation-resistant microorganisms entered a viable but non-culturable state (VBNC). Further investigations indicated that some members of the microbial community were able to regain culturability after a resuscitation treatment at 4°C. Two representative EPS-producing isolates were exposed to the same radiation regime in sand microcosms to evaluate their radiation tolerance/resistance. This was done as exopolymer has recently been shown to provide other protective advantages for microorganisms in nature (i.e. protection from predation and antimicrobial substances, desiccation resistance and nutrient trapping). When considered alone, EPS-producing isolates showed increased radiation-resistance when compared
to the entire community.

As it is expected that heat generated by buried radioactive waste will drive water out of the near-field area in the repository, a long-term desiccation (8 month) experiment was conducted to evaluate effects on the indigenous microbiota. Natural rock microcosms were used in these studies to closely simulate in situ conditions. While some loss in culturability and metabolic capacity occurred in the desiccated rock samples, it was minimal, which indicated that desiccation did not significantly affect bacterial survival. MIC-implicated bacterial groups were isolated from the most desiccated samples which showed they are well adapted and potentially may survive this repository condition.

Yucca Mountain sulfate-reducing, iron-oxidizing and exopolysaccharide-producing bacteria were tested for their actual corrosion potential under simulated laboratory conditions. This was accomplished through construction of an "artificial electrochemical cell" which contained a material currently being considered for outer canister construction (i.e. 1020 carbon steel) in a soft R2A agar medium. Microorganisms were tested both alone and in
combinations using polarization resistance methodology to determine corrosion rates. Analysis indicated that all microbial types, alone or in combination, could corrode carbon steel at a rate significantly higher than that observed in an abiotic control. The electrochemical cells containing all three microbial types exhibited the highest rates of corrosion.
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the love and support that are essential for success. This
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CHAPTER 1

INTRODUCTION

The ecology of microbially-influenced metal corrosion (MIC) is complex and dependent on many biotic and abiotic factors. It involves adhesion of microorganisms to a metal or metal alloy surface, metabolism within an established colony, and direct bacteria-metal interactions (Ford and Mitchell, 1990). The collective effects of these phenomena results in deterioration of the underlying metal substratum and potential structural failure. Recent estimates made by the National Bureau of Standards projected the national cost of metal corrosion to exceed $167 billion per year (Jones, 1992). Biocorrosion is known to significantly contribute to this economic nightmare and so has become a research interest worldwide. In the United States, research on MIC is of particular importance because of the proposed storage of high-level radioactive waste in
metal canisters at Yucca Mountain, Nevada Test Site.

**Microbially-Influenced Corrosion**

Evaluation of microbially-influenced corrosion requires a multi disciplinary approach including consideration of metallurgy, electrochemistry, and microbiology.

Metallurgy is concerned with producing metals and alloys, converting them into usable products, and improving their performance (Borenstein, 1993). It allows us to understand the chemical, physical, and mechanical behavior of metallic materials under various conditions. It also allows us to make predictions regarding the susceptibility of a particular metal to biotic forces of corrosion. Some factors which affect the corrosion rate of a particular metal include oxidative stability of the metal or alloy, surface characteristics of the metal (i.e., roughness), and abiotic and biotic surface deposits.

Electrochemical corrosion is best described as a series of reactions occurring at a metal surface in contact with an electrolyte-containing aqueous phase.
These reactions are driven by a difference in electrical potential between two points on a metal surface or between two metal surfaces (Jones, 1992). This potential difference may be attributed to both abiotic (i.e., environmental pH or temperature) and biotic factors (i.e., microbial or fungal metabolism). The potential difference causes a current of electrons to flow through the metal from the area of lower potential (the anode) to the area of higher potential (the cathode). The rate of electron flow through the metal is the corrosion current. Corrosion is governed by the oxidation-reduction reactions that take place at each electrode, and the system is termed an electrochemical or galvanic cell. The corrosion process is limited by the rate at which the electrons are accepted by the liquid medium at the cathode. The corrosion rate is also a function of the ability of ions and electrons to be conducted through the aqueous phase and participate in the cathodic chemical reactions (Borenstein, 1993).

Electrochemical corrosion may be associated with the growth of microorganisms. The critical factors that
limit or promote MIC are those that affect the growth of the causative microorganisms rather than factors affecting the electrochemical reactions (Borenstein, 1993). Microorganisms can influence corrosion processes by directly inducing anode formation or by affecting the existing anode-cathode reaction through their metabolism. Due to the complex nature of these processes, a variety of bacterial types may be involved. Complex assemblages of microorganisms form consortia which effectively and efficiently corrode virtually any metal surface (Ford and Mitchell, 1990, Geesey, 1993). These assemblages are termed "biofilms". In the context of MIC, four groups of microorganisms have historically been considered important: 1) the sulfate-reducing bacteria; 2) the iron-oxidizing bacteria; 3) the acid-producing bacteria and 4) the exopolysaccharide-producing bacteria (Ford and Mitchell, 1990, Geesey, 1991). Each of these bacterial types induces corrosion in a way that is dependent on their metabolism or growth characteristics. The end result, however, is similar in that they all promote localized or pitting corrosion.
Bacterial Groups Associated with Corrosion

Perhaps the most well-documented cases of MIC involve the sulfate-reducing bacteria (SRB) (Ford and Mitchell, 1990, Hamilton, 1985, Philp et al., 1991). Corrosion of metals caused by SRB occurs under strict anaerobic or alternating aerobic/anaerobic conditions and is often dependent on other biofilm microbial types for optimal activity (i.e. cross feeding of nutrients and decreased oxygen tension) (Costerton and Boivin, 1991, Ford and Mitchell, 1990). SRB oxidize organic substances (often provided by fermentative heterotrophs in the biofilm) to organic acids and CO₂ by anaerobic sulfate respiration (Hamilton, 1985). They can grow in soil, freshwater or saltwater and can tolerate a wide pH range (Borenstein, 1993). SRB are involved in several reactions which accelerate corrosion. Through the action of an active hydrogenase, these organisms can prevent the accumulation of atomic or molecular hydrogen at the cathode, thus causing cathodic depolarization (Geesey, 1991). The metal sulfides formed as a result of Sulfate respiration also may contribute to the corrosive
activity of SRB. Iron sulfides may act as effective anodic or cathodic depolarizers or may facilitate the accumulation of atomic hydrogen at the metal surface, causing hydrogen embrittlement (Geesey, 1991).

Iron-oxidizing bacteria oxidize iron from the ferrous (soluble) to ferric (insoluble) form under aerobic conditions. Ferric ions can then attract chloride ions and produce ferric chloride, an extremely corrosive compound that pits carbon steel and stainless steel (Borenstein, 1993). Other corrosion products which exacerbate corrosion include ferric hydroxides. These compounds deposit on the metal surface as tubercles, causing anaerobic conditions beneath them (Ford and Mitchell, 1990). The result is breakdown of the protective oxide coating (observed on most metals) and localized corrosion. Iron-oxidizers can be classified as neutrophilic (i.e., those which optimally grow at neutral pH) or acidophilic (those which optimally grow at low pH).

Some microorganisms exhibit modes of metabolism that cause them to produce and excrete large amounts of
acid (Geesey, 1991). The acid may be of either an inorganic or organic nature. In biofilm communities the acid may accumulate, and under conditions of decreased oxygen tension, cause localized pH gradients at the metal surface. These pH gradients allow for cathodic depolarization, and thus acceleration of corrosion (Ford and Mitchell, 1990).

The last important bacterial group contain those microbes that produce exopolysaccharide (EPS). EPS provides the "glue" which aids in surface adhesion and stabilization of biofilm communities. EPS-producing microbes are ubiquitous in nature and may both directly and indirectly cause MIC (Costerton and Boivin, 1991). These microorganisms directly cause MIC through formation of differential aeration cells (Geesey, 1991). Differential aeration cells are created by heterogeneous colonization of a metal surface. The bacterial cells replicate on the surface and produce EPS which allows for the formation of an oxygen concentration gradient in the forming biofilm. The respiratory activity in the biofilm causes the area under the microcolony to become
anaerobic and thus, anodic to the metal surface exposed in the bulk aqueous phase. The net result is the formation of an electrochemical cell and corrosion. EPS-producing microbes can also directly affect corrosion processes by binding metal ions and establishing a metal concentration cell (Ford and Mitchell, 1990, Geesey, 1991). The binding capability results in anodic depolarization and accelerated corrosion rates.

EPS-producing microorganisms may contribute indirectly to corrosion by establishing microniches within the biofilm. Microniches are important in the formation of diverse bacterial consortia within biofilms (Costerton and Boivin, 1991). The creation of an anaerobic zone during differential aeration cell formation allows for the colonization of anaerobic SRB near the metal surface. pH gradients which develop due to accumulation of acid metabolites in the biofilm may create the correct environment for acidophilic iron-oxidizers. While scientists have classified the bacteria implicated in MIC in the above discussed groups
for descriptive convenience, one division is not mutually exclusive of another. For example, many EPS-producing microorganisms are in fact capable of metal oxidation.

**Bacterial Growth in Biofilms**

One factor that greatly impacts MIC is that bacteria preferentially grow in a sessile or biofilm mode of growth (Costerton et al., 1995). This growth mode promotes many of the corrosion mechanisms previously described by allowing highly structured, physiologically cooperative communities, to form on surfaces (Videla and Characklis, 1992). There are direct analogies between biofilm communities and the tissues formed by eukaryotic cells (Costerton et al., 1995).

Biofilms usually form in response to the concentration of nutrients at a surface (conditioning film) (Costerton, 1987). The phenomenon was first noted by Zobell (1943) who observed that marine bacteria preferentially attached to the walls of sampling containers containing seawater. In addition to the nutritive benefits gained by biofilm formation, it
appears that this mode of growth is a universal bacterial strategy for survival in many ecosystems (Costerton et al., 1987, Lawrence et al., 1995, Marshall, 1992, Marshall, 1988, Roszak and Colwell, 1987). The polysaccharide matrix which stabilizes the biofilm appears to impart several protective influences on the bacterial consortium. Bacteria within biofilms are notably more resistant to predation, pH fluctuations, bacteriophage infection, toxic chemicals, and antibiotics (Costerton et al., 1995, Geesey et al., 1992). Additionally, biofilm bacteria have been shown to exhibit desiccation resistance due to the highly hydrated nature of exopolymer (Ophir and Gutnick, 1994, Roberson and Firestone, 1992). This may serve as a selective survival advantage to microbes which exist in arid ecosystems (Marshall, 1988). Lastly, biofilm microbiota continue to have a nutritional advantage over planktonic bacteria even after establishment of the initial community. The EPS matrix serves as a nutrient trap under oligotrophic conditions which are often encountered in nature (Costerton et al., 1987).
**MIC in Aquatic Systems**

Virtually all electrolytic aqueous environments can support the growth of at least some of the microorganisms now known to be involved in MIC. Thus, as biofouling and subsequent MIC is a problem in many aqueous environments, most work has been done under these conditions (Little et al., 1991, Videla and Characklis, 1992). Geesey et al. (1978) developed several quantitative recovery methods to enumerate biofilm bacteria in a pristine mountain stream. Results indicated that biofilm bacteria predominated and their metabolic activity exceeded that of planktonic members of the community. These methods have now been applied to a large number of aquatic environments in natural and industrial ecosystems (Costerton and Boivin, 1991, Costerton et al., 1994). Data obtained from these studies, as well as others, indicate that biofilm populations predominate in virtually all nutrient-sufficient aqueous systems and the biofilm microbiota present result in significant metabolic activity (Costerton et al., 1995). Although some of these
investigations were not directly related to MIC, but rather were geared towards biofilm detection, they all have predictive importance in evaluating the potential for MIC. The potential is based on the observed dependence of MIC on biofilm development.

**MIC at the Proposed Yucca Mountain Nuclear Waste Repository**

Nuclear testing has been conducted at the Nevada Test site since the 1950's. Both above ground and below ground nuclear tests resulted in the production of several kinds of nuclear waste. Much of the solid waste is currently stored in metal canisters on-site, awaiting permanent disposal. In addition, commercial nuclear power facilities and military installations have also contributed to the nuclear waste stockpile since the 1960's. Currently, storage methods of high-level waste vary widely depending on storage location and include metal encasement, ceramic encasement, and submersion (Geesey, 1993).

The current strategy in the United States is to standardize disposal and storage by constructing a
government-controlled repository. This would allow the waste to be isolated during the 300-1000 year period required to reduce the radioactivity to a safer level (Geesey, 1993). The waste package design must consider this time frame and provide an effective barrier to leakage of radioactive material to the subsurface environment. Currently, a double-shell container is being considered with the inner shell being composed of titanium alloy and the outer shell of 1020 carbon steel (Geesey, 1993).

Yucca Mountain is the current repository site under consideration in the United States. It is located on the Nevada Test Site, approximately 125 miles northwest of Las Vegas, Nevada. The planned repository is to be located approximately 350 meters or more below the land surface and 225 m above the present static water table in the unsaturated, densely welded, devitrified, rhyolitic tuff of the Topopah Spring Member (Geesey, 1993).

The subterranean mineralogy of the Yucca Mountain site is thought to include several inorganic nutrients
required for microbial metabolism (i.e. nitrogen, phosphorus, iron and manganese) as well as low amounts of organic carbon (0.15-0.55 mg/L of rock) (Geesey, 1993). Though generally unsaturated, the tuff does contain a small amount of water (4%) (Chapter 2). Due to the lithotrophic existence of Yucca Mountain microbes under natural conditions, bioavailability of endogenous nutrients and water is probably limiting metabolic activity and the potential for MIC. However, microorganisms have been discovered that are able to survive under sub-optimal and often extreme environmental conditions (McCabe, 1990, West et al., 1985). During repository characterization and construction, the natural environment will be drastically altered by tunnel boring activities and introduction of exogenous carbon and water (Geesey, 1993). This could potentially create an ideal environment for MIC by providing necessary factors such as water, electrolytes and nutrients for indigenous microbes and those introduced during construction phases (Christofi and Philp, 1991).
In the early 1980s, interest in the presence and activities of indigenous subsurface microbial communities resulted in the establishment of the Department of Energy's Subsurface Science Program. This program has significantly contributed to current knowledge of subsurface microbial ecology by evaluation of many subterranean sites in the United States (Geesey, 1993). In addition, other countries which utilize nuclear power have also evaluated potential sites for radioactive waste storage (Arter et al., 1991, Bachofen, 1991, Rosevear, 1991, West et al., 1985, West et al., 1982). These studies have demonstrated the existence of viable microorganisms from 30-2800 m depth (Phelps et al., 1989). In association with the subsurface science program, Boone et al. (1995) has cultured viable bacteria from >9000 ft deep.

Rainier Mesa at the Nevada Test Site was one of the sites chosen for contribution to the Department of Energy Subsurface Science Program. This site is in geographic proximity to Yucca Mountain and was created by the same volcanic event, and thus, is considered a
structural analog. Results from Rainier Mesa indicated the presence of a viable and diverse microbial community (Amy et al., 1992, Haldeman and Amy, 1993, Haldeman et al., 1993). Therefore, the activity of the microbial community at Yucca Mountain should be carefully evaluated to determine the potential threat of MIC to the storage canisters. Microbiological evaluations should include determining presence of microbiota capable of MIC and evaluating the effects of repository conditions (i.e., gamma radiation, desiccation and high temperature) on the microbes (McCabe, 1990). Designing such experiments is daunting, as the most difficult parameter to evaluate in the study of radioactive waste disposal is the long time frame necessary to observe measurable change (Rosevear, 1991).

**Microbial growth and survival under conditions of gamma irradiation**

Over the last two decades, scientists have identified a large number of microorganisms which defy the usual assumption that biological life is destroyed under extreme conditions (Kushner, 1978). Ionizing
radiation provides no exception as microbes have been discovered which can withstand significant exposure. *Micrococcus radiodurans* and its relatives are very resistant to the lethal and mutagenic effects of ionizing radiation (Anderson et al., 1956). This organism shows no loss of viability at doses up to 500 krad (Moseley, 1983). Radiation resistance has also been elucidated in other microorganisms such as *Escherichia coli*, *Salmonella sp.* and *Listeria monocytogenes* (Thayer et al., 1990, Farag et al., 1990, Francia et al., 1985, Nasim and James, 1978). Much of the research mentioned above has been conducted for the food industry using pure cultures grown under laboratory conditions. These experiments have helped in the development of procedures to control microbial contamination in food products using gamma-radiation.

**Microbial life and survival under desiccating conditions**

When assessing water relations in natural systems, the most widely used parameter is water activity (*a_w*) (Brown, 1990). Water activity describes the water
content of a solution or porous medium and is a function of water potential (Gee et al., 1992). These measurements are much more useful in describing water relations than determinations of gravimetric water content, as they can be related to the energy microorganisms expend for water assimilation (Skujins, 1984). Microbial growth under water stress varies widely with little relationship to classical taxonomy ($a_w = 0.998-0.62$) (Harris, 1981), however, it appears that the growth limit for most Gram positive and Gram negative eubacteria is $a_w = 0.90$ (Brown, 1990). An exception to this rule occurs in the archaebacterial halophiles. Due to their unique "osmotic" physiology, halophiles are capable of growth at water activities of 0.75. Fungi are considered to be the most desiccation-resistant of all soil microbes ($a_w = 0.60$), however, their presence and abundance in arid systems varies widely (Skujins, 1984, Durrell and Shields, 1960).

While low water activities may severely impact growth and activity either directly (low matric potential) or indirectly (decrease nutrient transport),
many microbes are capable of survival and retained viability when exposed to long periods of desiccation (Kieft et al., 1990, Boylen, 1973). While it was once thought that the most desiccation-resistant bacteria were those that could form spores or cysts (i.e., Bacillus sp., actinomycetes), many vegetative cells have now been shown to withstand water stress for prolonged periods of time (Balkwill, 1989, Kieft et al., 1990, Boylen, 1973, Chen and Alexander, 1973, Elwan and Diab, 1970) Elwan and Diab (1970) noted that in sand which contained 0.95-2.5% water and was nutrient poor (0.09-0.033% organic carbon), 84% of the bacteria were in the vegetative form. Xerotolerant, non spore-forming microbes have been isolated from both Gram negative (Acinetobacter sp.) and Gram positive (Arthrobacter sp.) genera, in both soil and subsurface ecosystems (Kieft et al., 1990, Cacciari and Lippi, 1987).

Mechanisms of desiccation-resistance utilized by soil or endolithic microbes have not been thoroughly explored (Skujins, 1984, Harris, 1981). However, it is well recognized that the basic response of a
microorganism to water stress is dominated by the biophysical need of the organism to attain water potential equilibrium with its environment (Brown, 1990, Harris, 1981). Scientists have begun to evaluate relationships between growth/survival and ionic transfer/osmoregulation as possible resistance mechanisms (Cacciari and Lippi, 1987, Chen and Alexander, 1973).

An additional link to understanding desiccation resistance in soil/rock ecosystems may be found in exopolysaccharide-production. Exopolymer material elaborated by soil microbes has been shown to retain large quantities of water under desiccating conditions (Ophir and Gutnick, 1994, Roberson and Firestone, 1992). Mugnier and Jung (1985) demonstrated retained bacterial viability after more than 3 years at a water activity of 0.069 when cells were entrapped in polysaccharide gels. DeVault et al. (1990) demonstrated that low water activity actually stimulated EPS production in Pseudomonas aeruginosa. All of these observations indicate that EPS may serve in promoting survival under
Overview

The work presented in the following chapters of this dissertation represents the first microbial characterization conducted at Yucca Mountain. Special attention was given to MIC-implicated microbial groups and the effects of repository conditions on the indigenous microbial community. The following is a brief chronological introduction to the microbial studies at Yucca Mountain. The chapters are manuscripts prepared for submission to various journals. The appendices contain additional manuscripts based on work relevant but not directly related the dissertation topic.

Pilot Investigation- Chapter 2

The initial microbiological analysis of rock material from Yucca Mountain addressed community-level parameters as well as the presence and numbers of MIC-implicated microorganisms. Additionally, a physical analysis of the rock was conducted to assess environmental conditions. Because access to Yucca
Mountain was difficult to achieve alternate locations on the Nevada Test site (i.e. Rainier Mesa and Yucca Flat) were characterized to develop sampling protocols. Additionally, it was of interest to determine whether one of the alternate sites could be used as a structural analog for microbiological analysis in the event that Yucca Mountain access remained restricted. Comparisons made between Yucca Mountain, Yucca Flat and Rainier Mesa, indicated that while some similarities occur, the different sites were unique in many ways. However, a potential for MIC was documented at each site.

Characterization of Exopolysaccharide-Producing Microbes Isolated from Yucca Mountain- Chapter 3

Due to the importance of exopolysaccharide-producing (EPS) microorganisms in biofilm formation and subsequent MIC, all culturable isolates from the original characterization study with this colony morphology were further characterized. This included MIDI (fatty acid methyl ester) analysis for bacterial identification, extraction and purification of EPS for
compositional analysis, and EPS-production capability on various carbon sources. Additionally, attempts were made to directly extract EPS from crushed rock material. The results indicated diverse EPS-producing microbes.

The Effect of Gamma-radiation on the Indigenous Microbial Community at Yucca Mountain- Chapter 4

The effect of gamma-radiation on the indigenous microbiota present at Yucca Mountain was determined through a long-term time course experiment designed to closely simulate repository conditions. Microcosms were constructed and the dose rate of gamma radiation was selected based on current estimates of emissions from the proposed canister surface. Special emphasis was placed on determining the survival of microorganisms implicated in MIC. Results indicated that microorganisms became non-culturable after 24 hours of radiation exposure but retained viability as determined by other direct methods. Additionally, an experiment was conducted on pure cultures of two EPS-producing isolates to determine their radiation tolerance. Results indicated increased radiation resistance of the two EPS-
producers as compared to the whole microbial community.

Resuscitation of Viable but Non-Culturable Microorganisms Exposed to Gamma-radiation- Chapter 5

This experiment was conducted as a direct consequence of the results obtained in Chapter 4. A resuscitation regime at 4°C was performed on the irradiated rock and traditional culturing techniques were then utilized to determine whether members of the microbial community could regain culturability. Results indicated that a proportion of the original community was able to survive and resuscitate after gamma-radiation exposure. However, the only MIC-implicated group capable of this was the EPS-producers.

The Effect of Long-Term Desiccation on Indigenous Microbial Communities at Yucca Mountain-Chapter 6

Due to the high temperatures likely to occur near the repository site, water is expected to be driven away for many years, followed by local recondensation. Thus, a long-term experiment was conducted to assess the effects of desiccation on indigenous microbiota with
emphasis on MIC-implicated bacterial groups. After an eight month incubation under low humidity, microbial numbers did not radically drop as was expected. It appears, however, that the microbial community structure changed appreciably with desiccation. Very adherent microbial types, including EPS-producing microbes, dominated after extended desiccation. In addition, sulfate-reducing bacteria were detected after long periods of desiccation. These results indicate that MIC-implicated populations of desiccation-resistant microbes exist at Yucca Mountain as members of the indigenous community.

Corrosion of 1020 Carbon Steel by Yucca Mountain Bacterial Isolates- Chapter 7

As representative MIC-implicated isolates were obtained from Yucca Mountain, it became important to determine their corrosion capabilities. An electrochemical cell was designed to test their activity in a laboratory experiment using 1020 carbon steel. Sulfate-reducing bacteria and iron-oxidizing bacteria, obtained from enrichment cultures as well as purified
cultures of EPS-producers, were inoculated alone and in combination in the cells. Corrosion rates were determined using polarization resistance methods and compared to an abiotic control. The results indicated that bacterial isolates were capable of corrosion of 1020 carbon steel under the prescribed laboratory conditions.

Discussion- Chapter 8

The discussion of this dissertation summarizes the results of research characterizing the microbiota at Yucca Mountain and the effects of some potential repository conditions on these microorganisms. It includes issues related to presence of MIC-implicated populations of microorganisms as well as their survival under sub-optimal and extreme conditions. Lastly, the corrosion capabilities of Yucca Mountain MIC-implicated isolates are compared and discussed.

References


CHAPTER 2

COMPARISON OF THE MICROBIOTA IMPORTANT IN MICROBially-INFLUENCED CORROSION FROM THREE SUBSURFACE ENVIRONMENTS AT THE NEVADA TEST SITE: RAINIER MESA, YUCCA MOUNTAIN AND YUCCA FLAT

This chapter has been prepared for submission to Applied and Environmental Microbiology as a note and is presented in the style of that journal. The complete citation is:


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The potential for microbially-influenced corrosion (MIC) depends on the presence of specific microbes as well as proper environmental conditions for growth and activity. Three deep subsurface locations on the Nevada Test Site (Rainier Mesa, Yucca Mountain, and Yucca Flat) were characterized microbiologically and physically to assess the potential for MIC at the proposed Department of Energy repository for high-level nuclear waste. Various parameters were compared to determine whether Rainier Mesa or Yucca Flat might serve as a structural analog to Yucca Mountain for microbiological investigations. While some similarities existed between the sites, each had unique microbiological and physical characteristics. MIC-implicated microbes were isolated from each of the three locations in varying numbers, i.e., sulfate-reducers, iron-oxidizers and exopolymer-producing strains.

Microorganisms have been implicated in the deterioration of organic, geologic and metallic materials in natural ecosystems (4). With metals, the
process is called microbially-influenced corrosion, or MIC. Historically, the bacterial communities associated with MIC can be divided into four metabolic categories: 1) sulfate-reducing bacteria; 2) iron-oxidizing bacteria; 3) exopolysaccharide-producing bacteria and 4) acid-producing bacteria (4, 5). To evaluate the potential for microbially-influenced corrosion in deep subsurface environments at the Nevada Test Site, where a long-term repository for high-level radioactive waste has been proposed, rock and water samples from three locations were characterized and compared. This study includes the first microbial characterization of both Yucca Mountain and Yucca Flat.

Sampling locations on the Nevada Test Site are depicted in Figure 1. Rainier Mesa samples (RM-R, RM-C, RM-W) were collected from the south drift of tunnel U12n at 400 m depth as previously described (1). Nearly saturated deep subsurface rock (RM-R) was collected from zeolitized tuff where recharge water had collected to form a perched water zone (7). Sample RM-C was obtained from a clay pocket located in the rubble of a free
flowing fracture, and sample RM-W from the subsurface fracture water (19 L/min). Yucca Mountain rock samples (YM-LF, YM-RF, YM-SR) were collected from an alcove located within the north portal exploratory shaft at 60 m depth. Rock obtained from this site was unsaturated welded tuff. As Yucca Mountain is geologically homogenous, the welded tuff obtained is characteristic of that expected at the repository horizon. Sample YM-SR was surface-impacted rock while the other two were separated from surface contamination. Yucca Flat samples (YF-SR, YF-R) were obtained from alluvial sediments at 305 m depth in the U1A shaft. Sample YF-SR was a surface-impacted sediment while sample YF-R represented a sample uncontaminated by surface microbiota; both were from an unsaturated zone in the alluvial deposit. All samples were collected aseptically and transported in coolers to the laboratory for analysis within 6 hrs.

Temperature measurements of sampling locations were taken in the field. pH of the rock/sediment samples was measured by the method of Janitsky (12) and gravimetric
water contents were determined before and after 24 hours of desiccation at 105°C. Rock samples were aseptically pulverized using a sterile mortar and pestle, and thoroughly homogenized before testing for microorganisms (1). Samples were subdivided into three subsamples and each was subsequently analyzed independently for determination of microbial heterogeneity. Analyses of variance with Tukey confidence intervals (family error rate 0.10) were conducted using Minitab Release 8.0 statistical software (Minitab, Inc., State College, PA) to determine significant differences among mean values for microbiological parameters.

Slurries of the homogenized samples were prepared for microbiological analysis as described previously (8, 9). Culturable counts were determined by serially diluting slurries in deionized water and spread-plating samples in triplicate on R2A agar (Difco) (16). R2A was chosen as it is a low-nutrient medium frequently used to isolate bacteria from the environment. However, the nutrient content of R2A is probably higher than that which occurs naturally in Yucca Mountain rock. Shannon-
Weaver diversity and equitability indices were calculated based on R2A colony morphology to determine bacterial heterogeneity (3). EPS-producing microorganisms were enumerated and isolated from R2A plates based on their colony morphology. Iron-oxidizing (2, 14) and sulfate-reducing (2, 10) bacteria were enumerated using a five-tube most probable number (MPN) technique. Phospholipid fatty acid (PLFA) analyses were performed on duplicate samples to quantify living biomass and assess community structure (6).

The physical characteristics of the samples from the three sites are listed in Table 1. Both the Rainier Mesa and Yucca Mountain rock samples were of volcanic origin, while the Yucca Flat samples were from an ancient alluvial floodplain. Moisture contents varied between sites, with Rainier Mesa exhibiting the highest content and Yucca Mountain the lowest. Field temperatures were similar for the Rainier Mesa and Yucca Mountain sites (16°C and 12°C respectively) but the Yucca Flat site exhibited a higher temperature of (20°C). pH values obtained from the rock or water
samples were within the physiological range for bacterial growth. However, the Yucca Mountain and Yucca Flat samples had higher values than those from Rainier Mesa.

The microbiological analyses are summarized in Table 2. Culturable counts from the samples were significantly different, with the exception of the RM-C and YM-RF samples. The highest culturable count was obtained from the YM-SR (surface rock) sample while the lowest was from the RM-W (water) sample. Of the rock samples analyzed, those from Yucca Flat exhibited the lowest recoverable microbial numbers, with YF-R below limits of detection. Diversity and equitability measurements showed significant variations with many comparisons statistically different; however, no distinct trends were noted. Diversity was highest in the YM-RF sample and lowest in the YM-SR. Equitability measurements were highest in RM-FW and YF-SR and lowest in YM-SR. More distinct colony morphotypes were isolated from the Yucca Mountain samples than from those obtained at other sites.
MIC potential was assessed by determining the community percentage of EPS-producing organisms and by enriching for and enumerating iron-oxidizing and sulfate-reducing bacteria. EPS-producing microbes were more abundant in Rainier Mesa samples, which could be due to the higher moisture content at this site. Iron-oxidizing bacteria were detected in six of the eight samples (75%) with the highest number in the Yucca Flat samples. The Yucca Flat alluvium was reddish in color, perhaps due to a high iron content. Sulfate-reducing microorganisms were detected in only three of the samples (RM-R, YM-SR, YF-R). However, they were detected at each sampling site, and the highest numbers were found in the RM-R and YM-SR samples. Although sulfate-reducing bacteria were detected in just three of the eight samples, they may have been present in more of the samples because it is known that there need not be a direct correlation between the detection of these bacteria and their activity in natural environments (10).

Phospholipid fatty acid (PLFA) viable biomass
estimates and community composition analysis were performed on four of the rock samples: two from Yucca Mountain, one from Rainier Mesa and one from Yucca Flat. The highest recoverable PLFA fraction was obtained from Yucca Mountain rock samples, followed by those from Rainier Mesa (Table 2). Extraction of the alluvial sediment samples from Yucca Flat yielded no detectable PLFA fraction by gas chromatography. The Yucca Mountain PLFA profile contained terminally-branched saturates, which may indicate the presence of sulfate-reducing bacteria or Gram positive genera such as Arthrobacter (15). The detection of a fatty acid (i17:1w7c) typical of a number of Desulfovibrio sp. supports the presence of sulfate-reducing bacteria. Monoenoic fatty acids were also detected, particularly 16:1w7c and 18:1w7c, which are the most abundant PLFAs in Gram-negative organisms (15). PLFA profiles from Rainier Mesa samples were similar to those from Yucca Mountain samples except that there was no detection of the Desulfovibrio-related fatty acid, i17:1w7c (6).

In comparing the three sample sites, it is clear
that while some similarities occur, the samples obtained from the different sites are unique in many ways. The microbiology of the three locations was impacted by both physical (i.e., water content) and geological (i.e., volcanic vs. alluvial origin) differences. Thus, it was determined that neither Yucca Flat or Rainier Mesa could serve as a structural analog for future MIC studies.

While many factors must be present to promote microbial activities associated with MIC (i.e., water and nutrients), the potential for biocorrosion in deep subsurface environments at the Nevada Test Site is apparent, as all implicated groups of microbes were present in each of the sites. The activity of the microbiota under natural conditions is probably limited due to their lithotrophic mode of existence (i.e., decreased bioavailability of water and nutrients). However, during repository construction, perturbation and addition of exogenous water and carbon will undoubtedly positively influence the activity level of resident and introduced microbiota. This has significant implications for the Yucca Mountain
repository as the current canister design under consideration for high-level nuclear waste storage has an outer layer of carbon steel and an inner layer of titanium alloy (13). MIC has been documented for carbon steel in industrial systems and thus, if proper conditions are created through human activity during repository construction, outer canister integrity would be in jeopardy (13).

We sincerely thank Roger Jacobson, Sam Hockett, and Nate Cooper from the Desert Research Institute, Las Vegas, NV, Chuck Costa and Carl Hawn at the Lyner Project, Yucca Flat, Dana Haldeman from University of Nevada, Las Vegas and Mr. Max Powell from the Yucca Mountain Project Office for sampling assistance. Thanks also go to Patricia Castro and Theodore Lagadinos for laboratory assistance. This work was supported by funding from the Yucca Mountain Project office through a cooperative agreement with UNLV (DE-FC08-90NV10872) and a National Science Foundation, State of Nevada EPSCoR Women in Science fellowship.
REFERENCES


Figure 1. Sampling site locations on the Nevada Test Site [adapted from Henne (11)].
Table 1. Physical analysis of samples

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Physical Description</th>
<th>Moisture (%)</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Sample Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM-R</td>
<td>Zeolitized tuff</td>
<td>12.9</td>
<td>16</td>
<td>8.0</td>
<td>8/3/93</td>
</tr>
<tr>
<td>RM-C</td>
<td>Clay Pocket</td>
<td>35.1</td>
<td>16</td>
<td>8.0</td>
<td>8/3/93</td>
</tr>
<tr>
<td>RM-W</td>
<td>Fracture Water</td>
<td>100</td>
<td>16</td>
<td>8.0</td>
<td>8/3/93</td>
</tr>
<tr>
<td>YM-LF</td>
<td>Welded volcanic tuff</td>
<td>4.3</td>
<td>12</td>
<td>8.7</td>
<td>12/9/93</td>
</tr>
<tr>
<td>YM-RF</td>
<td>Welded volcanic tuff</td>
<td>3.9</td>
<td>12</td>
<td>8.0</td>
<td>12/9/93</td>
</tr>
<tr>
<td>YM-SR</td>
<td>Welded volcanic tuff</td>
<td>3.9</td>
<td>12</td>
<td>8.8</td>
<td>12/9/93</td>
</tr>
<tr>
<td>YF-R</td>
<td>Alluvium</td>
<td>7.8</td>
<td>20</td>
<td>8.6</td>
<td>10/5/94</td>
</tr>
<tr>
<td>YF-SR</td>
<td>Alluvium</td>
<td>7.9</td>
<td>20</td>
<td>8.7</td>
<td>10/5/94</td>
</tr>
</tbody>
</table>

* RM-R, 400 m deep subsurface rock from Rainier Mesa; RM-C, 400 m deep clay pocket in fracture zone at Rainier Mesa; RM-W, 400 m deep free-flowing fracture water from Rainier Mesa; YM-LF, 60 m deep subsurface rock from left face of Yucca Mountain North Portal; YM-RF, 60 m deep subsurface rock from right face of Yucca Mountain North Portal; YM-SR, 60 m deep surface-impacted rock from Yucca Mountain North Portal; YF-R, 305 m deep alluvial sediment from Yucca Flat; YF-SR, 305 m deep surface-impacted alluvial sediment from Yucca Flat.
Table 2. Microbiological analysis of samples

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Culturable Counts*</th>
<th>Diversity (H')</th>
<th>Equitability (J)</th>
<th>Morphotypes</th>
<th>Community % EPS-Producersa</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM-R</td>
<td>5.11 (0.02)</td>
<td>1.99 (0.10)</td>
<td>0.78 (0.02)</td>
<td>12.7 (1.1)</td>
<td>28.0</td>
</tr>
<tr>
<td>RM-C</td>
<td>4.55 (0.04)</td>
<td>1.61 (0.12)</td>
<td>0.85 (0.05)</td>
<td>6.7 (0.6)</td>
<td>43.3</td>
</tr>
<tr>
<td>RM-W</td>
<td>1.39 (0.09)</td>
<td>2.29 (0.03)</td>
<td>0.93 (0.03)</td>
<td>11.7 (1.2)</td>
<td>31.6</td>
</tr>
<tr>
<td>YM-LF</td>
<td>3.56 (0.12)</td>
<td>1.99 (0.09)</td>
<td>0.72 (0.02)</td>
<td>16.3 (3.2)</td>
<td>2.7</td>
</tr>
<tr>
<td>YM-RF</td>
<td>4.65 (0.05)</td>
<td>2.59 (0.52)</td>
<td>0.66 (0.02)</td>
<td>18.7 (4.2)</td>
<td>4.3</td>
</tr>
<tr>
<td>YM-SR</td>
<td>5.54 (0.14)</td>
<td>1.49 (0.16)</td>
<td>0.61 (0.03)</td>
<td>12.0 (4.2)</td>
<td>8.2</td>
</tr>
<tr>
<td>YF-R</td>
<td>BLD*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td>YR-SR</td>
<td>2.96 (0.17)</td>
<td>2.20 (0.12)</td>
<td>0.90 (0.05)</td>
<td>11.7 (0.6)</td>
<td>5.7</td>
</tr>
</tbody>
</table>

* Sample names

* Culturable Counts in CFU/mL

H' Diversity, J Equitability, Morphotypes, % Community, EPS-Producers

BLD = Below Limit of Detection

ND = Not Determined
### Table 2 cont. Microbiological analysis of samples

<table>
<thead>
<tr>
<th>Sample*</th>
<th>Iron-oxidizing MPN&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sulfate-reducing MPN&lt;sup&gt;b&lt;/sup&gt;</th>
<th>PLFA Biomass (pmole/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM-R</td>
<td>0.6</td>
<td>2.5</td>
<td>0.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>RM-C</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>ND</td>
</tr>
<tr>
<td>RM-W</td>
<td>7.0</td>
<td>&lt;0.2</td>
<td>ND</td>
</tr>
<tr>
<td>YM-LF</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>0.9</td>
</tr>
<tr>
<td>YM-RF</td>
<td>2.3</td>
<td>&lt;0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>YM-SR</td>
<td>1.3</td>
<td>2.2</td>
<td>ND</td>
</tr>
<tr>
<td>YF-R</td>
<td>&gt;1.7 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.2</td>
<td>BLD&lt;sup*d&lt;/sup&gt;</td>
</tr>
<tr>
<td>YF-SR</td>
<td>&gt;1.7 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>&lt;0.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

Note. Mean values of three replicate samples are presented with standard deviations in parentheses.

* RM-R, 400 m deep subsurface rock from Rainier Mesa; RM-C, 400 m deep clay pocket in fracture zone at Rainier Mesa; RM-W, 400 m deep fracture water from Rainier Mesa; YM-LF, 60 m deep subsurface rock from left face of Yucca Mountain North Portal; YM-RF, 60 m deep subsurface rock from right face of Yucca Mountain North Portal; YM-SR, 60 m deep surface-impacted rock from Yucca Mountain North Portal; YF-R, 305 m deep alluvial sediment from Yucca Flat; YF-SR, 305 m deep surface-impacted alluvial sediment from Yucca Flat.

<sup>b</sup> Culturable counts are log-transformed cells/g dry weight for rock/sediment samples or cells/ml for water samples. MPN estimates are cells/g dry weight for rock/sediment samples and cells/ml for water samples.

<sup>c</sup> determined by morphology on R2A.

<sup>d</sup> test not done.

* test results below limits of detection.

<sup>e</sup> taken from Haldeman et al. (7).
CHAPTER 3

CHARACTERIZATION AND COMPARISON OF EXOPOLYSACCHARIDE-
PRODUCING ISOLATES FROM YUCCA MOUNTAIN

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52
ABSTRACT

Exopolysaccharide-producing (EPS) microbes have long been associated with biofilm production in natural environments. To examine their presence in the densely welded tuff at the proposed Yucca Mountain nuclear waste repository, three samples were obtained from an alcove within the north portal starter tunnel. One surface-contaminated sample (YM-SR) was compared to two samples (YM-RF and YM-LF) not impacted by contaminating microbiota. MIDI-FAME analysis of twelve distinct aerobic EPS-producing heterotrophs failed to identify nine which indicated that these microorganisms were unique and/or had not been previously described. A principal components analysis clustered all of the isolates into four groups with known organisms. Two isolates exhibited a unique chromatographic pattern previously found exclusively in anaerobes. EPS was isolated and purified from eight of the isolates after growth on R2A medium and compositional analysis was done by GC-mass spectrometry. The results indicated a diverse group of exopolymers whose carbohydrate
compositions varied greatly. Direct extractions of carbohydrates from the surrounding rock were below limits of detection, indicating low EPS-production or decreased persistence. To determine whether nutrient variability would stimulate or retard EPS-production, the isolates were plated on 11 different media. An average of 25% of the isolates from the YM-RF or YM-LF samples were able to produce EPS on one or more medium type, but 46% of the isolates from the YM-SR sample had this ability. Varying the carbon source affected both the ability of the microbes to produce EPS as well as the amount. These studies suggest that EPS-production by introduced heterotrophic microorganisms at Yucca Mountain will be positively impacted by the input of exogenous nutrient sources during repository construction.

INTRODUCTION

The characteristics that enable bacteria to survive and colonize rocks are diverse and poorly understood (Amy et al., 1993, Kaiser and Bollag, 1990, Lappin-Scott
and Costerton, 1990). Fluctuations of organic nutrients, and variations in water, oxygen, and pH on both large and small scales has led to microbial diversity within endolithic environments (Lappin-Scott and Costerton, 1990). However, the activity of endolithic microbes is relatively low as they are considered to be nutrient-limited, receiving only intermittent supplies of substances necessary for endogenous metabolism. Thus, any survival strategy that microbes could use to ameliorate the harsh conditions in rock would be beneficial.

Biofilms generally occur in areas with high surface to volume ratio favoring their formation in rock environments (Marshall, 1992, Busscher et al., 1987). Oligotrophic conditions present in lithotrophic environments may also cause alterations in bacterial hydrophobicity favoring biofilm formation (Marshall, 1992). This preferred mode of growth provides a selective advantage to microorganisms living in nature. Exopolysaccharide (EPS)-producing microbes have long been associated with biofilm production in natural
environments (Geesey et al., 1992, Marshall, 1992). EPS provides the structural matrix for biofilm community establishment. Microorganisms which produce EPS initially take advantage of the concentration of macromolecules that occurs at interfaces during biofilm production and later are able to trap and concentrate nutrients in the exopolysaccharide matrix (van Loosdrecht, 1990). EPS has also been suggested to function in desiccation resistance (Roberson, 1992) as well as microbial protection from predation, antimicrobial agents and other toxic substances (Geesey et al., 1992).

The recovery of EPS-producing microbes from endolithic environments thus, would not be unexpected. However, the presence of these microbes in the densely welded tuff of the proposed nuclear waste repository at Yucca Mountain may pose a serious problem in that EPS-producing microorganisms have been implicated both directly and indirectly in microbially-influenced corrosion processes (Costerton and Boivin, 1991, Geesey, 1991, Ford and Mitchell, 1990). It is, therefore, of
interest to characterize EPS-producing microbes from the site to better understand their physiological activities and metabolic capabilities.

MATERIAL AND METHODS

Bacterial isolates and cultivation techniques

Heterotrophic isolates from Yucca Mountain were obtained from an alcove located within the north portal exploratory shaft at 60 m depth. Three sites in the alcove were sampled including a surface-impacted rock area (YM-SR) and two rock faces separated from surface contamination (YM-RF and YM-LF).

Twelve different exopolysaccharide-producing (EPS) microorganisms were identified based on colony morphology on R2A (Difco Laboratories, Detroit, MI). Eight of these isolates were obtained from non-impacted rock (YM-1, YM-4, YM-9, YM-29, YM-33, YM-35, YM-37, YM-40) and four from the surface-impacted sample (SR-7, SR-8, SR-21, SR-25). All isolates were cultivated on R2A agar unless otherwise noted (Reasoner and Geldreich, 1985).
EPS-Production Potential of Heterotrophic Bacterial Isolates

All 92 distinct heterotrophic isolates from the three rock samples (YM-LF, YM-RF, YM-SR), regardless of original colony morphology, were spotted on eleven solid media containing different carbon sources or nutrient formulations. This was done to determine whether nutrient variability would stimulate/retard EPS production in these microorganisms. The media included R2A agar (Difco), 1% PTYG agar (peptone, 0.05 g; tryptone, 0.05 g; yeast extract, 0.1 g; glucose, 0.1 g; MgSO₄·7H₂O, 0.6 g; CaCl₂·2H₂O, 0.07 g; Bacto-agar, 15 g per liter, pH 7.0), Luria agar (tryptone, 10.0 g; yeast extract, 5.0 g; NaCl, 0.5 g; Bacto agar, 15 g per liter, pH 7.0) and Bushnell-Hawes agar (Difco) with 0.5 g yeast extract and a selected carbon source (1% glucose, 1% galactose, 1% glucose and galactose, 0.2% citrate and 0.08 g bromthymol blue, 0.2% acetate and 0.08 g bromthymol blue, 1% starch, 1% casamino acids and glucose or 1% Tween 80 (Polyoxyethylene sorbitan monooleate) and 0.02 g neutral red) per liter, pH 7.0.
All plates were incubated at 25°C for 14 days.

Those heterotrophic isolates capable of EPS-production (29%) were further characterized using a semi-quantitative split plate technique. EPS-producing isolates were grown to a standard concentration (10⁷ cells/ml) in R2B (yeast extract, 0.5 g; proteose peptone, 0.5 g; casamino acids 0.5 g; glucose, 0.5 g; soluble starch, 0.5 g; sodium pyruvate, 0.3 g; K₂PO₄, 0.3 g; MgSO₄·7H₂O, 0.05 g per liter, pH 7.2). They were inoculated on the 11 media described as a streak and a 10 ul spot. The morphology of the streak allowed for a qualitative determination of EPS-production based on appearance while measurement of the spot allowed for a semi-quantitative assessment. Plates were incubated as described above.

MIDI-FAME Analysis of Exopolysaccharide-Producing Isolates

For MIDI analysis of the 12 EPS-producing isolates, fatty acids were extracted and methyl esterified from bacterial cultures grown for 24 hours on trypticase soy
agar according to the specifications of the manufacturer (Microbial ID, Inc., Newark, NJ). MIDI provided identifications of microorganisms when similarity indices of EPS-producing isolates matched profiles of known microbes in the data base (TSBA aerobe library version 3.6), and provided a principal components plot showing relatedness of all EPS-producing isolates based on cluster analysis of fatty acid methyl ester profiles.

**EPS Extraction from Pure Cultures**

Exopolysaccharide was extracted from eight selected isolates (YM-4, YM-9, YM-33, YM-35, YM-37, YM-40, SR-21, SR-25). Approximately 100 R2A plates were used to cultivate each isolate. After incubation at 25°C for 14 d, the bacteria were gently harvested from the plates with a sterile rubber spatula and placed in 25 ml of phosphate-buffered saline (pH 7.0) (Henningson and Gudmestad, 1992). The mixture was vortexed briefly to disperse bacterial masses and then centrifuged at 33,000 x g for 10 minutes at 4°C in an Sorvall RC-2B refrigerated centrifuge (Brown and Lester, 1980). The
pellet was resuspended and sonicated for five minutes at 18 W with a Branson Cell Disruptor 200 (Vandevivere and Kirchman, 1993, Brown and Lester, 1980). The mixture was centrifuged for a further 10 minutes as described above and the supernatant filtered using a 0.2 um Acrodisc filter (Gelman Sciences) apparatus for highly viscous liquids. Exopolymer was precipitated with 3 volumes of ethanol at 4°C for 24 hrs (Garrote et al., 1992) and collected by centrifugation (4°C) at 12,000 x g for 20 minutes. The ethanolic supernatant was removed and the EPS was dissolved in a minimal amount of sterile deionized water and dialyzed exhaustively against deionized water at 4°C in 12,000 molecular weight cutoff dialysis tubing (Spectrapor) (Nesbit, 1991). The exopolymer material was frozen and lyophilized.

**EPS Purification**

EPS was purified by a modification of the method of Nesbit (1991). Crude lyophilized exopolymer was dissolved in 100 mM MgCl₂ at a concentration of 5 mg/ml. The pH of the solution was adjusted to between 7-7.5
with 0.05 M NaOH. Ribonuclease B-Type IIB and Deoxyribonuclease I (Sigma Chemical) were added to give a final concentration of 0.1 mg/ml and the solution slowly stirred in a 37°C waterbath for four hours. Pronase E (Sigma Chemical) was then added to give a final concentration of 0.1 mg/ml and incubated overnight at 37°C. Residual protein was removed by phenol extraction (Henningson and Gudmestad, 1992) and the extract was exhaustively dialyzed against deionized water at 4°C using 12,000 molecular weight cutoff dialysis tubing. The preparation was assayed for residual protein by the method of Lowry (1951), using bovine serum albumin as a standard and lyophilized.

**Compositional Analysis of EPS**

The eight purified exopolymers were sent to Dr. Russ Carlson at the University of Georgia, Complex Carbohydrate Research Center for compositional analysis. The glycosyl composition analysis was conducted by preparing the trimethylsilyl methylglycoside derivatives and using GC-mass spectrometry (electron impact and
chemical ionization) for detections. To hydrolyze the exopolymers and prepare the derivatives, the samples were treated with 1 M anhydrous methanolic-HCl for 16 hours at 80°C, then N-acetylated (methanol-pyridine-acetic anhydride) for 6 hours at room temperature. The resulting methyl glycosides were then derivatized with trimethyl silane reagent (Tri-Sil, Pierce Chemical Co.) and analyzed with a GC-mass spectrophotometer (Lindberg, 1990, York et al., 1985).

Carbohydrate Extraction from Yucca Mountain Rock

Direct extraction of carbohydrate from rock was performed by a modified soil extract method of Cheshire (1977). Yucca Mountain rock that was not surface contaminated was pulverized with a mortar and pestle under aseptic conditions. Crushed rock (300 g) was mixed with 1200 ml of 0.2 N NaOH and sonicated for 10 minutes at 50% power using a Branson Cell Disruptor 200 (Cheshire, 1979). The mixture was shaken at room temperature overnight at 150 rpm. After settling, the supernatant was removed and clarified by centrifugation.
The extract was filtered (0.2 um filter), evaporated to 100 ml, and exhaustively dialyzed against deionized water. The dialysate was subjected to ethanol precipitation as described above.

RESULTS

**EPS-Production Potential of Heterotrophic Bacterial Isolates**

When all heterotrophic isolates obtained from the three sample locations (YM-RF, YM-LF, YM-SR) were screened using the spotting technique, the following percentages were found to be capable of EPS-production on one or more media: YM-RF-(30%); YM-LF- (18%) and YM-SR- (46%).

The subset of EPS-producing isolates, identified by the replica plate assay, were then further characterized using the semi-quantitative split plate technique. The percentages of isolates capable of EPS-production on specific carbon sources is listed in Table 1. The results indicated that varying the carbon source or nutrient formulation affected both the ability of
microorganisms to produce EPS as well as the amount they produced. Some isolates appeared to be true oligotrophs because they were unable to grow on anything but a low nutrient medium (i.e., R2A). Relatively few isolates at any site were capable of growth or EPS-production on citrate, acetate or Tween 80. One unique isolate produced EPS only on galactose and casamino acids/glucose media. Lastly, of all the isolates tested only five did not produce EPS on the R2A media commonly used in our laboratory; however, they were capable of EPS-production on other carbon substrates.

*MIDI-FAME Analysis of Exopolysaccharide-Producing Isolates*

MIDI-FAME analysis of the 12 aerobic EPS-producing heterotrophs failed to identify 9 of them within the acceptable limit of 30% similarity used previously for subsurface microorganisms (Amy et al., 1992). Only YM-33, YM-37, and SR-25 had acceptable matches to *Rhodobacter capsulatus* (0.625), *Rhodococcus erythropolis* (0.496) and *Micrococcus luteus* (0.338), respectively.
Several other isolates matched to known organisms at less than 30% similarity.

A principal components analysis based on similarity between fatty acid methyl ester profiles, divided the isolates into four clusters (Fig. 1). Library entries are included to provide perspective to the plot. Two isolates, YM-29 and SR-21, exhibited chromatographic peaks not identifiable using the MIDI-FAME library. When further analyzed with GC-mass spectrometry, this sample were found to contain a series of long chain aldehydes (C18:0-C21:0) previously found in gram-positive anaerobes (White, 1995).

**EPS Extraction and Purification from Pure Cultures**

Yields of extracted EPS varied significantly among the isolates and some required a second extraction to obtain the amount necessary for compositional analysis. Protein content of all eight purified exopolymers was below limits of detection.

**Compositional Analysis of EPS**

Table 2 presents the glycosyl composition of the
eight purified exopolysaccharides. There was considerable diversity in the composition of the exopolymers, but most contained common monosaccharide components. One notable exception was the EPS from isolate SR-21, which contained a rare 2-amino-6-dideoxyhexose. Six naturally occurring 2-amino-6-dideoxyhexoses have been identified, including those with D- and L-gluco, D- and L- galacto, L-manno, and L-talo configurations (Lindberg, 1990). While the exact configuration of the sugar in the SR-21 EPS could not be determined, the D-gluco isomer could be ruled out as the retention time did not match the reference standard.

**Carbohydrate Extraction from Yucca Mountain Rock**

Although several attempts were made, no carbohydrate component could be directly extracted from Yucca Mountain rock. As much as 300 g of rock was extracted with no detectable results.

**DISCUSSION**

In tests evaluating EPS-production capability of heterotrophic microbes on a variety of carbon sources,
the surface-impacted isolates (YM-SR) exhibited the highest potential (46%) as compared to the isolates obtained from the non-impacted rock (25%). This result correlates with the fact that in the initial characterization studies of Yucca Mountain, the highest community percentage of EPS-producers was observed in the surface-impacted rock (4.2%). Additionally, the EPS-producing isolates from YM-SR possessed greater metabolic versatility when presented with a variety of carbon sources (Table 1). This suggests that contamination during repository construction could significantly enhance growth of introduced EPS-producing populations potentially capable of microbially-influenced corrosion.

Several subgroups of EPS-producing microorganisms were identified in the semi-quantitative assay (i.e., low production on citrate, acetate and Tween-80, enhanced production on low-nutrient media). This information will help to make predictions regarding metabolic behavior of these microorganisms when exposed to exogenous carbon and nutrient sources during
repository construction. Even if construction conditions attempt to limit external contamination, human activity and more importantly, water input (1000 gallons/ft.) will undoubtedly influence the metabolism and activity of these microbes and their subsequent contribution to microbially-influenced corrosion (Haldeman et al., 1995, Haldeman et al., 1994).

The characterization of specific EPS-producing isolates from Yucca Mountain has resulted in a more complete understanding of this group of microbes. MIDI-FAME analysis identified only 25% of the isolates, indicating that these microorganisms are unique and/or have not been previously described and placed in the CEB data base (the most comprehensive for subsurface microbes). Lack of identification has been observed in microbial analysis of other subsurface environments (Haldeman et al., 1993, Balkwill, 1989). The presence of long-chain aldehydes in two of the isolates (YM-29, SR-21) also illustrates the uniqueness of these subsurface EPS-producers. However, adequate characterization of subsurface microbes awaits
compilation of information in a comprehensive data base and confirmation of techniques such as MIDI-FAME (based on membrane fatty acids) with rDNA sequencing.

The compositional analysis of the eight exopolymers showed great diversity when the isolates were grown under standardized conditions on R2A medium (Table 2). These compositions may not necessarily correlate with EPS-production under natural conditions, since EPS composition has been demonstrated to change when microorganisms are provided with different carbon sources and other nutrients (Cerning et al., 1994, Buckmire, 1984, Duguid and Wilkinson, 1958). However, R2A medium contains small quantities of many substrates, which more likely stimulates the organic carbon makeup of the tuff than a glucose-rich high growth yield laboratory medium. The compositional analysis presented in this paper, is the first performed on subsurface bacterial exopolymers.

EPS-producing microbes have been documented to promote corrosion both indirectly, through biofilm formation and, directly by differential aeration cell
formation (Geesey, 1991). Additionally, various acidic
groups associated with the exopolymer composition might
also enhance corrosive effects. Five of the eight
exopolymers analyzed (YM-4, YM-33, YM-37 SR-21 and SR-
25) had compositions which included glucuronic and/or
galacturonic acid residues indicating that these
microbes might exhibit enhanced corrosivity.

Direct carbohydrate extractions from Yucca Mountain
rock were unsuccessful. If present, carbohydrate levels
were below limits of detection with the methods
utilized. This may be a reflection of low in-situ
activity of EPS-producing microbes in the endolithic
environment (Kaiser and Bollag, 1990, Lappin-Scott and
Costerton, 1990) or an indication of low EPS persistence
(i.e., rapid turnover) which has been observed in soil
ecosystems (Cheshire, 1979). The former hypothesis is
favored because deep subsurface microbes have been shown
to commonly exist under starvation-survival conditions
(Amy et al., 1993).
Acknowledgments

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Starvation-survival of deep subsurface isolates.


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(1990) Influence of interfaces on microbial


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Albersheim P (1985) Isolation and characterization
of plant cell walls and cell wall components.
*Methods Enzymol* 118:3-40.
Figure 1. Principal components analysis of Yucca Mountain exopolysaccharide-producing isolates.
| 33.93 | *Micrococcus roseus*  
|■ D O O C Q.  
|■ DO Q. Cg | • Rhodobacter capsulatus (YM-33)  
| 21.82 | • Archaeobacter globiformis  
|■ D O O C Q.  
|■ DO Q. Cg | • Archaeobacter atrocyaneus (SR-7)  
|■ D O O C Q.  
|■ DO Q. Cg | • Micrococcus luteus (SR-25)  
|■ D O O C Q.  
|■ DO Q. Cg | • Bacillus macerans (YM-1)  
|■ D O O C Q.  
|■ DO Q. Cg | • Clavibacter michiganense (YM-40)  
|■ D O O C Q.  
|■ DO Q. Cg | • Pseudomonas aeruginosa  
| 9.70 |  
|■ D O O C Q.  
|■ DO Q. Cg | • Nocardodes albus (SR-8)  
|■ D O O C Q.  
|■ DO Q. Cg | • no identification (YM-9)  
| -2.41 |  
|■ D O O C Q.  
|■ DO Q. Cg | • Acinetobacter johnsonii  
|■ D O O C Q.  
|■ DO Q. Cg | • Rhodococcus erythropolis (YM-37)  
|■ D O O C Q.  
|■ DO Q. Cg | • Gordona bronchialis (YM-29, SR-21)  
| -14.5 |  
|■ D O O C Q.  
|■ DO Q. Cg |  
| -26.7 |  
|■ D O O C Q.  
|■ DO Q. Cg |  

* represents FAME library entries to give perspective to the PCA plot.
Table 1. EPS-Production on Specific Carbon Sources

<table>
<thead>
<tr>
<th>Sample</th>
<th>YM-RF*</th>
<th>YM-LF*</th>
<th>YM-SR*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% PTYG Agar</td>
<td>33%</td>
<td>33%</td>
<td>31%</td>
</tr>
<tr>
<td>R2A Agar</td>
<td>92%</td>
<td>67%</td>
<td>85%</td>
</tr>
<tr>
<td>Luria Agar</td>
<td>75%</td>
<td>33%</td>
<td>69%</td>
</tr>
<tr>
<td>1% Casamino Acids/Glucose Agar</td>
<td>75%</td>
<td>77%</td>
<td>100%</td>
</tr>
<tr>
<td>0.2% Citrate Agar</td>
<td>8%</td>
<td>17%</td>
<td>8%</td>
</tr>
<tr>
<td>0.2% Acetate Agar</td>
<td>8%</td>
<td>17%</td>
<td>8%</td>
</tr>
<tr>
<td>1% Starch Agar</td>
<td>25%</td>
<td>50%</td>
<td>31%</td>
</tr>
<tr>
<td>1% Tween 80 Agar</td>
<td>17%</td>
<td>0%</td>
<td>15%</td>
</tr>
<tr>
<td>1% Glucose Agar</td>
<td>67%</td>
<td>67%</td>
<td>77%</td>
</tr>
<tr>
<td>1% Galactose Agar</td>
<td>50%</td>
<td>17%</td>
<td>77%</td>
</tr>
<tr>
<td>1% Glucose/Galactose Agar</td>
<td>67%</td>
<td>77%</td>
<td>77%</td>
</tr>
</tbody>
</table>

* YM-RF and YM-LF are rock samples not impacted by surface contamination; YM-SR is a surface-contaminated rock sample.
Table 2. Glycosyl composition (mole %) of eight Yucca Mountain exopolysaccharides

<table>
<thead>
<tr>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.0</td>
<td>8.7</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>65.8</td>
<td>69.3</td>
<td>21.2</td>
<td>45.1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>27.5</td>
<td>40.5</td>
<td>13.7</td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>1.8</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.3</td>
</tr>
<tr>
<td>Glucuronic Acid</td>
<td>2.3</td>
<td>0.8</td>
<td></td>
<td>1.6</td>
<td></td>
<td></td>
<td>1.5</td>
<td>0.4</td>
</tr>
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<td>Galacturonic Acid</td>
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<td></td>
<td></td>
<td></td>
<td>29.6</td>
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<td></td>
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<tr>
<td>Mannose</td>
<td></td>
<td></td>
<td>0.4</td>
<td>1.7</td>
<td>25.7</td>
<td>1.2</td>
<td>3.2</td>
<td>36.0</td>
</tr>
<tr>
<td>Galactose</td>
<td>23.5</td>
<td>25.3</td>
<td>28.5</td>
<td>2.6</td>
<td>5.6</td>
<td>2.8</td>
<td>34.9</td>
<td>11.0</td>
</tr>
<tr>
<td>Glucose</td>
<td>6.5</td>
<td>0.6</td>
<td>20.3</td>
<td>26.6</td>
<td>49.0</td>
<td>83.5</td>
<td>11.3</td>
<td>52.6</td>
</tr>
<tr>
<td>N-acetyl galactosamine</td>
<td>3.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-amino-6-deoxy hexose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8.4</td>
</tr>
</tbody>
</table>

*mole% is calculated from the GC-mass spectrophotometer (electron impact) total ion current peak areas of the trimethylsilyl methyl glycoside derivatives, with response factor correction.
CHAPTER 4

EFFECT OF GAMMA RADIATION ON INDIGENOUS MICROBES OF YUCCA MOUNTAIN, NEVADA TEST SITE

This chapter has been prepared for submission to Radiation Research and is presented in the style of that journal. The complete citation is:

ABSTRACT

Specific bacterial types that contribute to microbially-influenced corrosion, such as sulfate-reducing, iron-oxidizing, acid-producing and exopolymer-producing (EPS) bacteria are readily recovered from natural environments. As each group has been isolated from rock at the site of the proposed nuclear waste repository at Yucca Mountain, it is important to determine what long-term effects the environmental conditions at the facility will have on their survival. A timecourse experiment was conducted to evaluate the effects of gamma radiation on the indigenous microbiota present at this site. Microcosms were constructed by placing pulverized Yucca Mountain rock in polystyrene cylinders. A relatively low dose rate (163 rads/min) was used to mimic expected emission from an unbreached canister (200 mRem/hr over 20 m²) with continuous exposure (96 hrs). The microbial communities were characterized after receiving cumulative doses of 0, 9.8, 57.8, 233.1, 466.9, 700.9 and 934.4 Krads. Radiation-resistant microorganisms in the pulverized rock became viable but non-culturable (VBNC) after a 233.1 Krad
radiation dose. Two representative exopolysaccharide-producing isolates from Yucca Mountain were exposed to the same radiation regime in sand microcosms. One isolate was much more radiation-resistant than the other, but both had greater resistance than the entire community.

INTRODUCTION

In 1982, Congress passed the Nuclear Waste Policy Act, under which the federal government assumed responsibility for management of spent nuclear fuel from the nation's nuclear power plants (1). Several potential permanent disposal sites for high-level nuclear waste, were identified, but in 1987, the choice of sites was limited to Yucca Mountain, Nevada Test Site. Yucca Mountain has, therefore, become the focus of an extensive site-characterization project.

In the evaluation of long-term repository integrity at Yucca Mountain one factor which must be considered, is waste package design. The waste canister must provide an effective barrier to the surrounding environment for the expected 300 to 1000 year containment period (2). While the physical, chemical and radiation stability of the
proposed canister design have been considered, the effects of biotic forces have been neglected (2). Microbially-influenced corrosion (MIC) of metal and other materials, is caused by several metabolic types of bacteria, including sulfate-reducers, iron-oxidizers, acid-producers and exopolysaccharide-producers, all of which have been isolated from Yucca Mountain rock (2, 3). Therefore, it was important to determine how repository conditions (i.e., long-term gamma radiation exposure) impact survival and activity of indigenous microbes, including MIC-implicated groups (4-6).

Exopolysaccharide (EPS)-producing microbes require special consideration as they are responsible for biofilm formation that facilitates MIC (7, 8). The EPS matrix has been shown to provide microbes with protection from predation, antimicrobial agents and desiccation, and thus, their potential role in radiation-resistance should not be overlooked (7, 9).

The isolation of radiation-resistant microbes in environmental situations has mostly occurred in areas exposed to high radiation fields (i.e., nuclear power
facilities). Microorganisms in the reactor core after the Three Mile Island disaster were growing under radiation doses of 10 Gy/h (10). In addition, Mergeay et al. (11) discovered microbes in primary cooling systems of nuclear reactors undergoing a radiation flux of $5 \times 10^{14}$ neutrons/cm$^2$.s. However, efforts to evaluate the effects of gamma radiation on indigenous communities of microorganisms have not received much attention or has been poorly documented (12-14). Therefore, as the underground repository storage of nuclear fuel is imminent, it is imperative to evaluate the radiation effects on microbial communities under projected repository conditions. Results from this type of work could help scientists predict the stability of storage canisters to MIC.

MATERIALS AND METHODS

*Microcosm construction for microbial community irradiation*

Welded volcanic tuff (moisture content 4.0%) was obtained from an alcove at 60 m depth within the north portal exploratory shaft at Yucca Mountain. The sample
for this experiment was separated from surface contamination by careful aseptic sampling methods (15-17).

As culturable microbes were in low abundance \(10^2-10^3\) cells/gram dry weight (gdw) in the native rock, rock was aseptically pulverized using a sterile mortar and pestle, adjusted to 10% gravimetric water content with sterilized deionized water and held at 25°C for 14 days. The rock was then thoroughly homogenized before being subdivided into the 24 microcosms utilized for this experiment. Microcosms were constructed by placing 100 g of pulverized rock into sterile polystyrene graduated cylinders (Corning, Corning NY). All transfers were conducted in a laminar flow hood (Forma Scientific, Marietta, OH).

Microcosm construction for irradiation of exopolysaccharide producing isolates

Two exopolysaccharide (EPS)-producing isolates (YM-37 and SR-25) from the Yucca Mountain site were used. YM-37 was collected from rock separated from surface microbial contamination while SR-25 was obtained from a surface-impacted sample. YM-37 and SR-25 were acceptably identified by MIDI-FAME (Microbial ID, Inc., Newark, DE-
fatty acid methyl ester analysis) as *Rhodococcus erythropolis* (0.496) and *Micrococcus luteus* (0.338), respectively.

Microcosms for pure culture irradiation were prepared by placing 100 g of sterilized -50+70 mesh white quartz sand, (Sigma Chemical, St. Louis, MO) in sterile polystyrene graduated cylinders. Bacterial isolates (YM-37, SR-25) were grown at 25°C to stationary phase (10⁷ cells/ml), in R2B (yeast extract, 0.5 g; proteose peptone, 0.5 g; casamino acids, 0.5 g; glucose, 0.5 g; soluble starch, 0.5 g; sodium pyruvate, 0.3 g; K₂PO₄, 0.3 g; MgSO₄·7H₂O, 0.05 g per liter, pH 7.2). Microcosms were inoculated with 10 ml of culture suspension (to 10% sand water content) and thoroughly mixed. Microcosms were allowed to incubate at 25°C for 7 days prior to irradiation so that inoculated microbial populations could become accustomed to the microcosm environment.

Radiation regime

A ⁶⁰Co ionizing radiation source, located at EG&G (Las Vegas, NV), was used to expose the microcosms to gamma emissions. The dose rate was 163 rads/min at a
temperature of 24.5°C. Microcosms were randomly placed in the radiation field and periodically removed for analysis. The following were the cumulative radiation doses: 1 hour (9.8 Krads), 6 hours (57.8 Krads), 24 hours (233.1 Krads), 48 hours (466.9 Krads), 72 hours (700.9 Krads), and 96 hours (934.4 Krads). A preliminary check was made to ensure the calculated dose rate of 163 rads/min was uniformly irradiating each microcosm. Thermoluminescent dosimeters were placed in the center of 18 mock microcosms. The microcosms were then placed in the 60Co beam at a distance of 1 meter from the source and irradiated for 30 s.

Microbiological analysis of microcosms during irradiation

Sample preparation.

Slurries (1:10 w/v) in sterile 0.1% sodium pyrophosphate (pH 8.0), were shaken at 125 rpm for 1 hour at 24°C. For community analysis, slurries were prepared from each of three microcosms sampled at 0, 1, 6, 24, 48, 72 and 96 hrs. For EPS-isolate analysis, three microcosms were analyzed at 0, 48 and 96 hrs.
**Microbial cell counts.**

To measure total microorganism density, samples were prepared for acridine orange direct counts by the method of Haldeman et al. (18-19). This protocol was designed for use with subsurface rock samples.

To measure viable densities of microorganisms, the respiring cell count procedure of Rodriquez et al. (20) was modified such that 5.0 mM 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences, Inc.) was prepared in R2B.

Culturable counts were determined by serially diluting slurries in phosphate buffered saline (PBS), pH 8.0 and plating in triplicate on R2A agar (21). After 14 days incubation at 24°C, culturable counts were calculated, and EPS-producing microorganisms were identified by colony morphology, including large, glistening and/or runny appearance.

**Survival curve construction.**

To compare the radiation resistance of both the microbial community and the two EPS-producing isolates,
D$_{10}$ and D$_{10}$ values were calculated from survival curves based on culturable counts. D-values were calculated for both non-shouldered and shouldered survival curves as described by Harm (22). The equation used for a non-shouldered curve was:

1. \[ \frac{S}{S_0} = e^{-cF} \]

where \( S/S_0 \) is the ratio of survivors at dose \( F \); and \( -c \) is the slope of the exponential portion of the curve. The D-value is thus equivalent to \( F \) if the percent of survival and the slope are known. The equation used for a shouldered curve was:

2. \[ n = e^{n_{0.37}F \ or \ 0.10} \]

where \( n \) is the y-axis intercept of the regression line for the exponential portion of the curve; \( F_t \) is the threshold dose (the length of the shoulder); and \( F_{0.37} \ or \ 0.10 \) is the dose calculated in equation #1 which resulted in a 37% or 10% survival rate of the population. The D-value, in this case, is defined as the sum of \( F \) (from equation 1) and \( F_t \) (23).

**MPN (most probable number) tests.**

To estimate the number of acidophilic iron-oxidizing
bacteria, a five-tube most probable number (MPN) analysis was performed using a medium previously described (24). All MPN tubes were incubated at room temperature under aerobic conditions for 6 wk before scoring. Putative positive tubes were confirmed by subculture on a solidified medium (1.5% purified agar) with the same nutrient formulation (25). MPN tubes for sulfate-reducing bacteria were prepared with a lactic acid enrichment medium (24, 26) and incubated under anaerobic conditions.

**Metabolic analysis.**

Metabolic capability of the microbial community throughout the radiation regime was assessed using BIOLOG microtiter plates (27-28). Microtiter plates for both gram positive (GP) and negative (GN) organisms were utilized for this experiment, resulting in carbon source utilization profiles containing 128 unique compounds. Slurries were diluted (1:1, vol:vol) with filter-sterilized PBS (pH 8.0) prior to inoculation. Plates were incubated for 14 days at 24°C and read both manually and spectrophotometrically using a Titertek Multiscan Plus plate reader with a 590 nm filter. Positive wells were
distinguished by visible formazan formation (purple).

**Lipid analysis.**

Phospholipid fatty acid (PLFA) analyses were performed on the community-level experiments to assess viable community biomass, composition and stress status as described by Kieft et al. (29). Diglyceride fatty acids were also quantified to assess non-viable biomass by a previously described method (30, 31). Duplicate samples were analyzed before irradiation and at doses of 9.8, 57.8, 233.1, 466.9, 700.9 and 934.4 Krads.

**RESULTS**

*Microbiological analysis of microcosms during irradiation*

**Microbial cell counts.**

Microbiological cell counts for the community-level experiment are depicted in Fig. 1. Direct counts (AODC) and respiring counts (CTC) fluctuated over the radiation regime but remained within one order of magnitude of their original value ($10^9-10^4$ cells/gdw respectively). Culturable counts were one to two magnitudes lower than
direct and respiring counts at the onset of the experiment but quickly fell to below detectable limits after a cumulative dose of 233.1 Krads. The fact that a decrease in respiring counts (CTC) was never observed is consistent with the hypothesis that these microbes are entering a viable but non-culturable (VBNC) state. This pattern has been observed in other studies (32) that have employed direct methods to assess and detect cell viability. EPS-producing bacteria were detected and quantified in the non-irradiated community and rock that had received a radiation dose of 9.8 and 57.8 Krads, at values of 8.5 x 10^5, 3.18 x 10^5 and 2.65 x 10^3, respectively. While these numbers vary, EPS-producers always constituted approximately 4.0% of the culturable population.

**MPN tests.**

Most probable number enumerations of acidophilic iron-oxidizing microorganisms indicated their presence in the non-irradiated original community (0.32 +/- 0.11 cells/gdw) as well as at 9.8 Krads (0.41 +/-0.02 cells/gdw). Microbial numbers in the remaining irradiated samples were below limits of detection using this method.
Sulfate-reducing microorganisms were not detected either before or throughout the radiation regime.

**Metabolic analysis.**

Carbon source utilization of irradiated microbial communities was measured using BIOLOG plates (Table I). A decreasing trend in metabolic activity with increasing radiation dose was noted. The original non-irradiated community exhibited the highest metabolic diversity, using carbon sources in all major categories represented on BIOLOG plates (e.g., carbohydrates, carboxylic acids, polymers, amines/amides, amino acids). At higher radiation doses, utilization was limited to simple sugars, a few amino acids and very few carboxylic acids.

**Lipid analysis.**

PLFA abundance (viable cells) decreased and DGFA abundance (non-viable cells) increased over the course of the radiation regime (Figure 2). The PLFA/DGFA ratio shows a pattern of decline (five-fold) over the first three radiation dosages. The stabilization of the ratio over the last three timepoints, indicated the presence of
a viable but non-culturable (VBNC) radiation-resistant population of microorganisms.

The viable community composition, as determined by PLFA analysis, is depicted in Figure 3. In general, saturation of fatty acids increased with cumulative radiation dose while unsaturation decreased. The normal terminally branched and mid-chain branched saturates all increased in relative (mole) percentage. This pattern has been observed in other stressed subsurface environments as well as in studies utilizing pure cultures (29, 33).

Microbial cell counts for individual EPS-producing isolates.

Cell counts for the EPS-producing isolates, YM-37 and SR-25 are shown in Figure 4. While both respiring and culturable counts for YM-37 were initially equivalent (10⁸ cells/gdw), the culturable counts dropped several orders of magnitude by 466.9 Krads (Figure 4A). Culturable counts continued to drop to below limits of detection by the end of the radiation regime (934.4 Krads) but respiring counts remained relatively constant. Thus, it appeared that cells remained viable but non-culturable
(VBNC).

While respiring counts for SR-25 followed the similar stable pattern observed with YM-37, the culturable count pattern varied (Figure 4B). No divergence of culturable count was observed until after 466.9 Krads. After this dose, the culturable count dropped and VBNC cells appeared to predominate in the microcosms.

**Survival curves.**

D-values were calculated from constructed survival curves for the microbial community as well as the two EPS-producing isolates (Table II). The lowest D-value was obtained from the microbial community ($D_{10} = 32.9\text{Krads}$) as compared to the two EPS-producing isolates, YM-37 and SR-25 ($D_{10} = 135.4$ and $441.6\text{ kRads}$, respectively).

**DISCUSSION**

It is well documented that perturbations such as sampling, sample handling (i.e. grinding), and water addition redistribute nutrients in rock material making them more available to microbes for utilization (22-24). This information was used to construct microcosms, as
microbes were in low abundance in the native rock (10^2-10^3 cells/gdw) and it would have been otherwise difficult to document the expected response to gamma radiation.

The microbial community lost culturability and showed significantly reduced metabolic capacity after 24 hours of radiation exposure (233.1 Krads), however, other measurements of survival (i.e., respiring cell counts and PLFA abundance) indicated retained viability (Fig. 1, 2 and Table I). This showed that irradiated bacteria entered a viable but non-culturable (VBNC) state likely due to radiation injury incurred by the cells (34). VBNC bacteria are present in many natural environments (34-36) and various stress factors have been documented to induce entrance into this physiologic state (37).

The dramatic decrease observed in both culturability (Fig. 1) and carbon source utilization (Table I) exhibited by irradiated microbes is not unlike that observed in marine bacteria undergoing a starvation stress response (38). Additionally, this appears to be a consistent physiologic change noted in viable but non-culturable (VBNC) cells (39). Carbon source utilization profiles also

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changed drastically as the radiation dose increased. Bacterial members of the original non-irradiated community were capable of using a variety of carbon sources while the microbial community at higher dosages were limited to simple sugars and amino acids. This is undoubtedly a stress response, as microbes are attempting to funnel their energy into DNA repair rather than enzyme production for elaborate metabolic pathways.

While there were some fluctuations in the CTC respiring cell counts, no decrease in cell numbers (i.e. viability) was demonstrated as radiation dose increased (Fig. 1). Further supporting evidence for the VBNC hypothesis is provided by PLFA viable biomass estimates, which showed a five fold decrease (< one log of bacterial cells) by 233.1 Krads but remained constant as radiation dose increased. DGFA non-viable biomass estimates increased slightly as radiation dose increased. The discrepancy (< one log of cells) observed between CTC counts and PLFA estimates may be explained by abiotic reduction that may occur in the CTC assay. Abiotic reduction of CTC has been documented, either in reducing
environments or in the presence of reducing agents and can result in elevated cell counts (40). As environmental samples are largely undefined systems, CTC results must be interpreted with caution. Autoclaved controls run in tandem with each analysis detected no abiotic reduction.

PLFA functional group analysis has also been used to document responses of microbial communities to other environmental stresses such as desiccation (29). Similar to the response observed in this experiment (Fig. 3), Kieft et al. (29) noticed an increase in saturated fatty acids when bacteria were exposed to long-term desiccation. Changes in fatty acid saturation presumably allow the cells to maintain membrane fluidity as they enter a non-culturable state and may represent a survival response (39).

As part of the community analysis, specific microbial types implicated in MIC, were evaluated for survival to gamma radiation. Results indicated that all of these groups (i.e., EPS-producers, iron-oxidizers and sulfate-reducers) were in low numbers within the natural community and, therefore, could not be adequately assessed with the
cultural methods utilized.

The two EPS-producing isolates which were individually tested, demonstrated increased radiation resistance when compared to the whole community (Table II). SR-25 (Micrococcus luteus) showed the most significant survival pattern with a $D_{10}$ of 441.9 Krads. This value approached that previously observed in Deinococcus radiodurans ($640 \pm 180$ Krads), the most radiation resistant microbe currently known, and a close phylogenetic relative to Micrococcus luteus (23, 41). The shouldered curve (Fig. 4B) is an indicator that this microbe was capable of overcoming sublethal effects caused by gamma irradiation. YM-37 also showed a moderate resistance with a $D_{10}$ of 135.4 Krads (Fig. 4A). Whether the observed resistance to radiation by these isolates can be attributed to EPS-production or efficient DNA repair mechanisms is not known. In comparison, the calculated $D_{10}$ value for the microbial community was relatively low, at 32.9 Krads (Table II). This observation can be compared to similar values (17-26 Krads) obtained when individual subsurface bacterial isolates were tested by Arrage et al.
Comparisons of radiation-resistance based on D-values must be made with caution as dose rates and test conditions used often vary between experiments and can impact results. Historically, determinations of radiation-resistance are calculated from survival curves based on culturable counts (12, 41). While this approach may be acceptable under certain conditions, its application to results presented in this paper is not appropriate. If D-values had been based on viable but non-culturable cell determinations, then results would indicate a much more radiation-resistant community or isolate. These results have significant implications for the Yucca Mountain repository as they indicate that indigenous microbes are capable of surviving gamma irradiation in a VBNC state.

ACKNOWLEDGEMENTS

This research was supported by the University of Nevada, Las Vegas-Yucca Mountain Project Office Cooperative Agreement under contract (DE-FC08-90NV10872) with the United States Department of Energy. Support also came from the National Science Foundation Nevada EPSCoR Women in Science Program. We gratefully acknowledge the
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Thorngren, Mr. Henry Crossen and Ms. Patricia Castro for
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Figure 1. Log transformed microbiological cell counts/gdw for community-level gamma irradiation experiment.
Figure 2. Ratio of the abundance of PLFA (viable) to DGFA (non-viable) cells.
Figure 3. Microbial community composition based on PLFA (viable) functional group distribution.
Figure 4. Log transformed culturable and respiring cell counts/gdw for the EPS-producing isolates, YM-37 (A) and SR-25 (B).
Table I. Carbon source utilization by microbial communities before and throughout gamma irradiation*

<table>
<thead>
<tr>
<th>Radiation dose (Krads)</th>
<th>% Substrates utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>82.6%</td>
</tr>
<tr>
<td>9.8</td>
<td>22.0%</td>
</tr>
<tr>
<td>57.8</td>
<td>26.0%</td>
</tr>
<tr>
<td>233.1</td>
<td>12.1%</td>
</tr>
<tr>
<td>466.9</td>
<td>3.2%</td>
</tr>
<tr>
<td>700.9</td>
<td>1.1%</td>
</tr>
<tr>
<td>934.4</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

*Table entries represent the percentage of carbon sources (128 total) utilized by microbial communities throughout the gamma irradiation regime as determined by BIOLOG.
Table II. D-values* from bacterial survival curves to gamma radiation

<table>
<thead>
<tr>
<th>Sample</th>
<th>$D_{37}$</th>
<th>$D_{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yucca Mountain microbial community</td>
<td>14.2</td>
<td>32.9</td>
</tr>
<tr>
<td>YM-37</td>
<td>58.5</td>
<td>135.4</td>
</tr>
<tr>
<td>SR-25</td>
<td>190.8</td>
<td>441.6</td>
</tr>
</tbody>
</table>

* D-values are defined as the gamma radiation dose (Krads) which reduced the cell population to either 10% or 37% of the original number of cells. D-values were calculated from the regression line of the exponential slope of the survival curve as described in Materials and Methods.
CHAPTER 5

RESUSCITATION OF MICROBES FROM GAMMA IRRADIATED
YUCCA MOUNTAIN ROCK

This chapter has been prepared for submission to
Radiation Research and is presented in the style of that
journal. The complete citation is:

Pitonzo, B.J. and P.S. Amy. 1996. Resuscitation of
microbes from gamma irradiated Yucca Mountain rock.
ABSTRACT

Microbiological analysis of Yucca Mountain rock, which had been gamma-irradiated up to a dose of 934.4 Krads, indicated that some microorganisms became viable but became non-culturable (VBNC), and lost metabolic capacity as measured by BIOLOG microtiter plates. To investigate this phenomenon, portions of irradiated rock were placed at 4°C for 2 months to resuscitate the microbes to a culturable state. Culturable heterotrophs were enumerated and BIOLOG plates were inoculated to determine their metabolic capability. Culturable bacteria were found at all radiation doses which had previously been non-culturable. Only 9-10 distinct colony types were recovered compared to 26 from non-irradiated samples. BIOLOG plates indicated partial recovery of metabolic capacity in all the tested samples. MIDI-FAME of the recovered isolates yielded a dendrogram containing three distinct relatedness groups. All resuscitated isolates clustered with the original non-irradiated isolates at the genus level and 92% of them clustered at the species level. Resuscitation of
microbes from a viable but non-culturable state in irradiated rock has significant implications for the proposed nuclear waste repository at Yucca Mountain.

INTRODUCTION

Plate counts used to enumerate culturable microorganisms can dramatically underestimate the total number of bacteria present in samples taken from natural environments (1). In the late 1970's, development of several new protocols for determining cell viability indicated that many non-culturable cells were indeed viable and able to actively metabolize (2, 3). In response to certain environmental stresses (i.e., radiation, high/low temperature, osmotic shock, low-nutrients, etc.), some microorganisms lose the ability to grow on media with which they had been routinely cultured, entering a viable but non-culturable state (VBNC) (4, 5). The apparent reduction in metabolic and physiologic processes in VBNC microbes may allow energy reserves to be funnelled into necessary repair processes for recovery. A decrease in endogenous metabolism has
previously been shown to be a primary survival strategy of bacteria existing under starvation conditions (6). The list of bacteria which enter the VBNC state has increased rapidly and includes both gram-positive and gram-negative genera. However, most work on VBNC has been concentrated on gram-negative species or genera (7,8,9).

The VBNC state is currently thought to be reversible (10). Reversal or "resuscitation" appears to require an "inducing factor" (e.g., a temperature or osmotic change or nutrient addition) and is quite slow, often requiring days or weeks (4, 6, 11, 12). If the VBNC state is truly a survival strategy invoked by stressed microbes, then resuscitation would be an essential component to the process. Whereas entry into the non-culturable state may in some manner protect the cell against one or more environmental stresses, resuscitation to a state of potential rapid metabolism would allow the cell to actively compete in the environment.

In the case of gamma irradiation, extensive DNA
damage induces the SOS response, the SOS response includes decreased metabolic activity and inhibition of cell division, resulting in a temporarily induced VBNC state (5, 13). If microbial cells contain an effective DNA repair mechanism (e.g., Deinococcus radiodurans) (14) it is plausible to consider that recovery or "resuscitation" might occur after a period of time. In this paper, we describe the survival and subsequent resuscitation of gamma-irradiated indigenous microbiota at Yucca Mountain. This phenomenon may have significant implications for evaluation of potential biotic influences (i.e., microbially-influenced corrosion) in the repository environment as it indicates that microbes may be capable of surviving sub-optimal conditions caused by radioactive waste burial.

MATERIALS AND METHODS

Sample exposures

Irradiated rock samples that exhibited no culturable heterotrophic count on R2A were chosen for resuscitation. These samples had received the following
cumulative doses of gamma radiation: 24 hours (233.1 Krads), 48 hours (466.9 Krads), 72 hours (700.9 Krads) and 96 hours (934.4 Krads). A non-irradiated sample was also tested for comparison. Immediately following gamma-irradiation rock samples were placed at 4°C for 2 months, under sterile conditions (7).

Microbiological analysis

Rock samples were analyzed immediately after removal from refrigerated conditions. Slurries prepared (1:10 w/v) in sterilized 0.1% sodium pyrophosphate (pH 8.0), were shaken at 125 rpm for 1 hour at 24°C.

Culturable counts were determined by serially diluting slurries in phosphate buffered saline (PBS), pH 8.0 and plating in triplicate on R2A agar (Difco) (15).

Metabolic capability was assessed using BIOLOG microtiter plates (16, 17). Microtiter plates for both gram positive (GP) and gram negative (GN) were utilized for this experiment, resulting in carbon source utilization profiles containing 128 unique compounds. Slurries were diluted (1:1, vol:vol) with filter-
sterilized PBS (pH 8.0) prior to inoculation. Plates were incubated for 14 days at 24°C and read both manually and spectrophotometrically using a Titertek Multiscan Plus plate reader with a 590 nm filter. Positive wells were distinguished by visible formazan formation (purple).

Fatty acid methyl ester (FAME) analysis was performed on all 12 resuscitated isolates as well as 26 isolates obtained from the non-irradiated original community. For MIDI analysis, fatty acids were extracted and methyl esterified from bacterial cultures grown for 24 hours on R2A according to the specifications of the manufacturer (Microbial ID, Inc., Newark, NJ). A dendrogram was generated by the MIDI system that clusters isolates into relatedness groups on the basis of their membrane fatty acid content. Their relatedness was measured by Euclidean distance. Although no formal interpretation of the Euclidean distance scale relating to microbial taxonomy exists, results obtained by others (18, 19) suggest that samples linked below 10 and 25 Euclidean distance units are from...
the same species and genus, respectively.

RESULTS

Culturable cell counts

After 2 months resuscitation, culturable counts were detected in rock samples at all doses which were previously non-culturable (Fig. 1). The counts were several orders of magnitude lower \((10^3 - 10^4)\) than those observed in the non-irradiated sample \((10^7)\) (Fig. 1). Nine to ten distinct microbial types were isolated from the resuscitated samples, based on colony morphology, compared to the 26 isolated from the original non-irradiated sample \((19)\). This indicates that a portion of the original microbial community was capable of resuscitation from the VBNC state induced by gamma radiation injury.

A constant culturable count was obtained \((10^7\) cells/gdw) in the non-irradiated sample (before and after resuscitation), indicating that the resuscitation regime did not affect microbial numbers positively or negatively. In addition, the non-irradiated community
composition after resuscitation, as determined by observed colony morphology, was not significantly different from that observed before resuscitation. This supports the idea that the microorganisms resuscitated from the treated samples were representative of the original community and not a result of outgrowth of a few organisms selected for under low temperature conditions.

**BIOLOG carbon utilization assay**

Carbon utilization by gamma-irradiated resuscitated microbial communities, as determined by BIOLOG analysis, is presented in Table I. All resuscitated samples showed recovery of metabolic activity compared to samples tested immediately after gamma irradiation (Table I). This pattern of partially recovered physiological response is indicative of resuscitation from the VBNC state (10). However, the percentages of carbon source utilization in resuscitated samples (23-31%) did not approach that in the original non-irradiated community (80%). This suggests that the
metabolic response is due to a portion of the original community which survived and persisted after gamma irradiation.

While the original non-irradiated community used carbon sources in all major categories represented in BIOLOG plates (i.e., carbohydrates, carboxylic acids, polymers, amines/amides and amino acids), the resuscitated communities predominately used carbohydrates and amino acids. Few polymers (e.g., glycogen, dextrin, Tween-40 and Tween-80) were used by resuscitated communities was also noted.

*MIDI-FAME analysis of resuscitated isolates*

The three main bacterial relatedness groups determined by MIDI analysis are shown in Table II. Microorganisms were resuscitated from all three groups and these values approximated the percentages observed in the original community (i.e., gram-positive- 50%, gram-negative- 30% and Gordona/Rhodococcus- 20%). All of the resuscitated isolates clustered with isolates obtained from the non-irradiated community at the genus
level, with 92% of them clustering at the species level. This strongly suggest that the original microorganisms were resuscitated from a VBNC state induced by gamma radiation exposure.

**DISCUSSION**

Resuscitation of microorganisms from a viable but not culturable (VBNC) state remains a contested issue in microbiology (1, 10). Many resuscitation studies have relied on nutrient additions to stimulate VBNC cells to culturability (1, 10, 12, 20). Whether true resuscitation of VBNC cells occurred under these conditions, or whether a few culturable cells below limits of detection with bacteriological culture methods multiplied as a result of the added nutrient is difficult to prove. To circumvent the problems associated with introduced nutrients, Nilsson et al. (6) described the resuscitation of bacteria from microcosms without nutrient addition through a temperature upshift. The experimental design used in this paper parallels Nilssons work. At no time were nutrients added to the
irradiated rock and culturability was restored after incubation at a temperature (4°C) below ambient rock temperature (18°C).

The culturable heterotrophs detected in previously non-culturable samples, were low in number compared to the non-irradiated control. Resuscitation appears to require a considerable length of time as a more rapid recovery might be detrimental, in that a microbe may not be able to survive a second round of stress (10).

The return of metabolic capacity appeared to parallel the pattern observed in the culturable count (Table I). While there was a definite increase in metabolic capacity compared to the samples tested immediately after irradiation, recovery to values observed in the non-irradiated community was never attained. This might be explained by the fact that resuscitation is a slow process. In addition, recovery to the levels observed in the non-irradiated sample may never occur because some microbes are killed during the radiation regime. Thus, the final percentages and patterns observed may reflect the survival of more...
radiation-resistant members of the community. The observation that carbohydrates and amino acids were primarily utilized may also reflect the slow repair processes which occur in injured cells. The simpler carbon sources can be more easily incorporated (less energy cost) than more complex sources that require additional enzymatic processing before use.

Comparisons of fatty acid profiles between the original community and resuscitated isolates provided additional evidence supporting resuscitation. That 92% of the resuscitated isolates clustered at the species level with isolates of the original community makes it hard to argue that the results are due to external contamination.

The microorganisms recovered after the resuscitation regime were likely radiation-resistant members of the original microbial community. However, whether the presence of these cells can be attributed to a few "culturable microbes" present in the rock immediately after gamma irradiation remains unknown. Even though there was no exogenous nutrient addition, regrowth of a
few cells might occur due to nutrients released by moribund or dead cells present in the population (21). This scenario is a possibility as the detection limit used in the gamma irradiation experiment was 10 cells/gram of rock. However, nine replicate culturable counts (at each timepoint) were performed at the minimal dilution with no detectable colonies observed at any time.

Whether "true resuscitation" of VBNC occurred or a few undetectable culturable microorganisms survived the radiation regime, the implication for the Yucca Mountain repository remains clear. Microorganisms can survive a long-term gamma radiation exposure and, under proper conditions, regain metabolic activity and the ability to become functional members of the microbial community.

ACKNOWLEDGEMENTS
This research was supported by the University of Nevada, Las Vegas-Yucca Mountain Project Office Cooperative Agreement under contract (DE-FC08-90NV10872) with the United States Department of Energy. Support also came

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from the National Science Foundation Nevada EPSCoR Women in Science Program. We gratefully acknowledge Ms. Patricia Castro, Ms. Bethanie Thorngren and Mr. Theodore Lagadinos for their laboratory contributions.

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Fig. 1. Log transformed culturable counts of gamma irradiated rock samples after two months resuscitation at 4°C.
Table I. Carbon source utilization by resuscitated microbial communities

<table>
<thead>
<tr>
<th>Radiation dose (Krads)</th>
<th>% Substrates utilized</th>
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<td>0</td>
<td>80.6%</td>
</tr>
<tr>
<td>233.1</td>
<td>31.5%</td>
</tr>
<tr>
<td>466.9</td>
<td>23.4%</td>
</tr>
<tr>
<td>700.9</td>
<td>28.2%</td>
</tr>
<tr>
<td>934.4</td>
<td>29.0%</td>
</tr>
</tbody>
</table>

*Table entries represent the percentage of carbon sources (128 total) utilized by irradiated microbial communities after resuscitation at 4°C for 2 months as determined by BIOLOG.*
Table II. Percentage of non-irradiated and resuscitated bacterial isolates in each of three relatedness groups generated by MIDI-FAME analysis.

<table>
<thead>
<tr>
<th></th>
<th>Mixed Gram +</th>
<th>Mixed Gram -</th>
<th>Gordona/Rhodococcus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-irradiated isolates (26 total)</td>
<td>47.8%</td>
<td>30.5%</td>
<td>21.7%</td>
</tr>
<tr>
<td>Resuscitated isolates (12 total)</td>
<td>50.0%</td>
<td>33.3%</td>
<td>16.7%</td>
</tr>
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</table>
CHAPTER 6

EFFECT OF LONG-TERM DESICCATION ON INDIGENOUS MICROBES
OF YUCCA MOUNTAIN, NEVADA TEST SITE

This chapter has been prepared for submission to Applied and Environmental Microbiology and is presented in the style of that journal. The complete citation is:


145
ABSTRACT

Microbially-influenced corrosion (MIC) of storage canisters at the proposed nuclear waste repository at Yucca Mountain depends on the survival and subsequent activity of those indigenous microbes typically implicated in corrosion processes. Sulfate-reducing, iron-oxidizing, acid-producing and exopolysaccharide (EPS)-producing bacteria have been previously isolated from Yucca Mountain rock and now must be evaluated for survival under the extreme repository conditions that will occur with nuclear waste burial (i.e., radiation, high-temperature, desiccation). A timecourse experiment was conducted to evaluate the effects of desiccation on the indigenous microbiota present in Yucca Mountain, with emphasis on MIC-implicated groups. Triplicate natural rock microcosms from two humidity regimes (30% and 99%) were sampled at 28, 73, 141 and 234 days and the microbial communities compared to those of rock samples analyzed before treatment. While some loss in culturability and metabolic capacity occurred in the desiccated samples, it was minimal. Other methods of
assessing microbial biomass (PLFA/DGFA) and activity (respiring cell counts) also indicated that bacterial survival was not significantly affected by long-term desiccation. The absence of PLFA stress markers indicated that these microbes were well adapted to long-term desiccation. Acid-producing and EPS-producing bacteria were detected throughout the desiccation regime, with EPS colony morphotypes peaking at 28 days. Acidophilic iron-oxidizing bacteria were only detected at time 0 and 28 days desiccation while sulfate-reducing bacteria were found only after 141 and 243 days desiccation. Therefore, microorganisms present in Yucca Mountain, including MIC-implicated groups, are well adapted to long-term desiccation and potentially may survive this repository condition.

INTRODUCTION

While there is no doubt that microbial populations exist in the subsurface (1-3), many scientists in the field of radioactive waste management have predicted that the near-field environment around a waste
repository would be sterile due to extreme environmental conditions such as gamma radiation, high-temperature, and low water content. However, this position has been repeatedly challenged as microbes have been found in many environments which had previously been considered uninhabitable (4-7).

Water availability may be the most important factor influencing microbial growth and activity in unsaturated subsurface environments (8, 9). The natural water activity at Yucca Mountain is approximately 0.65, therefore, microbial growth and activity under these conditions would be expected to be minimal (10). This minimal activity level can probably be attributed to indirect effects of water availability (i.e., nutrient transport). The additional impact of buried nuclear waste will undoubtedly exacerbate this effect due to direct reduction in matric potential. Thermal loading models predict that heat generated by the radioactive decay processes in the canisters will dry the near-field rock by evaporation of water in the rock matrix. This will allow a flow of water vapor through fractures to
cooler regions where it will condense (10). However, as the canister temperature decreases over time, formation water will return and condense on their surfaces. Thus, while microbial activity may be temporarily arrested, it is evident that if bacteria possess the ability to survive desiccating conditions, they may eventually recolonize the repository environment and cause microbially-influenced corrosion.

MATERIALS AND METHODS

Sampling. Welded volcanic tuff was obtained from an alcove at 60 m depth within the north portal exploratory shaft at Yucca Mountain. The rock sample for this experiment was separated from surface contamination by careful aseptic sampling methods (11).

Microcosm construction. Culturable microbes were in low abundance (10^2-10^3 cells/gdw) in the native rock. To increase the number, rock was aseptically pulverized using a sterile mortar and pestle, adjusted to approximately 10% gravimetric water content with sterilized deionized water and placed at 25°C for 14
days. The rock was then thoroughly homogenized and subdivided into 27 microcosms each containing 100 g of pulverized rock in sterile glass petri dishes with elevated lids. All transfers were conducted in a laminar flow hood (Forma Scientific, Marrieta, OH).

**Desiccation regime.** Autoclavable desiccators with shelves (Nalgene, Rochester, New York) were sterilized and the bottom reservoir was filled with either sterilized deionized water (99% humidity) or a sterilized saturated solution of MgCl₂ (30% humidity) (12). Microcosms were placed on top of the desiccator shelves and the desiccators were sealed (air tight). A thermometer/hydrometer (Oakton, Japan) was placed in each desiccator to monitor temperature and humidity throughout the timecourse. Temperature was maintained at 24°C throughout the desiccation regime. Microcosms were maintained at 99% and 30% humidity for as long as 234 days. Triplicate microcosms from each humidity level were removed on days 28, 73, 141, and 234 for testing. Rock samples were also tested before treatment.
Physical characterization. Gravimetric water content was determined before and after 24 hours of drying at 105°C for microcosm rock analyzed throughout the desiccation treatment. Water activity was determined using the Decagon Aqua Lab CX-2 Water Activity Meter as described by Gee et al. (13).

Microbiological characterization. Slurries of homogenized rock (1:10 w/v), in sterile 0.1% sodium pyrophosphate (pH 8.0), were shaken for 1 hour at 24°C (125 rpm) from each of three microcosms sampled at 0, 28, 73, 141 and 234 days.

Total direct counts were prepared using the acridine orange technique described by Haldeman et al. for use with subsurface rock samples (14, 15).

To determine respiring cell counts a procedure of Rodriguez et al. (16) was modified such that 5.0 mM 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences, Inc.) was prepared in R2B (yeast extract, 0.5 g; proteose peptone, 0.5 g; casamino acids, 0.5 g;
glucose, 0.5 g; soluble starch, 0.5 g; sodium pyruvate, 0.3 g; K$_2$PO$_4$, 0.3 g; MgSO$_4$·7H$_2$O, 0.05 g per liter, pH 7.2).

Culturable counts were determined by serial dilution of slurries in phosphate buffered saline (PBS), pH 8.0 and spread plated, in triplicate, on R2A agar (Difco) (17). After 14 days incubation at 24°C, the results of the triplicate plates were averaged and Shannon-Weaver diversity and equitability indices were calculated (18). EPS-producing microorganisms were identified by colony morphology, including large glistening and/or runny appearance.

To estimate the number of acid-producing heterotrophic microorganisms, a five-tube most probable number (MPN) analysis was performed. MPN tubes were prepared with a modified triple sugar iron enrichment medium designed for microorganisms cultivated from low-nutrient environments (19). The medium formulation was as follows: yeast extract, 0.5 g; proteose peptone, 0.5 g; casamino acids, 0.5 g; glucose, 1.0 g; lactose, 1.0 g; sucrose 1.0 g; soluble starch, 0.5 g; sodium...
pyruvate, 0.3 g; K$_2$PO$_4$, 0.3 g; MgSO$_4$ \cdot 7$H_2$O, 0.05 g; Na$_2$SO$_3$ \cdot 5$H_2$O, 0.47 g; ferric citrate, 0.3 g; phenol red, 0.025 g per liter, pH 7.5). All MPN tubes were inoculated with slurry dilutions and were incubated at room temperature for 2 wk before interpretation.

Large scale enrichment cultures were performed to detect acidophilic iron-oxidizing and sulfate-reducing bacteria. Rock (10 g) from microcosms was added to 100 ml of appropriate medium and allowed to incubate at room temperature for 6 wk before qualitative interpretation. For detection of acidophilic iron-oxidizing bacteria, an enrichment medium that has been previously described was used (19). Flasks were gently rotated (125 rpm) under aerobic conditions. Putative positive flasks were confirmed by subculture on a solidified medium (1.5% purified agar) with the same nutrient formulation (20). For sulfate-reducing bacteria, a lactic acid medium (19, 21) was used and samples were incubated in an anaerobic chamber (Labline, Melrose Park, IL).

Metabolic capacity of the microbial community was assessed using BIOLOG microtiter plates (22, 23).
Microtiter plates for Gram positive (GP) and Gram negative (GN) organisms were utilized for this experiment, resulting in carbon utilization profiles for 128 unique compounds.

Phospholipid fatty acid (PLFA) analyses were performed to assess viable community biomass, composition and stress status as described by Kieft et al. (24). Duplicate samples were analyzed at 0, 28, 73, 141, and 234 days from both humidity regimes. Diglyceride fatty acid (DGFA) estimates were also done to assess non-viable biomass (25, 26).

**Statistical analysis.** Two-sample t-tests were conducted using Minitab Release 8.0 statistical software (Minitab, Inc., State College, PA) to determine significant differences between the mean values of tested microbiological and physical parameters (alpha = 0.05).

**RESULTS**

**Physical analysis.** Gravimetric moisture content and water activity of the rock samples are depicted in Fig. 1. While initial water content and activity were 13.4%
and 0.999, respectively, values rapidly diverged between the microcosms maintained at 99% humidity and 30% humidity. By 28 d of desiccation water content and activity dropped to 0.38% and 0.360, respectively. Water contents at both humidities then remained constant throughout the rest of the treatment. Water activity continued to slightly decrease in the rock maintained at 30% humidity but was stable in the samples kept at 99% humidity.

**Microbiological characterization.** Direct, respiring and culturable counts are presented in Fig. 2A-C. Direct counts were 2 to 3 orders of magnitude higher \((10^8-10^9)\) than culturable counts \((10^5-10^7)\) throughout the treatment period. Fluctuations were noted in total cell counts at 99% humidity, but values remained within one magnitude of their original value \((10^9)\) (Fig. 2A). In microcosms maintained at 30% humidity, a slight decrease (< 1 order of magnitude) in cell numbers was noted on day 141 but values then stabilized over the remainder of the timecourse.
While respiring counts were initially similar to culturable counts at day 0 (10^7 cells/gdw), a one magnitude increase was noted in the 99% humidity samples at day 28 (Fig. 2B). Counts then remained relatively stable over the remainder of the regime. In microcosms maintained at 30% humidity, respiring counts showed an increasing trend from day 0 and peaked at day 141 (10^6 cells/gdw). However, by day 234 cell numbers had returned to values close to their original value.

Culturable counts in microcosms maintained at 99% humidity fluctuated slightly over the timecourse but remained close to the original value obtained on day 0 (10^7 cells/gdw) (Fig. 2C). In the samples kept at 30% humidity, a decreasing trend was observed by day 28, resulting in a 1 order of magnitude loss in culturability by day 141 (10^6 cells/gdw). Values appeared stabilized over the remainder of the timecourse.

Community characteristics of the microcosms are presented in Table 1. Diversity and equitability indices were highest at day 0 (non-desiccated) with
values of H' - 2.50 and J - 0.76. With the exception of DES-T28 (30%), all other values were significantly lower, indicating microbial response to the imposed humidity regimes.

The number of distinct bacterial colony morphotypes was also highest on day 0, however, no distinct trends were noted over the exposure period. A community shift was observed based on R2A colony morphology in the desiccated microcosms (30% humidity); by day 73 spreading, extremely adherent colony types predominated as compared to the well-defined, easily removed colonies observed at day 0 and 28. The odd spreading, adherent colonies were only observed in the desiccated sample after 28 days. Fungi and actinomycetes were not observed at any timepoint with either treatment, nor were spores or hyphae noted on fluorescent microscopic counts.

The desiccation response of MIC-implicated bacterial groups showed that the community percentage of BPS-producing microorganisms was relatively constant in all samples (1-3%) with one significant exception, (DES-
T28 (30%). This desiccated sample exhibited a dramatic increase in the presence of EPS-producers with a value of 23.9%. Sulfate-reducing bacteria were detected on days 141 and 234 at both humidity levels. Conversely, acidophilic iron-oxidizers were detected only early in the experiment, at days 0 and 28 (30% humidity). Estimates of heterotrophic acid-producing microorganisms are depicted in Fig. 3. Acid-producing microbes were significantly higher in microcosms maintained at 99% humidity ($10^3$ cells/gdw) compared to those held at 30% humidity ($10^1$-$10^2$ cells/gdw) on days 28 and 73. However, on day 141, a ten fold increase in cell numbers was observed in the desiccated microcosms (30%) while values continued to decline in the non-desiccated rock (99%). By day 234, cell numbers were approximately equal in microcosms maintained under both humidity regimes.

Carbon source utilization by non-desiccated and desiccated microbial communities is presented in Table 2. The non-dessicated community showed the highest metabolic diversity, using carbon sources in all major
categories represented on BIOLOG plates (e.g., carbohydrates, carboxylic acids, polymers, amines/amides, amino acids). Metabolic diversity exhibited a decreasing trend over the desiccation treatment. The loss in diversity appeared due to the inability of the community to utilize methylated and phosphorylated sugars and polymers.

Viable biomass estimates as determined by PLFA analysis are depicted in Fig. 4. PLFA increased with time under 99% humidity, but ended on day 234 at a level equal to that observed at 30% humidity. PLFA/DGFA ratios (data not shown) changed little over, or between, the two timecourses.

Microbial community compositions in non-desiccated (99% humidity) and desiccated (30% humidity) samples, based on PLFA functional group distribution, are depicted in Fig. 5. These data showed only small differences in PLFA profiles between non-desiccated and desiccated treatments. A principal components analysis indicated that the desiccated community increased in Gram negative associated lipids while non-desiccated
samples showed lipids found in Gram positive bacteria.

PLFA stress signatures were also analyzed to document effects of the humidity treatments on the microbial communities over time. Results from these analyses (data not shown) indicated that microorganisms were not under physiological stress at either humidity level.

**DISCUSSION**

To construct microcosms for this desiccation experiment, rock grinding and water addition were used to stimulate resuscitation and growth of the microbial community. The culturable cell numbers increased from $10^2$-$10^3$ cells/gdw in the native rock to $10^7$ cells/gdw. Sampling and sample handling (i.e., grinding) appears to redistribute nutrients in the rock material, making them more available to microbes for utilization (11, 27, 28). Addition of sterile water also aids in this redistribution process and provides a resource limiting microbial growth (29). This observation had been made in other low matric potential subsurface environments that
have undergone similar water addition (9).

Perturbation and water addition will occur during repository construction through human activity and water input during mining operations (1000 gallons/ft.). While it is expected that heat generated from the radioactive decay process will "drive" water out of the near field environment, it is also predicted that water will return to encroach on the storage area after the repository cools. Thus, if indigenous microbes can survive the severe desiccation conditions predicted, it is plausible that their growth and activity may be positively impacted by the return of water.

In this experiment, microbial communities were exposed to a long-term (8 month), severe desiccation stress which reduced the water activity ($a_w = 0.22$) well below normally observed values under natural conditions ($a_w = 0.65$) (Fig.1). The water activity level resulted in a "direct" reduction in matric potential and created the "indirect" effects of water loss, loss of water activity and available nutrients.

Microbial community response to the desiccation
regime was not as dramatic as one might expect. Culturable counts in the desiccated microcosms, showed an initial one order of magnitude decrease and subsequently stabilized over time (Fig. 2C). This pattern is similar to that observed in a typical starvation-survival curve (30, 31). The surviving culturable cells appeared to be vegetative non-spore forming bacteria, with no fungi or actinomycetes. This is consistent with other subsurface microbial characterization studies where vegetative cells were the typical surviving types (1, 31).

The increase observed in PLFA biomass in the desiccated samples suggests that members of the microbial community were well adapted to sub-optimal conditions (Fig. 4). The discrepancy observed between culturable counts and PLFA biomass may be explained by entrance of microbes into a viable but non-culturable state (VBNC) (Fig. 2C and 4). This mode of life is frequently observed in microbes living under sub-optimal environmental conditions and appears to be a survival response (32, 33). That these microbes were not overly
stressed by the desiccation treatment was also indicated by the absence of PLFA stress signatures (Fig. 5) (24, 34).

Metabolic activity and diversity as determined by BIOLOG plates also showed that microbial communities retained most of their metabolic capabilities, only losing the ability to utilize methylated or phosphorylated sugars and some polymers (Table 2). This may indicate an initial response to the stress, as microbes may be funnelling their energy into cellular maintenance processes rather than enzyme production for elaborate metabolic pathways unnecessary during starvation conditions. Observations of decreased metabolic capacity have been made in marine bacteria undergoing a starvation response (35) and this pattern appears to be a consistent physiological change noted in VBNC cells (33).

The ten-fold increase in respiring count observed in the microcosms held at 99% humidity (day 0-28), may be a result of perturbation, or more likely due to adaptation to increased nutrient and water availability.
The increasing trend in respiring counts observed in the desiccated microcosms mimics that observed in PLFA biomass estimates (Fig. 2B and 4). However, the culturable count remained consistently 1-2 orders of magnitude lower at all timepoints. Thus, respiring and culturable counts support the VBNC hypothesis.

The increase in acid-producing microorganisms and the detection of sulfate-reducing bacteria on day 141 in the desiccated rock indicated a potential change in redox status (aerobic to anaerobic) in the microcosms (Fig. 3 and Table 1). Acidophilic iron-oxidizers detected on day 0 and 28 support this hypothesis (Table 1). The redox phenomenon may be linked to a peak in EPS-producing microbes (23.9%) observed on day 28 in the desiccated series (Table 1). EPS-production has been shown to be stimulated by desiccation and may help microorganisms maintain water potential under desiccation stress (36-38). Thus, elaboration of exopolymer is likely contributing to the creation of anaerobic zones in the microcosms by means of biofilm
formation (39-41) and by providing a stress survival tactic for the microbial community. These conditions select for microorganisms with anaerobic metabolism (i.e., sulfate-reducers and fermentative acid-producers).

Thus, while severe desiccation may alter microbial community composition, it is evident that some members of the indigenous microbiota present in Yucca Mountain can survive and/or be resuscitated by water addition. All groups of bacteria implicated in MIC were capable of surviving desiccation and growing under appropriate redox conditions. In addition, the desiccation inflicted on microorganisms in this experiment did not cause undue stress, as microbial responses were not dramatic. This survival response supports the hypothesis that these microbes have been present in the volcanic rock for extended periods of geologic time and were selected as "survivors" in sub-optimal environmental conditions (26). This observation has significant implications for the Yucca Mountain proposed waste facility and should not be discounted in the
repository design. While microbial activity may initially be impacted by repository conditions, survival as shown by the results in this study, ensures their capacity to potentially recolonize and become active in the near-field environment (MIC) when conditions once again become favorable.

ACKNOWLEDGEMENTS

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REFERENCES


Figure 1. Percent moisture and water activity (a<sub>w</sub>) measurements for microcosms exposed to 30% and 99% relative humidity.
Figure 2. Acridine orange direct counts/gdw (A), CTC resiping cell counts/gdw (B), and culturable cell counts/gdw (C) for microcosms exposed to 30% and 99% relative humidity.
Figure 3. MPN estimates/gdw of heterotrophic acid-producing microbes for microcosms exposed to 30% and 99% relative humidity.
Figure 4. PLFA (viable) biomass estimates for microcosms exposed to 30% and 99% relative humidity.
Figure 5. Microbial community composition based on PLFA (viable) functional group distribution, throughout the timecourse, for microcosms exposed to 30% and 99% relative humidity.
Community Composition (30% humidity)

Community Composition (99% humidity)

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Table 1. Microbiological analysis of desiccated (30% humidity) and non-desiccated (99% humidity) rock microcosms.

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>DIVERSITY (H')</th>
<th>EQUITABILITY (J)</th>
<th>MORPHOTYPES</th>
<th>COMMUNITY % EPS-PRODUCERSb</th>
</tr>
</thead>
<tbody>
<tr>
<td>DES-T0</td>
<td>2.50 (0.06)</td>
<td>0.76 (0.02)</td>
<td>27 (1.0)</td>
<td>2.5%</td>
</tr>
<tr>
<td>DES-T28 (30%)</td>
<td>2.14 (0.15)</td>
<td>0.75 (0.09)</td>
<td>18.0 (1.0)</td>
<td>23.9%</td>
</tr>
<tr>
<td>DES-T28 (99%)</td>
<td>1.78 (0.08)</td>
<td>0.57 (0.02)</td>
<td>22.0 (3.0)</td>
<td>3.2%</td>
</tr>
<tr>
<td>DES-T73 (30%)</td>
<td>1.47 (0.12)</td>
<td>0.46 (0.05)</td>
<td>25 (4.0)</td>
<td>1.6%</td>
</tr>
<tr>
<td>DES-T73 (99%)</td>
<td>1.44 (0.04)</td>
<td>0.50 (0.02)</td>
<td>18 (4.0)</td>
<td>0.6%</td>
</tr>
<tr>
<td>DES-T141 (30%)</td>
<td>1.67 (0.03)</td>
<td>0.56 (0.02)</td>
<td>20 (1.0)</td>
<td>2.8%</td>
</tr>
<tr>
<td>DES-T141 (99%)</td>
<td>1.65 (0.05)</td>
<td>0.67 (0.01)</td>
<td>12 (1.0)</td>
<td>3.0%</td>
</tr>
<tr>
<td>DES-T234 (30%)</td>
<td>1.62 (0.05)</td>
<td>0.57 (0.02)</td>
<td>18 (3.0)</td>
<td>2.1%</td>
</tr>
<tr>
<td>DES-T234 (99%)</td>
<td>1.23 (0.04)</td>
<td>0.50 (0.01)</td>
<td>11 (1.0)</td>
<td>1.0%</td>
</tr>
</tbody>
</table>
Table 1 cont. Microbiological analysis of desiccated (30% humidity) and non-desiccated (99% humidity) microcosms.

<table>
<thead>
<tr>
<th>SAMPLE*</th>
<th>PRESENCE OF SULFATE-REDUCING BACTERIA\textsuperscript{c}</th>
<th>PRESENCE OF IRON-OXIDIZING BACTERIA\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>DES-T0</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DES-T28 (30%)</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DES-T28 (99%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DES-T73 (30%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DES-T73 (99%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DES-T141 (30%)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>DES-T141 (99%)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>DES-T234 (30%)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>DES-T234 (99%)</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{*} DES-T0: original microcosm (T0) not exposed to any humidity treatment; DES-T28- microcosms held at the designated humidity for 28 days; DES-T73- microcosms held at the designated humidity for 73 days; DES-T141- microcosms held at the designated humidity for 141 days; DES-T234- microcosms held at the designated humidity for 234 days.

\textsuperscript{a} determined by morphology on R2A.

\textsuperscript{c} qualitatively determined by enrichment culture; limit of detection 10 cells/gram of rock.
Table 2. Carbon source utilization by non-desiccated and desiccated microbial communities

<table>
<thead>
<tr>
<th>Period of desiccation (days)</th>
<th>% Substrates utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>88%(^b)</td>
</tr>
<tr>
<td>28</td>
<td>85%</td>
</tr>
<tr>
<td>73</td>
<td>83%</td>
</tr>
<tr>
<td>141</td>
<td>76%</td>
</tr>
<tr>
<td>234</td>
<td>70%</td>
</tr>
</tbody>
</table>

\(^a\) Table entries represent the percentage of carbon sources (128 total) utilized by non-desiccated and desiccated microbial communities as determined by BIOLOG.

\(^b\) As the value obtained on day 0 and all of the non-desiccated samples was constant it is represented by one table entry.
CHAPTER 7

MICROBIA LLY-INFLUENCED CORROSION CAPABILITY OF
YUCCA MOUNTAIN BACTERIAL ISOLATES

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Biofouling and is presented in the style of that
journal. The complete citation is:

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Yucca Mountain bacterial isolates.

191
ABSTRACT

Microorganisms implicated in microbially-influenced corrosion were isolated from the deep subsurface at Yucca Mountain. Corrosion rates of iron-oxidizing, sulfate-reducing, and exopolysaccharide (EPS)-producing bacteria were examined in constructed electrochemical corrosion cells for periods up to 109 days. The test system consisted of a 1020 carbon steel coupon immersed in soft R2A agar prepared with simulated groundwater. A 1% KCl bridge was used to connect the test to a reference calomel electrode and a potential was applied with a platinum counter-electrode. The corrosion process was measured by polarization resistance methodology (Gamry Instruments, Inc.). Average corrosion rates were measured in milli-inches per year (mpy) against time. Purified cultures of EPS-producing bacteria and enrichment cultures of iron-oxidizing and sulfate-reducing bacteria were tested separately and in various combinations. An uninoculated control cell was prepared to assess abiotic corrosion. The corrosion rates peaked at 35 days at 1.2 mpy (control), 2.3 mpy (iron-oxidizing
bacteria), 3.30 mpy (sulfate-reducing bacteria), and 2.8 mpy (EPS-producing bacteria) before stabilizing. Various microbial combinations demonstrated higher corrosion rates (3.1-4.8 mpy) than single groups and peaked at 30 days. The results indicate that Yucca Mountain microorganisms, alone and in combination, are capable of causing corrosion of 1020 carbon steel.

INTRODUCTION

Corrosion has traditionally been regarded as a sequence of electrochemical reactions at a metal surface in contact with an aqueous electrolyte-containing solution (Geesey, 1991). This results in the dissolution of metal from anodic sites with subsequent electron acceptance at cathodic sites (Ford and Mitchell, 1990). However, it is now known that corrosion reactions may actually be induced or enhanced by microbial activity (Costerton and Boivin, 1991, Ford and Mitchell, 1990). Microorganisms implicated in microbially-influenced corrosion include iron-oxidizing, sulfate-reducing, acid-producing, and exopolymer-producing bacteria.
(Geesey, 1991, Ford and Mitchell, 1990). When a metallic surface is immersed in an aqueous solution, a conditioning film forms which attracts bacteria, algae, and other microorganisms to the surface, initiating biofilm formation (Borenstein, 1993). The biofilm contributes to the formation of an oxygen gradient, creating anaerobic zones in the portion of the biofilm in contact with the metal surface. This provides ideal conditions for the development of corrosion processes and leads to metal dissolution and pit formation (Borenstein, 1993).

The microorganisms utilized in this research project were previously isolated from rock samples obtained from the proposed nuclear waste repository site at Yucca Mountain. It is imperative to understand what effects microbial metabolism may have on materials chosen for site construction and waste containment. Loss of integrity of the metal canisters at this site could result in leakage of radioactive waste into the surrounding environment. One of the metals being considered for the outer barrier is 1020 carbon steel.
The corrosive effects of microbial activity on this metal were the focus of this research.

MATERIALS AND METHODS

Electrochemical corrosion cell

The corrosion cell design developed for these studies is depicted in Fig. 1. Test cells consisted of 40 X 130 mm flat-bottomed glass cylinders (Corning, Corning, New York) filled with 100 ml of a modified soft R2A agar prepared with simulated Yucca Mountain groundwater (J-13). This low-nutrient medium was chosen to simulate in-situ conditions, however, the organic content still exceeds what is present at Yucca Mountain. The modified agar was prepared by adding 0.5% Bacto-agar to R2B base (yeast extract, 0.5 g; proteose peptone, 0.5 g; casamino acids, 0.5 g; glucose, 0.5 g; soluble starch, 0.5 g; sodium pyruvate, 0.3 g; K₃PO₄, 0.3 g; MgSO₄·7H₂O, 0.05 g per liter J-13 water, pH 8.0). The simulated (J-13) groundwater contained per liter,

- CaSO₄·2H₂O, 19.90 mg
- CaCl₂·2H₂O, 12.92 mg
- Ca(NO₃)₂·4H₂O, 19.23 mg
- MgSO₄·7H₂O, 17.80 mg
- FeCl₂·4H₂O, 0.14 mg

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AlCl₃·6H₂O, 0.270 mg; Li₂SO₄·H₂O, 0.553 mg; MnSO₄·H₂O, 0.003 mg; KHCO₃, 13.57 mg; NaHCO₃, 179.4 mg; HF acid (49%), 2.21 mg; Na₂SiO₃·9H₂O, 303.6 mg, pH 8.0). A 1020 carbon steel coupon with 9.7 cm² exposed surface area and a platinum counter electrode were immersed in the soft agar. The platinum electrode was used for application of a potential to the metal coupon. A calomel reference electrode was immersed in another cylinder containing a 5% KCl solution. The test and reference cells were connected by a salt bridge composed of 1% KCl and 1.5% Bacto agar. All parts of the test cell and solutions were autoclaved prior to assembly to ensure sterility. The test apparatus was assembled in a laminar flow hood (Forma Scientific, Marrieta, OH), using sterile technique, and monitored for contamination for three days prior to inoculation.

**Microbiological cultivation and inoculation**

Yucca Mountain iron-oxidizing, sulfate-reducing, and exopolymer-producing (EPS) bacteria were cultivated separately in appropriate media. Iron-oxidizing bacteria
were grown in an enrichment medium and gently shaken (75 rpm) under aerobic conditions (Atlas, 1993). Sulfate-reducing bacteria were subcultured in a lactic acid medium (Atlas, 1993, Hamilton, 1985) and incubated in an anaerobic chamber (Labline, Melrose Park, IL). Enrichment cultures were used as the corrosion cell inoculum. EPS-producing microorganisms were grown in R2B medium under aerobic conditions. A set of four EPS-producing isolates were used in the corrosion cell inoculum. The four isolates were grown separately to stationary phase and then combined in equal numbers prior to inoculation. All bacterial inocula were adjusted to an optical density (OD) of 0.1 (600nm), which represented a standard concentration of approximately 10^7 cells/ml.

Test cells were inoculated with 1 ml of the selected cultures, in triplicate, with a sterile glass pipet along the length of the metal coupon. One set of three cells was inoculated with iron-oxidizing bacteria, a second set with sulfate-reducing bacteria, and a third set with a mixture of four exopolymer-producing
bacteria. Sets of three cells were also inoculated with the following combinations of microorganisms:
exopolysaccharide-producing and sulfate-reducing bacteria (EPS/SRB), exopolysaccharide-producing and iron-oxidizing bacteria (EPS/FeOX), sulfate-reducing and iron-oxidizing bacteria (SRB/FeOX), and exopolysaccharide-producing, sulfate-reducing and iron-oxidizing bacteria (EPS/SRB/FeOX). An uninoculated control cell was constructed to monitor abiotic corrosion. All manipulations of the test system were conducted in a laminar flow hood using sterile technique.

Electrochemical monitoring

Polarization resistance measurements were conducted by conventional potential scan methods using electrochemical hardware from Gamry Instruments, Inc. attached to an IBM PC compatible computer (Jones, 1992). A plot of potential change versus applied current was automatically generated as a result of a potential sweep from approximately -10 mV anodic to +10 mV cathodic to
the open-circuit. The slope of this plot at the origin is the polarization resistance, $R_p$, which is inversely proportional to the corrosion rate ($i_{corr}$) according to the Stearn-Geary relationship: $R_p = b_a b_c / i_{corr} (b_a + b_c)$, where $b_a$ and $b_c$ are the anodic and cathodic Tafel constants, respectively. $b_a$ and $b_c$ were measured independently for the conditions of these experiments using the Gamry Tafel analysis program. The computerized instrument system automatically performed a curve-fit analysis to extract the $R_p$ value and calculate the corrosion rate from each polarization curve in milli-inches per year (mpy) against time. Corrosion cells were monitored for up to 109 days.

RESULTS

*Electrochemical parameters*

Anodic and cathodic constants were determined during preliminary optimization assays: $b_a = 0.16$ V/Decade and $b_c = 0.093$ V/Decade. A scan rate of 0.05 mV/sec was determined to be optimum for steady state polarization determinations. Above 0.05 mV/sec,
measured polarization resistance decreased with scan rate, but lower scan rates required appreciably longer measurement times with no change in polarization resistance.

Corrosion rates of electrochemical cells containing a single microbial metabolic type

The average corrosion rates for cells inoculated with a single microbial type (i.e., iron-oxidizers, EPS-producers or sulfate-reducers) are presented in Fig. 2A-C. In each case, the corrosion rates decreased rapidly but then stabilized. There was a peak of activity on approximately day 35. Iron-oxidizing bacteria showed the lowest average corrosion rate at 2.3 mpy (Fig. 2A) followed by EPS-producing microbes at 2.8 mpy (Fig. 2B) and sulfate-reducing bacteria at 3.3 mpy (Fig. 2C). At all timepoints the values obtained from the test cells were significantly higher than those obtained in the abiotic control (1.2 mpy).
Corrosion rates of electrochemical cells containing combinations of microbial metabolic types

The corrosion rates for cells containing combinations of microbial types are depicted in Fig. 3A-D. The initial drops in corrosion rate were not as pronounced as that observed when corrosion cells were inoculated with a single metabolic type. With the exception of the EPS-producer/sulfate-reducing bacteria mixture, all rates peaked at about 30 days, prior to stabilization. The EPS-producer/iron-oxidizer combination exhibited the lowest peak rate at 3.4 mpy (Fig. 3A), followed by the iron-oxidizer/sulfate-reducer and EPS-producer/sulfate-reducer combinations at 4.1 mpy (Fig. 3B-C). The highest corrosion rate obtained in this study was from the cells containing all three metabolic groups (4.6 mpy) (Fig. 3D).

Discussion

These results indicate that all microbial types obtained from Yucca Mountain were capable of inducing corrosion of 1020 carbon steel coupons. Corrosion rates
were always significantly higher than those in the abiotic control cells.

The initial drop in corrosion rates, in some experiments, can be attributed to passivity of the metal surface caused by formation of a thin, protective, hydrated oxide film that acts as a barrier to the anodic dissolution process (Jones, 1992). However, this passive film is fragile and can be disrupted by surface-associated microbial activity as was observed in these studies. Corrosion rates peaked at 30-35 days probably reflecting the time necessary for colonization and initiation of localized corrosion processes (Ford and Mitchell, 1990).

Of the cells containing a single microbial type, the iron-oxidizing bacteria showed the lowest corrosion rate. This is not surprising, as the metabolism of these microorganisms is very slow even under optimum pH conditions (pH 2-3). That these bacteria can exhibit any corrosive activity in a pH 8.0 environment is interesting. Conversely, the cells which contained sulfate-reducing bacteria exhibited the highest
corrosion rates. Sulfate-reducing bacteria have been documented to show aggressive corrosion with many metals under anaerobic conditions (Borenstein, 1993, Philp et al., 1991, Hamilton, 1985).

Electrochemical cells containing combinations of microbial types always exhibited higher corrosion rates than those with a single microbial type. These results illustrate how important bacterial consortia are to microbially-influenced corrosion (Borenstein, 1993, Costerton and Boivin, 1991, Ford and Mitchell, 1990). Consortia appear to be particularly important to anaerobic corrosion processes (i.e., sulfate-reducing bacteria). EPS-producing bacteria can provide anaerobic microniches through biofilm formation on the metal surface and promote corrosion by sulfate-reducers.

The highest corrosion rate was observed in the electrochemical cells containing all three microbial types. While in situ conditions and microbial interactions are difficult to model in laboratory studies, it is clear that if proper conditions exist, the greatest risk for microbially-influenced corrosion
of carbon steel occurs when all three groups are present. Perhaps what is even more revealing, is the fact that all three metabolic groups have previously been isolated from the same Yucca Mountain rock sample. While a low-nutrient agar (R2A) was chosen to more closely simulate the organic carbon content of the rock, it is clear that this selection also provided a much more aqueous environment than would normally be encountered in situ. Thus, while corrosion rates obtained from these studies provide convincing evidence that Yucca Mountain microbes possess corrosive capabilities, rates may be artificially elevated. Further laboratory studies, using rock slurries prepared with minimum water addition, should be conducted so more accurate predictions of in situ corrosion rates might be made.

Acknowledgements

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References


Figure 1. Schematic representation of electrochemical corrosion cell apparatus.
1. Metal sample  
2. Platinum counter electrode  
3. 1% KCl-Agar salt bridge  
4. Calomel reference electrode  
5. R2A and microorganism(s)  
6. 5% KCl solution  
7. Rubber stopper
Figure 2. Average corrosion rates (mpy) of electrochemical cells inoculated with a single microbial type: iron-oxidizing bacteria (A), exopolymer-producing bacteria (B), and sulfate-reducing bacteria (C).
Figure 3. Average corrosion rates (mpy) of electrochemical cells containing combinations of bacterial types: exopolysaccharide-producing (EPS) and iron-oxidizing (FeOX) bacteria (A), iron-oxidizing (FeOX) and sulfate-reducing (SRB) bacteria (B), exopolysaccharide-producing (EPS) and sulfate-reducing (SRB) bacteria (C), and exopolysaccharide-producing (EPS), sulfate-reducing (SRB) and iron-oxidizing (FeOX) bacteria (D).
CHAPTER 8

GENERAL DISCUSSION

The chapters presented in this dissertation describe the first microbial characterization of Yucca Mountain subsurface rock. It is also the only study of Yucca Mountain which describes the potential for bacteria present in this environment to promote microbially-influenced corrosion (MIC). The topics addressed in these chapters encompass:

1. Characterization of the microbial community of Yucca Mountain,
2. Characterization of exopolysaccharide (EPS)-producing Yucca Mountain isolates,
3. Analysis of the effects of proposed repository conditions such as gamma radiation and desiccation on the survival and activity of Yucca Mountain microbiota, and
4. Study of biocorrosion capability of Yucca Mountain isolates on 1020 carbon steel. All chapters have been prepared for submission to peer-reviewed journals.

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Microbial characterization of Yucca Mountain

Results from the microbial characterization of Yucca Mountain rock indicated the presence of a diverse group of microorganisms including both heterotrophic and autotrophic microbes. Culturable numbers were typically low in the native rock ($10^2$-$10^3$), as observations previously made in other subsurface environments (Balkwill, 1989, Haldeman et al., 1994, Haldeman and Amy, 1993, Haldeman et al., 1993). The low abundance of microorganisms can best be explained by their physical environment. Analysis of Yucca Mountain rock indicated a low water content (4.0%) and low naturally occurring organic carbon (0.15-0.55 mg/l). These factors, in addition to the lithotrophic existence of many microbes, probably limited their in situ activity significantly. The microbial community was likely in a starvation-survival mode of life under natural conditions (Henis, 1987, Roszak and Colwell, 1987).

The most significant finding in this initial study was the detection of all bacterial groups typically implicated in MIC. While their activity is probably
limited under natural conditions the presence of these microbes poses a potential threat of MIC for the proposed repository. During repository construction, it is estimated that human activity and tunnel boring processes will introduce exogenous carbon and up to 1000 gallons/ft of water into the rock (Geesey, 1993). Drilling fluids, fossil fuel emissions and human activity is expected to potentially provide microbes with new carbon resources. This alteration of the environment will undoubtedly influence microbial activity by allowing indigenous and introduced microbes access to water and carbon sources (Haldeman et al., 1995, Haldeman et al., 1994). Thus, microbial potential for corrosion may be realized due to the impact of repository construction.

Physical and microbiological comparisons were made between Yucca Mountain, Yucca Flat and Rainier Mesa to determine whether other locations might serve as structural analogs for MIC studies. However, differences in water content and geologic origin among sites suggested none would serve as acceptable analogs of the
others for future investigations. The microbiology of the sites was impacted by the differences mentioned above, both in bacterial numbers and community composition.

Characterization of Exopolysaccharide (EPS)-producing Bacterial Isolates

EPS-production by microbes has been shown to be affected by the carbon sources available to the cell (Cerning et al., 1984, Fett, 1989). Under some conditions, microbes produce copious amounts of exopolymer, while under others, production is limited. Because of the importance of EPS-producing microbes to biofilm formation and subsequent MIC, the capability of heterotrophic isolates to produce EPS on a variety of carbon sources was assessed. The most significant result from these studies was that isolates from surface-contaminated rock possessed the highest potential for EPS-production on the greatest number of carbon sources. This suggests that perturbation of the natural environment during repository construction could
significantly enhance EPS-production of introduced populations potentially capable of MIC.

Many subsurface bacterial isolates have not been identified by conventional identification systems because many of the data bases currently in use were developed for microorganisms of medical importance. (Amy et al., 1992, Haldeman et al., 1993). The EPS-producing isolates recovered in this study were no exception, as only 25% were identified with any level of certainty by MIDI-FAME analysis. This result indicates that these microorganisms are either unique or have not been previously catalogued. MIDI-FAME analysis did provide a means by which to compare isolates. The isolates clustered into four distinct groups (based on principal components analysis) but the fatty acid profiles were all different, indicating a diversity of EPS-producing microbes in Yucca Mountain.

The EPS composition of many microorganisms has been determined, mostly for strains associated with medicine or industry (Cerning et al., 1994, Lenoir et al., 1988, Sutherland, 1985). This research provided the first
determination of EPS composition from subsurface bacterial isolates and the beginnings of a data base for future studies. Results from the EPS compositional analysis of the Yucca Mountain isolates demonstrated once again the bacterial diversity at this site.

**Effect of gamma-radiation on indigenous microbes at Yucca Mountain**

Previous experiments evaluating the effects of gamma radiation on natural microbial communities were limited and not well-documented (Popenoe and Eno, 1962, Stotzky and Mortenson, 1959). Dose rate or evidence of container penetration were not given. Thus, the experiment presented in this dissertation attempted to define thoroughly and control the treatment parameters as to simulate *in situ* conditions. Pulverized native rock in microcosms were used because current repository design at Yucca Mountain, includes the use of crushed rock as backfill material after waste burial (Geesey, 1993). The dose rate was based on expected radiation emission from unbreached canisters (Culbreth, 1995). The
only comparable study to this one was performed by a Canadian research group evaluating radiation resistance of microbial populations in bentonite (Stroes-Gascoyne et al., 1995). However, these investigations used artificially created composite microbial communities with significantly higher dose rates than those expected at the canister surface.

Under the experimental conditions used in this research, there was a complete loss in bacterial culturability after 24 hours of radiation exposure (233.4 Krads). However, other methods used to assess the microbial community (PLFA analysis, respiring cell counts) indicated the organisms retained viability. These observations support the idea that microorganisms entered a viable but non culturable state, probably due to cell damage incurred during radiation exposure.

Attempts to resuscitate injured microbes resulted in partial recovery of the community. Some microbes regained culturability and metabolic capacity as determined by BIOLOG carbon source utilization assays, although, numbers never equalled pre-radiation values.
Resuscitation is a controversial topic in microbiology because thorough documentation of the phenomenon is difficult (Oliver, 1993). Whether true resuscitation of injured microbes occurred in this experiment or a few cells survived in a culturable state below limits of detection, the implication for the Yucca Mountain repository is clear: members of the microbial community can survive and recover after exposure to gamma radiation. This result adds to a body of literature that indicates microorganisms in a starvation-survival mode have enhanced capability to endure environmental stress (Amy, 1996, Rockabrand et al., 1995, Henis, 1987, Roszak and Colwell, 1987).

EPS has been shown to perform many protective functions for microbes in sub-optimal environments as well as providing the “glue” necessary for biofilm formation. Multiorganism biofilms form a commonly encountered mode of existence for microbes in environmental situations (Ford and Mitchell, 1990, Geesey, 1993). The exopolysaccharide may serve as a nutrient trap, may protect the bacteria from predation,
and may aid in resisting desiccation (Costerton and Boivin, 1991, Roberson, 1992). The potential role of EPS in radiation-resistance should not be discounted. Two EPS-producing isolates that were individually exposed to gamma radiation showed increased radiation-resistance when compared to the entire community. Whether the observed resistance to radiation can be attributed to EPS-production or efficient DNA repair mechanisms is not known. It can be hypothesized that the EPS matrix may help to ameliorate the indirect effects of gamma radiation by serving as a barrier and decreasing the interaction of reactive species with DNA. However, this experiment provides some of the first survival values for EPS-producing microorganisms exposed to gamma radiation under simulated natural conditions. Radiation-resistant isolates could be selected over time in the repository radiation field, and if conditions became more hospitable for activity, they could then colonize the canisters and promote MIC.
Effect of long-term desiccation on indigenous microbiota at Yucca Mountain

Vegetative microbes can withstand long periods of desiccation in both soil and rock environments (Kieft et al., 1994, Kieft et al., 1990, Caccieri and Lippi, 1987, Boylen, 1973). The current investigation supports previous observations. Vegetative microbes were detected over an eight month desiccation treatment, but in contrast to previous studies, the response to desiccation was not very dramatic. No significant loss in viability/culturability was noted, which indicated that these microorganisms were not under extreme stress. Community composition over the desiccation treatment indicated that populations of "tolerant" microbes were selected. These microbial types were likely present in the microcosm volcanic rock for extended periods of geologic time and were selected as "survivors" under sub-optimal environmental conditions (Haldeman et al., 1994). The fact that indigenous microbiota exhibit pronounced survival capabilities when exposed to severe long-term desiccation, has significant implications for
the proposed repository at Yucca Mountain. Heat generated from waste canisters is expected to "drive out" water from the near-field area for thousands of years. However, when temperatures cool, water is expected to be drawn back into the repository bringing with it nutrients. Thus, if bacteria are capable of long-term survival under periods of desiccation, they could potentially recolonize the storage area and promote microbially-influenced corrosion.

**Microbially-influenced corrosion capability of Yucca Mountain bacterial isolates**

Carbon steel is currently being considered for the outer canister of high-level nuclear waste containers at Yucca Mountain and so it is imperative to determine whether indigenous MIC-implicated microbes are capable of effective corrosion under *in situ* conditions. MIC of 1020 carbon steel has been well-documented in aqueous industrial systems (Geesey, 1993, Jones, 1992). Data collected from corrosion studies conducted with Yucca Mountain isolates, indicated that MIC bacteria (i.e.,

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sulfate-reducers, iron-oxidizers and EPS-producers), both alone and in combination, could effectively corrode 1020 carbon steel under the conditions of the experiment. The most significant corrosion rate was obtained when all three microbial types were placed in a single corrosion cell and may indicate the importance of consortium formation to MIC (Ford and Mitchell, 1990). While a low-nutrient agar (R2A) was used to simulate the organic carbon content of the rock, the water content of the system was higher than would normally be encountered in situ. Thus, while these studies provide convincing evidence that Yucca Mountain microbes possess corrosive capabilities with 1020 carbon steel, rates may be artificially elevated. Further laboratory studies, using rock slurries prepared with minimum water addition, should be conducted to more accurately predict in situ corrosion rates.
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APPENDIX I

ADHESION CHARACTERISTICS OF A SUBSURFACE BACTERIAL EXOPOLYSACCHARIDE
OBJECTIVES

The objective of this analysis was to evaluate the adhesive characteristics of eight Yucca Mountain subsurface exopolysaccharides utilizing an attenuated total reflection Fourier transform infrared (ATR-FTIR) methodology. This method was developed by Dr. Peter Suci and Dr. Gill Geesey at the Center for Biofilm Engineering at Montana State University and has been used to look at adhesive properties of other polysaccharides such as alginate (Suci and Geesey, 1995). The long-range goal of this investigation was to correlate adhesive properties of the exopolymers to the compositional data previously obtained (Chapter 3). Upon completion, this investigation would provide the first set of data documenting the relevance of structural components to adhesion in bacterial exopolysaccharides.

EXPERIMENTAL DESIGN

Exopolysaccharide (EPS) was extracted and purified from the eight Yucca Mountain isolates described in Chapter 3. Due to unforeseeable problems with equipment,
only one of the exopolysaccharides (SR-21) was analyzed by ATR-FTIR. This isolate was chosen for initial adhesion studies due to its unusual EPS composition (i.e., presence of a 2-amino-6-dideoxyhexose).

Adsorption experiments were done using a stainless steel flow chamber (Circle Cell, Spectra Tech) fitted with a cylindrical germanium (Ge) internal reflection element. A simple flow through system was used to deliver solutions of EPS into the flow chamber for adsorption assays.

A binding curve, necessary to calculate adhesion parameters, was obtained as a step isotherm. Lyophilized, purified EPS was redissolved in deionized water to a stock concentration of 1 mg/ml. At EPS bulk concentrations of 0.01-0.20 mg/ml, a separate adsorption reaction (on germanium) was performed for 60 minutes under quiescent conditions followed by a 30 minute rinse with deionized water. During the course of each experiment infrared (IR) spectra were acquired periodically using a Perkin-Elmer Model 1800 Fourier transform infrared (FT-IR) spectrophotometer.
The binding curve generated, was fit to a Langmuir model. The equation describing Langmuir adsorption is:

\[ A = A_s \left[ \left( \frac{1}{Kc_b} + 1 \right)^{-1} \right] \]

where \( K \) is the binding (association) constant, \( c_b \) is the concentration of the substance in bulk solution, \( A \) is the absorbance (or band area in abs cm\(^{-1}\)) and \( A_s \) is the (estimated) saturation value of the absorbance (projected plateau for large bulk concentrations). Best fits for the parameters \( K \) and \( A_s \) were obtained by non-linear regression using Sigma Plot software (Jandel Scientific, Corte Madera, CA). \( A_s \) is then converted to surface coverage (\( \Gamma \)). Surface coverage (\( \Gamma \)) was estimated by comparing absorbances obtained in transmission mode with those obtained in the ATR mode using a previously published expression (Suci et al., 1995).

**RESULTS AND DISCUSSION**

The binding curve generated for EPS from isolate SR-21 is depicted in Fig. 1. A Langmuir fit to the binding curve was used to calculate the stickiness of SR-21 EPS with respect to Ge. This approach allows for
determination of two parameters, surface coverage ($\Gamma$) and bulk concentration to reach half saturation ($K^{-1}$), where a high ($\Gamma$) and low $K^{-1}$ indicate a relatively sticky substance. Adhesive parameters for SR-21 EPS are listed in Table 1. To put the obtained parameters for SR-21 in context, Table 1 also includes previously obtained ($\Gamma$) and $K^{-1}$ values for a muscle adhesive protein (MAP) (obtained from M. edulis) and an acidic polysaccharide, kelp alginate (Frolund et al., 1995). The low $K^{-1}$ (0.056 mg/l) obtained for SR-21 EPS indicate that this substance is relatively sticky. Additionally, the observed ($\Gamma$) for SR-21 EPS was also quite low (0.01 ug/cm$^2$). The low surface density of adsorbed EPS indicated by $\Gamma$ was not expected based on past analyses of other materials using this method. In general, tested material with low $K^{-1}$ values exhibited high $\Gamma$ values. However, interpretation of the adsorption parameters should be made with caution, as not many bacterial exopolysaccharides have been assessed with this technique. The adsorption parameters must be replicated and compared to other bacterial exopolysaccharide values.
to more fully interpret the obtained results.

Plans have been made to complete the analysis on the remaining exopolysaccharides in the fall of 1996. This work will provide new and important information on the adhesive characteristics of subsurface bacterial exopolymers.

REFERENCES


Figure 1. Binding curve of SR-21 adsorbed on germanium.
Band area C-O stretch region (abs x cm⁻¹ x 10) vs conc (mg/ml)
Table 1. Binding parameters for Langmuir fit to binding curves of SR-21 exopolysaccharide, muscle adhesive protein (MAP) and alginate to Ge. A lower value of $K^{-1}$ and a higher value of $\Gamma$ implies greater stickiness.

<table>
<thead>
<tr>
<th></th>
<th>SR-21</th>
<th>MAP</th>
<th>Alginate</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Gamma$ (ug cm$^{-2}$)</td>
<td>0.01</td>
<td>0.305</td>
<td>0.039</td>
</tr>
<tr>
<td>$K^{-1}$ (mg l$^{-1}$)</td>
<td>0.056</td>
<td>0.024</td>
<td>0.738</td>
</tr>
</tbody>
</table>
APPENDIX II

METHODS FOR MICROBIAL COMMUNITY ANALYSIS

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Fluorescent cell count methods.

Acridine orange direct counts (AODC) are used to assess total bacterial numbers (both viable and non-viable) in a sample.

Samples for AODC are prepared by mixing 9 ml of slurry with 1 ml of molten, filter-sterilized 1% Noble agar (Difco), and fixing the bacteria by addition of 135 ul of 37% formaldehyde. Two 5 ul portions are spread evenly over 1 cm² circles on glass slides previously heated to 550°C (>8 hrs) to remove residual organic material and bacteria from the glass surface. After drying, 100 ul of filter-sterilized 0.1% aqueous acridine orange solution containing 0.5 ug 4',6-diamidino-2-phenylindole (DAPI) dihydrochloride/ml is applied to the smears for 2 min. The stained smears are then rinsed gently for 1 to 2 min with a stream of filter-sterilized 0.2 M NaCl followed by deionized water, and examined immediately. Twenty fields on each of four replicate slides are counted (using a Nikon Optiphot microscope fitted with a Nikon Fluor 100X
objective lens, a 100 W mercury bulb, and a UV-1A filter cube). Cell counts are calculated by the following formula: \( \text{cells/ml} = (\text{average number of cells per field}) \times (\text{number of fields/1 cm}^2 \text{ diameter circle}) \times (\text{sample dilution}) \)

To measure viable densities of microorganisms, a respiring cell count procedure was used. 5.0 mM 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences, Inc.) is prepared in R2B (yeast extract, 0.5 g; proteose peptone, 0.5 g; casamino acids, 0.5 g; glucose, 0.5 g; soluble starch, 0.5 g; sodium pyruvate, 0.3 g; \( \text{K}_2\text{PO}_4 \), 0.3 g; \( \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \), 0.05 g per liter, pH 7.2). Equal volumes of slurry and 5 mM CTC (1ml:1ml) are added and mixtures are shaken in the dark for 4 hours at 200 rpm and 24°C. The cells are then fixed with 243 ul of filter-sterilized 37% formaldehyde. Samples are prepared for microscopy by filtering 50 ul through a 0.22 um black polycarbonate filter (Poretics) and mounting the dried filter on a glass slide with a coverslip. Twenty fields on duplicate slides are counted using a Nikon Optiphot microscope fitted with a Nikon
Fluor 100X objective lens, a 100 W mercury bulb, and a UV-1A filter cube. Cell counts are calculated by the following formula: cells/ml = (average number of cells per field) x (number of fields/area of filter) x (sample dilution).

**Microbial species diversity indices.**

Several mathematical indices can be used to express biological species diversity. These indices have rarely been applied to microbial communities because of the technical difficulties in speciating large number of microorganisms. Often microbiologists estimate community diversity by qualitative observation such as by colony morphology on plate counts.

Species diversity indices relates the number of species and the relative importance of individual species. There are two major components of species diversity, the species richness or variety component and the evenness or equitability component. Species richness can be expressed by simple ratios between total species and total numbers. It measures the number of
species in the community but not how many individuals are represented by each species. Equitability measures the proportion of individuals among the species present; equitability indicates whether there are dominant populations.

A widely used measure of diversity is the Shannon-Weaver index. This is a general diversity index that is sensitive to both species richness and relative species abundance. Caution must be used in interpreting the Shannon-Weaver index since it is sensitive to sample size, especially with small samples. Equitability, which is independent of sample size, can be calculated from the Shannon-Weaver index.

The Shannon-Weaver index of diversity \( (H) \) is calculated as follows:

\[
H = C/N (N \log_{10} N - \Sigma n_i \log_{10} n_i)
\]

where, \( C = 2.3 \), \( N = \# \) of individuals and \( n_i = \# \) of individuals in the \( i_{th} \) species.

Equitability \( (J) \) can be calculated as follows:

\[
J = H/H_{\text{max}},
\]

where \( H = \) Shannon-Weaver diversity index and \( H_{\text{max}} = \) theoretical maximal Shannon-Weaver diversity index for
the population examined (assumes each species has only 1 member).

**Phospholipid fatty acid and diglyceride fatty acid analysis to assess biomass and community composition.**

Phospholipid fatty acid (PLFA) analysis measures intact membrane phospholipids and thus, represents viable biomass. Diglyceride fatty acid (DGFA) analysis measures dephosphorylated or neutral lipids and is representative of non-viable biomass.

Total lipids are extracted from 75 gram rock samples with 300 ml of a single phase mixture of chloroform: methanol:water (1:2:0.8, v/v/v). After mixing and sonification for one minute, the samples are extracted at room temperature overnight. The liquid extractants are then transferred to separatory funnels and the phases are separated by adding one volume of water and chloroform. The organic phase was dried under vacuum using a rotary evaporator.

The total extracted lipid is then separated into three lipid classes by silicic acid column
chromatography (Unisil, 100-200 mesh). Samples are applied onto the columns with 150 ul of chloroform using disposable glass micro-pipets and the extracts are fractionated into neutral (DGFA), glyco- and phospholipids (PLFA) by elution with 1 ml of chloroform, 4 ml of acetone, and 1 ml of methanol. The methanol fraction containing the phospholipids was dried under N$_2$. Diglyceride fatty acids (DGFA) are isolated from the chloroform fraction by using thin-layer chromatography (60A, 250 mm thickness, Whatman, Clifton, NJ). The plate is then developed in hexane:diethyl ether (80:20, v/v) and a band just above the origin (corresponding to the elution of a standard, 1,2-dipalmitoyl-sn-glycerol, Sigma Chemical Co., St. Louis, MO) is discarded. Diglyceride fatty acids are eluted in 5 ml CHCl$_3$:MeOH (1:1, v:v).

Fractions containing both PLFA and DGFA are placed in 0.3 ml microvials with 150 ul chloroform and capped with a teflon-lined septa. Methyl nonadecanoate (50 pmoles) is added as internal standard and the solvents are evaporated under N$_2$. Transesterification is
performed by a mild alkaline methanolysis by adding 25 ul of methanol:toluene (1:1, v/v) and 25 ul 0.2M KOH in methanol. The microvials are heated at 37°C for 15 min, and after cooling, the pH is adjusted to 7 by adding 10ul of 1M acetic acid. The methyl esters are extracted with 100 ul of hexane:chloroform (4:1, v/v) and the organic solvents are evaporated under N₂.

Analysis of the fatty acid methyl ester preparations is performed using a Hewlett Packard 5880 gas chromatograph (GC). The column is a 50 m x 0.2 mm i.d. fused silica column coated with a nonpolar cross-linked methyl silicone phase (HP-1, Hewlett Packard). The initial oven temperature was 80°C for 1 minute, then increased at 10°C/min to 150°C, then at 3°C/min to 240°C, and finally at 5°C/min to 300°C. Hydrogen at a linear gas flow rate of 80 cm/sec was used as the carrier gas. Injections were performed in the splitless mode. Gas chromatography allows for the separation of the types of fatty acid methyl esters derived from bacterial PLFA and DGFA. Fatty acid nomenclature used designates the total number of carbon atoms:number of double bonds with the
position of the double bond closest to the methyl (\(\delta\)) end of the molecule indicated. This is followed by the suffix "c" for cis and "t" for trans configuration of monoenoic fatty acids. The prefixes "i", "a" and "br" refer to iso, anteiso and methyl branching of unconfirmed position, respectively. Cyclopropane rings are indicated with the prefix "cy" with the ring position indicated from the aliphatic end of the molecule.

The end result of the analysis is a profile of viable (PLFA) fatty acids and non-viable (DGFA) fatty acids which can be used to make biomass estimates, analyze community composition and assess stress responses of bacteria. PLFA/DGFA community composition is described by individually quantifying functional groups of fatty acids (i.e., normal saturates, tertiary branched saturates, mid-branched saturates, monoenoics and polyenoics) and observing specific trends in their abundance. In addition, the presence of particular fatty acids has been shown to serve as "biomarkers" for specific microorganisms in a community. Stress
responses have been documented by high cis/trans ratios of particular monoenoic fatty acids as well as a high concentration of cyclopropane fatty acids.

**MIDI-FAME (fatty acid methyl ester) analysis**

This method may be used for bacterial identification and/or to compare differences between bacterial strains using fatty acid profiles.

Bacterial cells (40 mg wet weight) are harvested from tryptic soy agar (TSA) plates, placed in glass screw top tubes and processed according to the MIDI protocol (Microbial ID, Inc., Newark, DE). Processing included saponification, methylation and extraction.

Saponification uses a strong methanolic base (45 g NaOH, 150 ml methanol and 150 ml water) combined with heat and pressure to kill and lyse the cells. Fatty acids are cleaved from the cell lipids and are converted to their sodium salts. 1 ml of the methanolic base is placed into the extraction tube, tightly sealed and vortexed for 5-10 sec. The tube(s) is then placed into a 100°C water bath for 30 min. Tubes are cooled to room
temperature in water prior to methylation.

Methylation converts the fatty acids (as sodium salts) to fatty acid methyl esters for GC analysis. This step uses a solution of 325 ml 6N HCl and 275 ml of methyl alcohol. Add 2 ml of methanolic HCl to each tube and place in an 80°C water bath for 10 min. Remove and quickly cool to room temperature in water.

Fatty acid methyl esters are then removed from the acidic aqueous phase and transferred to an organic phase with a liquid-liquid extraction procedure. Add 1.25 ml of a 1:1, v/v solution of hexane and methyl tert-butyl ether to each tube. Tubes are tightly sealed and placed on a laboratory rotator for 10 min. The aqueous (lower) phase is then removed and discarded with a Pasteur pipet.

A mild base wash (10.8 g NaOH in 900 ml distilled water) is added to the samples to remove free fatty acids and residual reagents from the organic extract. 3 ml of base wash were added to each tube, sealed tightly, and rotated for 5 min. The upper solvent phase was transferred to a microvial with a teflon crimp cap.
Extracted fatty acid methyl esters are then separated and identified using a gas chromatograph (Model 5890 Series II, Hewlett Packard Co.) fitted with a 25 m x 0.2 mm cross-linked 5% phenylmethyl silicone phase capillary column (Ultra 2), an auto injector (Model 7673, Hewlett Packard), and a flame ionization detector. The temperature of the auto injector is set at 250°C and the initial oven temperature of 170°C is raised at a rate of 5°C/min to 270°C. The flame ionization detector is set at 300°C. Hydrogen is the carrier gas, nitrogen is the makeup gas, and air is used to support the flame. The Microbial Identification System uses an external calibration mixture. The standard is a mix of the straight chain saturated fatty acids from 9 to 20 carbons in length and has five hydroxy acids. All compounds are added quantitatively so that the GC performance may be evaluated by the software each time the calibration mixture is analyzed. Retention time data obtained from injecting the calibration mixture may be converted to equivalent chain length (ECL) data for bacterial fatty acid naming. The
ECL value for each fatty acid can be derived as a function of its elution time in relation to the elution times of a known series of straight chain saturated fatty acids. Thus, it is possible, by comparison to the external standard, to compute the ECL value for each compound following analysis.

MIDI databases consist of more than 60,000 analyses of strains obtained from primarily medical and industrial culture collections. The cultures were collected from around the world to avoid potential geographic bias. Where possible, 20 or more strains of a species or subspecies were analyzed to make the entry. Analysis of an unknown results in an automatic comparison of the composition of the unknown strain to a stored database using a covariance matrix, principal component analysis and pattern recognition software. The covariance matrix takes into account the mole-for-mole relationship of conversion of one fatty acid to another (e.g., 16:0 to 16:1 due to action of a desaturase) which might occur in relation to a temperature shift, or in age differences. The pattern
recognition software uses calculations of cross terms (e.g., ratios between fatty acid amounts) in addition to the principal component base.

When comparing fatty acid profiles from bacterial isolates to determine relatedness, a dendrogram or principal components (PCA) 2-D plot may be generated with the MIDI software. The dendrogram program uses cluster analysis techniques to produce unweighted pair matchings based on fatty acid compositions. The use of the Euclidean distance scale provides a quick determination of the relatedness of entries at the genus, species and subspecies levels (approximately 25, 10, and 6 Euclidean distances respectively). Euclidean distance is the distance in two-dimensional space between two strains when their FAME compositions are compared.

A PCA 2-D plot uses a principal components analysis of FAME profiles to group entries in a two dimensional space. The x-axis represents the difference which can be explained by principal component 1, and the y-axis represents that which can be explained by principal
component 2. While it is known that the software utilizes the whole data set to generate the PCA plot, the exact algorithm used to determine the principal components is proprietary. The PCA plot is most useful for finding relationships among large numbers of organisms, or for visualizing the relationships of distantly related organisms.

Metabolic fingerprinting of microbial communities using BIOLOG microtiter plates

The BIOLOG system (BIOLOG Inc., Hayward CA) consists of a 96-well microtiter plate with 95 different carbon sources and a control well without a carbon source. Each well also contains nutrients, salts, a small amount of peptone and a tetrazolium violet redox dye. The dye is reduced during respiratory activity, and insoluble formazan accumulates inside the cells. Carbon substrates contained in the BIOLOG plates can be categorized into carbohydrates, carboxylic acids, polymers, amines/amides, amino acids and a few miscellaneous compounds.
By inoculating the BIOLOG plates with entire microbial communities it is possible to assess both metabolic activity and carbon source utilization patterns under natural and altered conditions. Slurries of rock/soil or water samples are diluted with phosphate buffered saline (1:1) and inoculated into the microtiter plate. Plates are incubated for 14 days at 24°C and read both manually and spectrophotometrically using a Titertek Multiscan Plus plate reader with a 590 nm filter. Positive wells were distinguished by visible formazan formation (purple).

Glycosyl composition analysis of bacterial exopolysaccharides

Purified bacterial exopolysaccharide material (20 mg) as well as sugar standards are transferred to 13 x 100 mm test tubes. 250 ul of 1M HCl in methanol is added, and the resulting solutions are heated at 80°C for 16 hr. [The 1M HCl in methanol can be prepared either by slowly adding acetyl chloride to methanol or by bubbling HCl gas from a gas cylinder into methanol,
determining the HCl concentration by titration, and then diluting to the proper concentration.] This converts the polysaccharide into a mixture of methyl glycosides and methyl ester methyl glycosides of the glycosyluronic acids. The methanolic HCl is removed by adding 100 ul of t-butyl alcohol and then evaporating with a stream of filtered air at room temperature. The methyl glycosides were then N-acetylated using methanol-pyridine-acetic anhydride (1:1:1 v/v/v) for 6 hrs at room temperature. The methyl glycosides and methyl ester methyl glycosides are then silylated using 0.5 ml of pyridine, 0.1 ml of hexamethyldisilazane, and 0.05 ml of trimethylchlorosilane, which can be purchased conveniently in these proportions as Tri-Sil (Pierce Chemical Co.). The samples are heated to 80°C for 20 min., and the silylating reagent is gently evaporated at room temperature. The derivatives are redisolved in 1 ml hexane and insoluble salts are allowed to settle. The supernatant is transferred to a clean test tube and carefully evaporated. The residue is dissolved in 100 ul of hexane, and 1 ul of this solution is analyzed by
gas chromatography (GC).

The GC analyses are performed on capillary columns; a fused silica DB1 (J and W Scientific) 30 meter by 0.25 mm i.d. is preferably used. Injections are made in the split mode (split ratio 10:1). The following oven temperature program is used: an initial temperature of 140°C, then an immediate increase to 180°C at a rate of 2°C/min. Then the column is conditioned for the next injection by increasing the oven temperature at a rate of 30°C/min to 275°C and maintaining this temperature for 10 min. As each sugar affords several derivatives, the peak areas of the major derivatives of each sugar must be added together before response factors can be calculated and glycosyl compositions determined.

**Most-probable number method for microbial populations.**

The most probable number (MPN) method permits estimation of population density without an actual count of single cells or colonies. The MPN technique is based on a determination of the presence or absence of microorganisms in several individual aliquots of each of
several consecutive dilutions of rock or other material. A prerequisite of the method is that the microorganism whose population is to be determined must be able to bring about some characteristic and readily recognizable transformation in the medium into which it is inoculated. A positive reading means only that at least one microorganism was present initially in the aliquot used for inoculation. Whether one, several or many cells were actually present is immaterial insofar as the reading of the positive response is concerned. However, for a valid estimate of the most probable number of organisms in the initial sample, it is necessary that single cells be capable of initiating growth in the medium used.

On the basis of probability theory, it is possible to calculate, from the numbers of positive and negative tubes, receiving a certain quantity of inoculum, the most probable number of microbes in that quantity of inoculum. By further elaboration of this theory, it is possible to combine the results from different dilutions in such a way that a single value is obtained for the
most probable number of organisms. This is done by using a statistically constructed MPN table which lists the most probable number of microbes associated with various combinations of observed values of three consecutive 10-fold dilutions. Five tubes at each of three consecutive 10-fold dilutions is commonly used.

**Medium formulation for culturing acidophilic iron-oxidizing bacteria.**

Liquid enrichment medium for iron-oxidizing bacteria is prepared by combining two separately sterilized solutions: Solution A - 44.22 g FeSO$_4$.7H$_2$O in 300 ml of deionized water, mix and autoclave; Solution B - 3.0 g (NH$_4$)$_2$SO$_4$, 0.5 g K$_2$HPO$_4$, 0.5 g MgSO$_4$.7H$_2$O, 0.1 g KCl, 0.01 g Ca(NO$_3$)$_2$, 1.0 ml H$_2$SO$_4$ (10N), pH between 3.0-3.6 and autoclave at 121°C for 20 min. Remove solutions from autoclave and cool to room temperature. Add Solution A and B and mix well before dispensing.

Solidified medium used to confirm the bacterial identification of *Thibacillus ferrooxidans* is prepared by combining three separately sterilized solutions:
Solution A- 33.4 g FeSO₄·7H₂O in 300 ml deionized water, adjusted to a pH of 2.5 with 6M H₂SO₄, stirred until almost colorless, and filter sterilized; Solution B- 6.0 g (NH₄)₂SO₄, 0.2 g KCl, 1.0 g MgSO₄·7H₂O, 0.02 g Ca(NO₃)₂, in 550 ml deionized water, adjusted to pH 3.0 and autoclaved at 121°C for 20 min.; Solution C- 7.0 g purified agar in 150 ml of deionized water, and autoclaved. Solutions B and C should be removed from the autoclave and allowed to cool for 5 min. at ambient temperature, and solution B added to C with gentle mixing. Solution A is then added to this combination and mixed well. The mixture is then poured into sterile petri dishes.

Medium formulation for culturing sulfate-reducing bacteria.

Medium for culturing sulfate-reducing bacteria is prepared by combining three separately sterilized solutions: Solution A- 3.5 ml sodium lactate (70% solution), 2.0 g MgSO₄·7H₂O, 1.0 g NH₄Cl, 1.0 g Na₂SO₄, 1.0 g yeast extract, 0.5 g K₂HPO₄, 0.1 g CaCl₂·2H₂O in
980 ml of deionized water, mix, pH to 7.4 and autoclave for 20 min. at 121°C; Solution B- 0.5 g FeSO₄·7H₂O in 10 ml of deionized water, mix and autoclave; Solution C- 0.1 g ascorbic acid, 0.1 g sodium thioglycollate in 10 ml of deionized water, mix and autoclave. Remove solutions from autoclave and allow them to cool to room temperature. Add Solution B and C to Solution A and mix before dispensing.