ABSTRACT

Title of Document: HARVESTING LIFE'S ENERGY: INCREASE IN THE AEROTOLERANCE OF THE ELECTROGENIC ANAEROBE GEOBACTER SULFURREDUCENS DUE TO OVER-EXPRESSION OF SUPEROXIDE DISMUTASE AND CATALASE

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Geobacter-based microbial fuel cells are becoming increasingly viable as an alternative energy source. Current research and commercial application have been slowed by the inability of *Geobacter* species to tolerate the presence of oxygen. Oxidative stress protection enzymes play a key role in protecting cells from oxygen damage. This project hypothesizes that the over-expression of two important oxidative stress protection enzymes, catalase and superoxide dismutase, can increase aerotolerance. These genes were amplified from the genome of *Geobacter sulfurreducens* and cloned into plasmid pRG5 behind the ptaclac promoter. This plasmid was transformed into *E. coli* and *G. sulfurreducens* to examine oxygen tolerance, gene expression, and enzyme activity. Preliminary data show increases in enzyme activity in *E. coli* and an increase in aerotolerance in *G. sulfurreducens* for both genes. This seems to be the first example of an increase in an obligate anaerobe's aerotolerance due to the intentional introduction of oxidative stress genes.

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Thesis submitted in partial fulfillment of the requirements of the Gemstone Program
University of Maryland, College Park
2009

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Acknowledgments

Team iGEM would like to thank:

Dr. David O'Brochta, our team mentor, for all of his advice, encouragement, and unsurpassed patience throughout the course of our project. He provided us with supplies when we didn't have funding and both he and the rest of his lab, particularly Kristina Pilitt, aided us immeasurably in completing our project.

Dr. Zvi Kelman, for use of his anaerobic chamber, gas set up, taking the time to teach us proper anaerobic technique, and his expertise with anaerobes.

Nozomi Sakakibara, for teaching us anaerobic technique and tolerating repeated requests for reinstruction.

Supida Piwkhow, for providing us with *Geobacter metallireducens* and aiding in the formulation of our growth medium.

Dr. Derek Lovley and the rest of his lab, with special thanks to Dr. Richard Glaven, for providing us with *Geobacter sulfurreducens* cells, advice, and sharing their expertise with *Geobacter.*

Dr. Daniel Stein, for providing us with a lab to get started.

Dr. Ilia Baskakov, Dr. Joseph Kao, Dr. Zvi Kelman, Dr. Srinivasa Raghavan, and Dr. Daniel Stein for serving on our thesis panel and for their helpful feedback and advice on our research and on our thesis.

Thomas Harrod, our team librarian, for always being willing to lend a helping hand with anything that we needed.

We would also like to thank Becton-Dickinson and the Howard Hughes Undergraduate Research Fellowship for their financial assistance of our project.

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Chapter 1: Introduction

1.1 Overview

The motivation to search for alternative sources of energy is fueled by an ever-changing climate, current dependence on rapidly depleting fossil fuel sources, and an exponential forecast of global demand for energy. The conflicting agendas of many of the world's governing powers greatly impede society's progress in reaching an equitable and cohesive solution. Consequentially, the temerity of reliance on limited carbon-based fuels demonstrates the need to develop a comprehensive long-term solution to the energy crisis.

1.2 Global Demand for Energy

According to the United Nations Population Division, the world population in 2005 was estimated to be 6.5 billion and increasing at a rate of 200,000 inhabitants per day. The future population of the world is projected to be approximately 9.2 billion by the year 2050, assuming current trends continue. Still, the average individual energy consumption per inhabitant worldwide over the past 30 years has remained at a constant 1.5 tons of oil equivalent (toe) per year (Hanjalić et al., 2008). This is principally due to a pair of counterbalancing effects that hold this ratio in check.

Countries with high individual consumption rates saw a relative decrease in population in contrast to countries with lower consumption rates. The aggregate population of countries with high rates climbed from 1.1 billion in 1975 to 1.4 billion in 2005, while the population of countries with low rates skyrocketed from 2.9 billion

to 5.1 billion respectively. The sharp contrast in growth rates between the two classifications resulted in a decrease in the average individual consumption. In contrast, the individual consumption rates of less developed countries climbed dramatically during this time period due to technological advancements and globalization in general.

Assuming the world population increases at a rate of 200,000 inhabitants per day with each inhabitant consuming 1.5 toe/yr, world energy consumption will grow at a rate of 300,000 toe/year per day (Hanjalić et al., 2008).

1.3 High Quality of Life

The consumption of energy throughout the world is heavily skewed as the result of an ever-present desire for a luxurious, 'American,' lifestyle. "Even the poorest and remotest in the 'global village' aspire to replicate the lifestyles enjoyed by Americans, Europeans and Japanese" (Barbir et al., 2008). But it is these lifestyles that involve extensive "extraction, processing and conversion of natural resources," both renewable and non-renewable, into goods that eventually become wastes and pollutants (Barbir et al., 2008). The United States, for instance, currently uses over 97 trillion joules annually; if everyone on the planet were to live an "energetic way of life" analogous to that of North America, the energy consumption of the world would multiply by more than five (Hanjalić et al., 2008).

Novel energy resources is critical to sustain such lifestyles, especially since expendable natural resources, most notably fossil fuels, are diminishing rapidly. In 2005, the U.S. also led with respect to natural gas consumption at 22,241 billion cubic feet. To put this into perspective, consumption of natural gas in North America as a

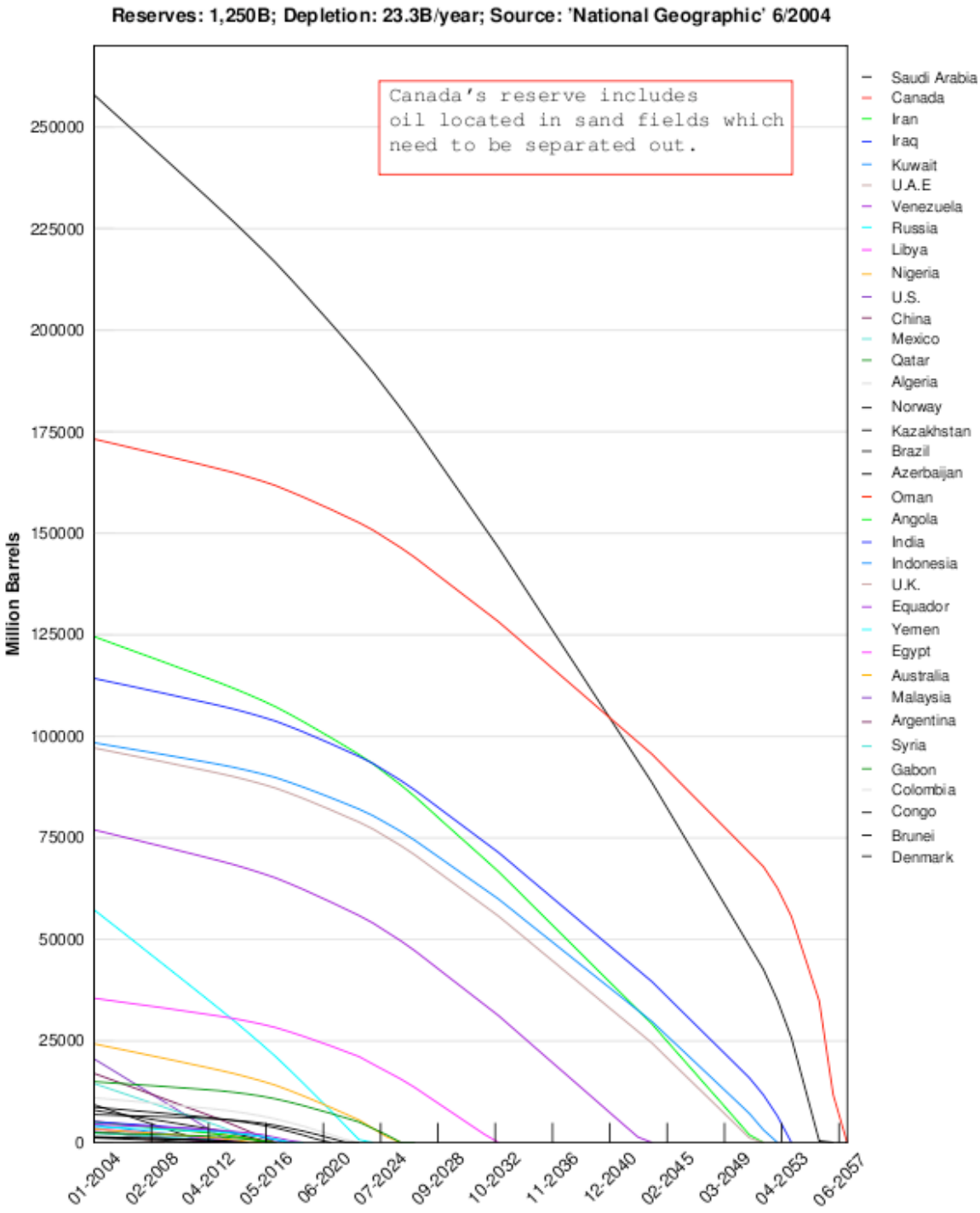
whole totaled 27,406 billion cubic feet. In 2007, the United States consumed 20.7 million barrels per day of petroleum products, crowning itself as the world's largest consumer of petroleum (EIA, 2009). Roughly one quarter of this consumption can be accredited to the transportation sector, where the convenience and reliability of petroleum is unparalleled (Wiser, 2000). Inexpensive fuel has unquestionably improved the quality of life of those fortunate enough to have access.

1.4 Fossil Fuels

Fossil fuels are the product of millions of years of intense pressure and heat acting upon preserved organic matter deep beneath the surface of the Earth. As the parameters of time, heat, and pressure cannot be effectively replicated by current technology, fossil fuels are considered finite natural resources. These fossil fuels, primarily petroleum, coal, and natural gas, are extracted and burned to release the chemical energy stored within hydrocarbon bonds. The relative abundance and motility of fossil fuels has made them the driving force behind the global economy since the dawning of the Industrial age. Unfortunately, humanity cannot sustain current levels of consumption, and a colossal shift in global infrastructure may be imminent.

Often the subject of extensive political and scientific debate, it is estimated that the world's supply of petroleum and natural gas will be depleted within the next 50 years. A recent estimate in 2004 recorded total world reserves at 1.25 trillion barrels and as *Figure 1* shows, with a daily global consumption of 85 million barrels,

Figure 1: World Oil Depletion Per Major Producer



petroleum depletion is estimated to transpire around the year 2057. In contrast, natural reserves of coal, which comprise 97% of proven fossil fuels, should last for up to 1500 years at the rate of the current depletion. However, if the consumption of coal should increase at a rate of 5% a year, this supply would last for only $1/15^{th}$ of the aforementioned estimate (EIA, 2009).

The combustion of fossil fuels releases a number of undesirable by-products, such as greenhouse gases, that contribute to global climate changes. "Nature is responding to the ever increasing consumption of fossil fuels and related greenhouse gas emissions with climate change and natural catastrophes, such as sea-level rise, extinction of species, floods, storms, droughts, heat waves, fires etc" with victims of such calamities increasing in tandem (Barbir et al., 2008). It is unclear how much irreversible damage has already been done to the atmosphere, but it is certain that the development of future energy technology must have a limited impact on the environment.

Instead of continuing to burn fossil fuels, it makes more sense to focus on energy conservation, both by actively limiting excessive use and considering alternative techniques such as carbon sequestration. Of the 97 trillion joules annually consumed in the United States, it is estimated that up to 32 trillion joules (33%) could be conserved if alternative technologies are implemented and conservation strategies are applied (Pimentel et al., 2004).

A plethora of such options are practicable, as long as proper incentives are established. Within the transportation sector for example, measures such as increased vehicle fuel economy, increased use of public transportation, and the use of trains for

commercial shipping could be employed to conserve fuel and energy. Likewise in the business sector, measures such as better insulation for heating and cooling, incorporation of solar window designs, and higher-efficiency furnaces and air conditioners could be implemented. Finally, with respect to equipment and appliances, conversion to fluorescent lighting and elevated energy standards would help contribute to more conservation conscious designs.

Energy conservation is a principal factor in contributing to global energy demands as they shift from fossil fuels to renewable and more environmentally-friendly sources. Alternative and renewable forms of energy such as hydropower, nuclear fission, solar power, wind power and biomass are currently under development. While these are promising, each has significant limiting factors and/or poses safety/health concerns.

1.5 Current Renewable Resources

1.5.1 Hydropower

Across the globe there are an estimated 45,000 large hydropower dams with a total generating capacity of 800 gigawatts. All together, they currently supply almost $1/5^{th}$ of the electricity consumed worldwide. In terms of electricity production, dams are second only to fossil fuels and generate 10 times more power than geothermal, solar and wind power combined. Over 160 countries around the world use hydropower as a source of electricity and in many developing countries, a single large dam may be the primary source of electricity. Industrialized nations with access to large rivers and bodies of water are the most successful at producing hydroelectricity;

Brazil, China, Canada, Russia and the United States alone account for more than half of the world's hydropower generation (Morton et al., 2008).

The concept behind hydroelectricity is straightforward: the potential energy within large quantities of water at high elevations is harnessed by directing the descending flow of water to spin turbines. As such, the limiting factors for hydropower production are the quantity and rate at which the water flows downhill. If all of the water sources in the world were turbined down to sea level, theoretically, 10 terawatts of power could be generated. It is important to note that this figure is strictly theoretical, as most often only 30% of a river's potential power is exploitable (Morton et al., 2008).

Hydropower dams are incredibly appealing because they have the capability to instantaneously respond to varying electricity demand without regard to the weather. The process by which they are activated is as simple as opening the dam and letting gravity do the rest. In addition, they require no fuel-extraction infrastructure or fuel transport.

Although they are a viable source of electricity, hydropower dams have certain limitations. Reservoirs occupy a lot of space and are expensive to construct in terms of both budget and labor. They also have significant environmental disadvantages, such as ecological effects on the ecosystems both upstream and downstream of the dam site. A dam might disrupt the migration patterns of fish as well as facilitate the accumulation of large deposits of sediment. In addition, biomass trapped within the reservoirs eventually decomposes and releases methane and carbon

dioxide in the same manner as fossil fuels. In some aspects, hydropower dams are almost as damaging to the environment as the combustion of fossil fuels.

1.5.2 Nuclear Fission

In 2006, there were 444 nuclear fission plants operating in 25 different countries around the world. As a whole, these plants generated 6% of the world's energy and 17% of the world's electricity. Currently, United States nuclear fission plants operate at 90% efficiency. However since 1973 no new reactors have been licensed for construction in the United States.

Nuclear fission-based power generation is the self-sustaining process of atomic splitting within a nuclear reactor core. This process uses nuclear fuels such as uranium. The heat generated from this process produces the steam that is used to spin turbines and generate electricity (Smith et al., 2008).

Nuclear fission is an appealing source of energy because it is clean and produces little, if any, greenhouse gases and gases that contribute to acid rain (Smith et al., 2008). Uranium, the principal fuel currently used in fission reactions, has a known reserve of 5.5 million tons. At current levels of extraction, this supply translates to about 80 years' worth of fuel (Morton et al., 2008). Additional elements that can be used as fission fuel include thorium (not yet proven) and plutonium created from uranium isotopes. Preliminary research shows that plutonium reactors could achieve up to 60 times more energy than current practices for each kg of uranium used (Morton et al., 2008).

Despite these promising factors, nuclear fission has severe limitations primarily due to safety and environmental risks. Catastrophes such as Three Mile

Island and Chernobyl have deterred the expansion of nuclear fission globally. Fission plants are economically efficient in the long-run, but they are expensive to construct and operate, so government subsidies are almost always necessary. Nuclear waste produced from the reaction needs to be securely stored in a remote location. Also, nuclear operations intrinsically bear the risk of an attack from terrorist organizations or an enemy state. While potentially practical and efficient, nuclear fission plants face an uphill battle for public acceptance.

1.5.3 Solar Power

The Earth's surface receives enough energy every hour from the sun to supply the world's energy needs for a year. Theoretically, all primary energy requirements could be fulfilled by the solar power that contacts less than $1/10^{th}$ of the area of the Sahara desert (Morton et al., 2008).

Renewable solar energy can be defined within two categories: photovoltaic and solar thermal power. The primary difference between the two is that while solar thermal power requires direct access to the sun, photovoltaic solar power can utilize moderately diffuse light that penetrates cloud cover. Even under optimal conditions, thermal solar panels can only convert 1% of the total amount of solar radiation captured into usable energy. In contrast, a typical photovoltaic solar panel can be anywhere from 12-20% efficient (Morton et al., 2008).

In concentrated solar thermal systems, sunlight is directed towards fluid filled turbines that begin to spin and generate electricity once the fluid has been heated to a certain temperature. The unique benefit to solar power is that, due to the wide-spread

availability of solar energy, it can be utilized off-grid and thus is accessible to areas lacking a well-developed infrastructure.

Solar energy is becoming increasingly economical, yet it remains subject to several limitations. Two of the greatest limitations of solar energy are cloud cover and manufacturing/installation costs (Morton et al., 2008). Large installations, particularly in areas with optimal sunlight and minimal cloud cover such as a desert, present the best cost-efficient solution. The remoteness of such locations creates the need for new distribution methods of high-power electricity to more densely populated areas. Even with these limitations, solar energy may be one of the most promising sources of renewable energy. However, before it can be globally incorporated, enhanced storage and distribution techniques need to be developed.

1.5.4 Wind

The movement of Earth's atmosphere generates vast amounts of energy. Research shows that at least 72 terawatts could be generated using 2.5 million of the larger turbines that are currently placed around the world with wind speeds of at least 6.9 meters/sec (Archer et al., 2005). Additionally, according to the Global Wind Energy Council, global wind capacity has risen by 25% in each of the past five years. As of January 2008, it is estimated that the world's installed wind capacity is 94 gigawatts, with the opportunity to triple over the next six years at a growth rate of at least 21% (Morton et al., 2008).

Wind power is inconsistent and intermittent, meaning that measures must be taken to account for such variability. For example, wind power favors windy seas, onshore breezes, and empty plains. It is also subject to the influence of climate

change patterns. For instance, temperatures surrounding a wind farm could rise as turbines slow down the wind to capture it. Researchers estimate that 2 terawatts of wind capacity could affect global temperatures by about 0.5 degrees Celsius, with warming around the mid-latitudes and cooling at the poles (Keith et al., 2004). While wind power is low-density, a relatively large amount of land is required to generate any substantial energy (usually less than 10 watts per meter^2) (Morton et al., 2008). In addition wind power is currently limited by the speed at which turbines can be manufactured.

The United States is taking an active stance to find ways to overcome these limitations. Competitive with coal in terms of costs, the only upkeep required is the maintenance of turbines and power lines. The United States added 5.3 gigawatts of wind capacity in 2007 with another 225 gigawatts in the planning stages. Currently, the country has more plans for wind-generating capacities than for coal and gas plants combined (Morton et al., 2008).

1.5.5 Biomass

Biomass was the world's first source of energy, and the largest until fossil fuels replaced it during the Industrial Revolution of the 20th century (Morton et al., 2008). Defined as anything that is currently or was previously alive, examples of biomass include trees, crops, garbage and animal wastes; the majority of the biomass used for energy is wood. The basis of biomass as a source of energy relies on the process of photosynthesis. Plants use this process to capture and store solar energy in the form of carbohydrates. Ultimately, these carbohydrates are combusted to create energy (Smith et al., 2008).

There are four different forms of biomass: biogas, solid bio-fuels, liquid bio-fuels and energy crops. Biogases such as methane are emitted through the decomposition of organic waste. Solid bio-fuels exist in the form of crops, hay, and wood while liquid bio-fuels include bio-diesel and ethanol. Finally, energy crops are grown specifically for use as solid and liquid bio-fuels; two prominent energy crops are corn and sugar cane.

Due to its versatile nature, "biomass can be used in any application that fossil fuels are used in" (Smith et al., 2008). In addition, biomass can supplement coal or gas in conventional power plants. In co-generation plants, biomass works at 85-90% efficiency by utilizing the waste heat (Morton et al., 2008). Biomass has surpassed hydropower as the largest source of renewable energy in the United States and researchers predict that worldwide production of energy from biomass has the "potential to provide for over half of the world's energy needs by the year 2050" (Smith et al., 2008). Due to the fact that it can be co-fired with fossil fuels, biomass has been cited by many experts "as the renewable energy that can help phase out fossil fuel use and smooth over the transition to cleaner energies such as wind and solar" (Smith et al., 2008).

Although a significant and viable source, biomass has its limitations. As far as electricity production is concerned, biomass may be limited to thinly populated areas due to the amount of land required to surround the proximity of the power plant. In addition, there is a limited availability of land to grow crops and sustain the forests necessary to harvest biomass. Finally, photosynthesis is not a 100% efficient process.

Since plant growth relies on a continuous water supply, abrupt changes in climate, such as a drought, could make land inarable.

1.5.6 Bio-electricity

Bio-electricity is an emerging form of renewable energy that encompasses electricity production from living organisms, particularly electrogenic (capable of producing electricity) microorganisms. These electrogenic bacteria produce electricity as part of their normal metabolism when placed in a microbial fuel cell. The specific designs are discussed below. The general goal of microbial fuel cells is relatively straightforward: to transform organic matter into electrical energy. *Geobacter sulfurreducens,* is an electrogenic bacterium that is used in microbial fuel cells to produce electricity.

Microbial fuel cells are considered to be extremely efficient and present no risk to the environment. However at the moment bio-electricity is not economically viable due to implementation costs and slow electrical production. Recent research has made great strides towards economic viability.

1.6 Summary

According to the 2007 report on Energy and Climate Change by the World Energy Council (WEC), "no single energy source can meet the energy needs of the world and its emission goals at the same time, nor can any single policy or measure provide the full solution" (Hanjalić et al., 2008). Each and every renewable resource discussed above is burdened by some limitation that discourages implementation on a large scale. Wind power, for instance, boasts cost-efficient and eco-friendly energy,

but is slow to manufacture and may contribute to climate change. Therefore, the solution to the impending energy crisis must be a collaboration of all the strengths and advantages of each resource.

Bio-electricity has the potential to play an important role in this multifaceted approach. It could eventually be utilized to aid in recycling of organic wastes in a productive manner as well energy production from biomass crops. While the scope of bio-electricity has yet to be determined, it is possible that with more efficient designs on a larger industrial scale, microbial fuel cells could significantly contribute to solving the energy dilemma.

Chapter 2: Literature Review

Useful Terms and Acronyms

- **Aerotolerance:** tolerance to oxygen
- **Aerotolerant:** tolerant to atmospheric levels of oxygen
- **CAT:** catalase
- **Eletrogenic bacteria:** bacteria which are capable of producing electricity in a microbial fuel cell
- **FAM:** Fumaric Acid Media
- **FAM-spec:** Fumaric Acid Media supplemented with spectinomycin
- **FCM:** Ferric Citrate Media
- **Microaerotolerant:** tolerant to small levels of oxygen, significantly below atmospheric levels
- **Oxidative Stress Protection Enzymes:** enzymes that catalyze reactions removing toxic oxygen species created by metabolism
- **pRG5CAT:** pRG5 containing a catalase gene as an insert
- **pRG5SOD:** pRG5 containing a superoxide dismutase gene as an insert
- **SOD:** superoxide dismutase

2.1 *Geobacter* Species

2.1.1 *Geobacter* Description

As previously stated, of particular interest for use in microbial fuel cells is the genus *Geobacter.* This is a genus of δ-Proteobacteria belonging to *Geobacteraceae*, a family which has unique metabolic capabilities. *Geobacter* species are capable of complete oxidation of complex carbon compounds in the absence of oxygen. This is accomplished by reducing metallic species at the terminal stage of the electron

transport chain. *Geobacter* species make up the largest portion of iron reducing bacteria in subsurface waters and other sediments and are highly involved in the recycling of oxidized metals like Fe(III) and Mn(IV).

The genus *Geobacter* has a number of unusual phylogenetic traits. For example both *G. sulfurreducens* and *G. metallireducens* have eukaryotic versions of the citrate synthase gene. Their metabolic genes are otherwise very similar to those of *Desulfovibrio vulgaris*, low-G+C firmicutes, and some Archaea.

Geobacter also have a wide variety of c-type cytochromes that are believed to be important in its ability to shuttle electrons outside the cell. These cytochromes are highly similar to those found in *Schewanella oneidensis,* another species capable of using external electron acceptors for metabolism, with 23 of these cytochromes common to both *G. sulfurreducens* and *S. oneidensis*. However, *G. sulfurreducens* has an additional 43 cytochromes not found in *S. oneidensis.*

S. oneidensis also shares the ability to produce electrically conductive pili with *Geobacter* species. This ability is likely critical to the high levels of electrical production that fuel cells based on either species are capable of. These pili are also found in a variety of other bacteria including photosynthetic species like Cyanobacteria.

There are a large number of potential biotechnology applications for *Geobacter* species. These species naturally occur in the interface between the oxic and anoxic layers in ground water. It is because of this that they can be used to reduce soluble metallic toxins in ground water. *Geobacter* precipitates these toxins out of

solution, making human water supplies and the environment in general much healthier.

Specifically, *Geobacter* have been used to remove uranium from ground water. This is accomplished with the addition of large quantities of acetate to stimulate growth of bacteria already present in the aquifer. As these bacteria oxidize the acetate they also reduce uranium (VI) to uranium (IV). Both species of uranium are radioactive, U(VI) is soluble in water, whereas U(IV) is not. Because U(IV) is precipitated out of the ground water it will not spread as easily from a spill site. It is possible that this method could be used to aid in mining uranium with significantly less environmental damage than is normally incurred (Lovley, 2002; Lovley, 2003).

In order to grow on electrodes, *Geobacter* utilizes an exterior metallic electron acceptor. Naturally, this can be used to establish a flow of electrons that travels from the anode, in which the bacteria are growing on, to a separate cathode. Thus *Geobacter* can grow by consuming simple sugars and produce an electrical current as a byproduct.

This discovery has allowed the development of a new generation of fuel cells using *Geobacter* and other similar species like *S. oneidensis* to break down carbon sources and produce electricity. At the moment the yield from this process is not high enough to justify the costs of constructing and operating *Geobacter* based fuel cells. However microbial fuel cells show some promise in wastewater treatment. Much of the energy spent in treating waste water is expended to remove biological contaminants. Microbial fuel cells could potentially remove these contaminants and

produce energy in the process instead of expending it. This would turn a process that is currently a net loss of energy into a net energy gain.

Another possible application for *Geobacter* is the development of electrically conductive pili. These pili can be thought of as nanowires and potentially could be used for the same kind of applications that carbon nanotubes are currently envisioned as serving. However because these structures are already produced in *Geobacter* naturally they could potentially be produced at much higher quantity and significantly lower cost than carbon nanotubes. Electrically conductive pili could be very useful in designing nanoscale computing and other non-biological nanomachines.

2.1.2 History of *Geobacter*

The first *Geobacter* species (initially designated strain GS-15) was isolated from the Potomac River, downstream from Washington D.C., in 1987 ("The *Geobacter* Project," 2004). This organism, known as *Geobacter metallireducens*, was the first organism found to oxidize organic compounds to carbon dioxide with iron oxides as the electron acceptor ("The *Geobacter* Project," 2004). *Geobacter metallireducens* is a rod-shaped, Gram-negative, anaerobic bacteria with both flagella and pili. The first *G. metallireducens* (initially known as strain GS-15) was isolated from freshwater sediment, and could gain energy through reduction of iron, manganese, uranium and other metals (Lovley & Phillips, 1988). *G. metallireducens* can also oxidize short chain fatty acids, alcohols, and monoaromatic compounds such as toluene and phenol using iron as its electron acceptor (Lovley et al., 1993). *Geobacter* species can transfer electrons onto the surface of electrodes. This ability

has allowed scientists to design new microbial fuel cells which can efficiently convert waste organic matter to electricity ("The *Geobacter* Project," 2004).

Geobacter can grow structures called pili, which can be used as nanowires that are 3-5 nanometers in diameter and up to 20 μm in length. By altering the genes of the organism, scientists can create many different pili with different qualities and functions (Reguera et al., 2005). "Such long, thin conductive structures are unprecedented in biology. This completely changes our concept of how microorganisms can handle electrons, and it also seems likely that microbial nanowires could be useful materials for the development of extremely small electronic devices (Reguera et al., 2005)."

There are currently 13 separate identified species of *Geobacter*: *Geobacter argillaceus, Geobacter bemidjiensis, Geobacter bremensis, Geobacter chapellei, Geobacter grbiciae, Geobacter hydrogenophilus, Geobacter metallireducens, Geobacter pelophilus, Geobacter pickeringii, Geobacter psychrophilus, Geobacter sulfurreducens, Geobacter thiogenes, Geobacter uraniireducens* each with their own properties (Euzéby, n.d.).. Team iGEM's research focused on *G. sulfurreducens.*

2.1.3 Eminent *Geobacter* researchers and accomplishments

The leading researcher in the study of *Geobacter* is Dr. Derek Lovley. Dr. Lovley discovered *Geobacter* in 1987. Upon his suggestion, the bacteria were named *Geobacter*. In 1984, Lovley joined the U.S. Geological Survey, where he focused on water resources. He first worked on water quality in the Chesapeake Bay, but later moved into groundwater studies. By 1987, Lovley believed that certain microbes existed which could "eat metals" or "breathe metals" since the Earth has elements

such as iron on which such microbes could live. While looking through sediments in the Potomac River in 1987, Lovley made his discovery. He found a previously unknown type of bacteria that survived by living off metals, which he called *Geobacter metallireducens*.

Geobacter metallireducens converted insoluble ferric oxide in non-oxygen environments into soluble iron. The iron was then able to travel in the environment, become re-oxidized, and return to its original insoluble state. In 1995, Lovley left the Geological Survey to take a professorship at the University of Massachusetts at Amherst. He focused his research on *Geobacter* and its biochemical composition. By 1997, Lovley was the head of the department, and in 2000, was named to a distinguished professorship. He also helmed what became known as the *Geobacter* Project. Lovley and his team continued to work on this idea for the U.S. Department of Energy, Naval Research, and the Defense Advanced Research Projects Agency.

In 2003, Lovley and one of his scientists discovered *Rhodoferax ferrireducens,* which had the potential to be a long-term energy producer. That same year, Lovley and his team built a fuel cell, or battery, that used bacteria to turn garbage into energy. The *Rhodoferax ferrireducens* bacteria in the battery create an electrical current by metabolizing carbohydrates. Though the initial fuel cell did not have a lot of power, there was much potential for growth of this idea.

2.1.4 Genome

The genome of *G. sulfurreducens* is 3.814 megabases and contains 3,466 open reading frames predicted to code for proteins. *G. metallireducens* has a similar genome with a total of 4.01 megabases encoding 3,621 genes. *G. metallireducens* also contains a 13.7kb plasmid coding 13 separate genes including a bacterial toxin and a gene for plasmid stabilization. In addition *G. metallireducens* is capable of chemotaxis using flagella in order to move towards soluble electron acceptors.

Both species of *Geobacter* have the enzymes necessary for glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway. These enzymes, with the exception of citrate synthase, are very similar to the typical bacterial homologs. As previously mentioned the citrate synthase is a eukaryotic type protein. With the addition of the *Geobacter* cytochromes this gives the bacteria the ability to completely oxidize sugars anaerobically and derive as much energy from each sugar as aerobic bacteria. In addition *Geobacter* species have all the components of the aerobic respiratory chain.

2.1.5 Membrane Properties

Geobacter species are gram-negative and so have an inner cell membrane, a cell wall with a periplasmic space, and an outer cell membrane. Member species have membrane-bound Fe(III) reductase enzymes. These enzymes are localized on the exterior membrane and, in conjunction with cytochromes in the same region, are responsible for the ability to reduce iron as the terminal stage of the electron transport chain.

The ability to reduce exterior metallic compounds is built on the interaction of a number of membrane proteins including the interactions of cytochromes and related proteins. For example in *G. sulfurreducens* the cytochromes OmcS and OmcE are both very important in the transport of electrons. However, transcription of both of these proteins is regulated, either directly or indirectly, by the cytochrome OmcF. Furthermore the entire structure of the periplasmic space is regulated by the protein OmpJ which is highly abundant in *G. sulfurreducens* membranes. Without OmpJ, fuel cells using *G. sulfurreducens* show dramatically decreased electrical production.

2.1.6 Motility and Attachment

Geobacter metallireducens express flagella and pili only when grown on insoluble Fe(III) and Mn(IV). Additionally, it demonstrates chemotaxis towards Fe(III) and Mn(IV) in solution. Regulation of flagella and pili formation seems to be driven by the lack of soluble terminal electron receptors. This is particularly important because pili appear to be necessary for the formation of biofilms and thus the transfer of electrons from the bacteria to an electrode in a microbial fuel cell. Not only are these pili probably necessary for attachment to the surface of the electrode, they are probably the direct means by which the electrons are transferred.

2.1.7 Aerotolerance

Geobacter was originally classified as an anaerobe. However, it has a number of unusual characteristics for such a species. Unlike a number of species of anaerobic bacteria which contain enzymes poisoned by the presence of even minute quantities of oxygen, *Geobacter* can tolerate oxygen for short periods of time. Thus, it would be

far more accurate to refer to it as microaerotolerant, or tolerant to sub-atmospheric levels of oxygen. In fact it has all the components necessary for the use of oxygen as a terminal electron acceptor.

Rather than enzyme poisoning, *Geobacter* seems to be unable to tolerate the presence of oxygen radicals for a long period of time, which are in part contributed by its own metabolic processes in the presence of oxygen. This is surprising because *Geobacter* contains homologues for many of the oxidative stress enzymes. Specifically, these species contain homologues for catalase, superoxide dismutase, and ruberythrin, as well as various other peroxidases. These attributes of *Geobacter* have significant implications in terms of oxidative stress and the capacity for the organism to potentially survive in oxygen.

2.2 Oxidative Stress

2.2.1 Function of Superoxide Dismutase and Catalase

Oxygen is required to support aerobic life, but key metabolic reactions can also result in byproducts that are toxic oxygen species. Examples of these species include the hydroxyl radical, the superoxide radical, and hydrogen peroxide, among others (*Figure 2*) (Bauman, 2007). Interference and destruction of the cell's functions occur through many different mechanisms. Radicals have an odd number of electrons, and have an unpaired electron in their valence shell. They are highly unstable, and due to their high reactivity, they are able to remove electrons from the outer shells of other species that are important to the cell's survival; this includes DNA, reduced riboflavins, 4Fe-4S clusters on dehydratases, and other necessary metabolites (Imlay, 2008).

Organisms that live in an aerobic environment produce enzymes that are capable of transforming these species into unreactive, harmless species. Two of the most important enzymes of this type are superoxide dismutase (SOD) and catalase (CAT). Superoxide dismutase converts superoxide radicals into hydrogen peroxide and molecular oxygen. As *Figures 2, 3 and 4* show CAT then converts this hydrogen peroxide into water and molecular oxygen (Bauman, 2007). SOD is one key enzyme involved in the protection of certain enzymes from destruction by oxygen radicals. Through enzymatic acitivity, like that of SOD, catalase is essential to breaking down hydrogen peroxide, which is formed in the environment and as a by-product of metabolic reactions (Zamocky et al, 2008).

Figure 2: (a) Hydroxyl radical, (b) Superoxide radical, and (c) Hydrogen peroxide

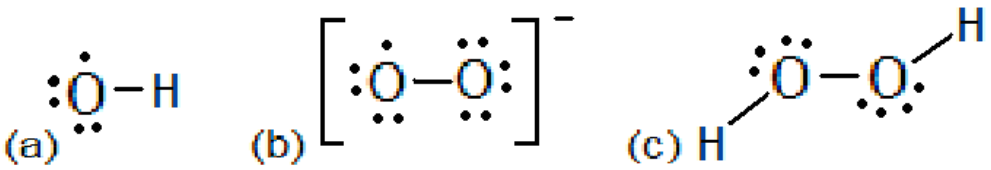


Figure 3: Reaction catalyzed by superoxide dismutase (SOD)

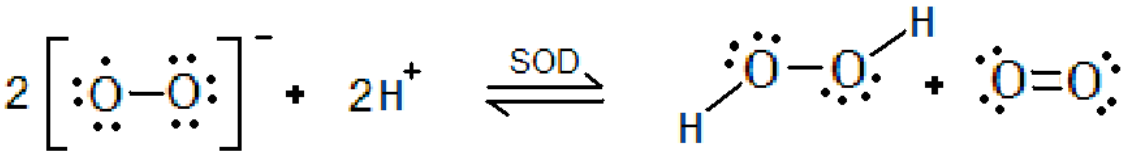
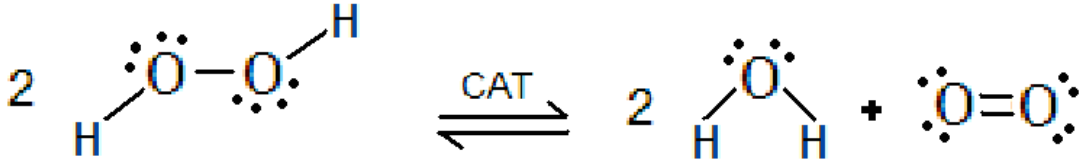


Figure 4: Reaction catalyzed by catalase (CAT)



Superoxide dismutase has been isolated from a variety of organisms, including both prokaryotes and eukaryotes. There are a total of four types, classified by their metal cofactor. The majority of SOD found in eukaryotes has been blue-green in color, with copper (II) and zinc (II) components, while the majority of SOD found in prokaryotes has been dark red in color, with two manganese (III) ions (Fridovich, 1972). Isolated from *E. coli,* a third type of SOD that is yellow in color and contains an iron (III) ion was discovered in 1973 (Yost et al, 1973). The last type of SOD was isolated in 1996 from various *Streptomyces* bacteria and contains a nickel (III) ion (Youn, 1996).

There are three different protein families of catalase: typical catalases, catalase-peroxidases, and manganese catalases. Typical catalases contain a heme prosthetic group and are the most abundant form of catalase. Organisms from all three domains of life have catalase from this family. Catalase-peroxidases also contain a heme group, but can catalyze the reduction of other peroxides in addition to hydrogen peroxide. They are not found in plants or animals. Manganese catalases have a dimanganese active site. They are found in a small number of bacterial species (Zamocky et al, 2008).

The active site of catalase (whether it is a heme or dimanganese) binds to one of the oxygen atoms on hydrogen peroxide, the second oxygen atom leaves as water with the two hydrogen atoms. This is also done with a second hydrogen peroxide molecule and the two oxygen atoms bound to the heme then join to form an O_2 molecule (Zamocky et al, 2008).

2.2.2 Previous Research on the Effects of Oxidative Stress on Obligate Anaerobes

There are five classifications of aerotolerance in bacterial species, obligate aerobes, facultative anaerobes, aerotolerant anaerobes, microaerotolerant anaerobes, and obligate anaerobes. Obligate aerobes require oxygen to survive, because it is used as the final electron acceptor in their metabolic pathways. Facultative anaerobes can use aerobic respiration or fermentation to produce the energy needed to survive, though aerobic respiration is preferred since it produces more energy. Aerotolerant anaerobes only use anaerobic respiration to produce the energy needed, however they can survive in aerobic environments due to enzymes that detoxify oxygen. Microaerotolerant anaerobes are able to survive in sub-atmospheric levels of oxygen but may not have the enzymes necessary for aerobic respiration. Obligate anaerobes only use anaerobic respiration and are unable to survive in environments with even low levels of oxygen (Bauman, 2007).

It was originally hypothesized that obligate anaerobes could not tolerate any oxygen in their environment due to the lack of enzymes such as superoxide dismutase and catalase, which protect the cell against the toxic affects of oxygen. However, recent research has shown that many organisms originally classified as obligate anaerobes do produce protective enzymes when confronted with oxidative stress.

Desulfovibrio desulfuricans, thought to be an obligate anaerobe, produces oxygen reductase, superoxide dismutase, and catalase in the presence of oxygen. In order to test the aerotolerance of this anaerobe, cultures were grown for seven hours under 5%, 15%, 18%, and 21% oxygen. The “strict” anaerobe was able to survive in up to 18% oxygen. In addition, the experiment showed that the growth curve was

similar under both aerobic and anaerobic conditions, and that cell division and motility were mostly unaffected. At 21% oxygen the cells stopped growing, but they did not die. Cells from the 21% oxygen tubes grew again after inoculation into anaerobic tubes; the exposure to oxygen did not permanently affect the viability of *D. desulfuricans* (Lobo et al, 2006).

Desulfovibrio vulgaris was also thought to be an obligate anaerobe. Samples of two different strains were flushed with pure oxygen and data was collected after 15, 30, and 60 minutes of exposure. One strain was wild type while the other lacked SOD. The cells without SOD had only ¼ the viability of the wild-type cells (Fournier et al, 2004).

Another species, from the same genus, *Desulfovibrio gigas*, has also been the center of recent oxidative stress research. It was originally thought that *D. gigas* was a strict anaerobe; however, evidence for substrate level phosphorylation was discovered, along with evidence that the bacteria produce higher amounts of ATP in the presence of oxygen. This shows that not only can *D. gigas* survive in the presence of oxygen, but may also be able to use oxygen in its metabolic pathways.

In addition, it has recently been discovered that the choice of growth media can increase the aerotolerance of *D. gigas*. This was done by growing the bacteria in fumarate/sulfate media as well as lactate/sulfate media. After adding NADH or succinate, oxygen was consumed by the fumarate/sulfate cultures (at similar rates to that of aerobic bacteria), and after adding catalase, water was produced. Oxygen was also consumed by the lactate/sulfate cultures, but the rate was lower by over one order of magnitude (Lemos et al, 2001).

Myxococcus xanthus has been one bacterial species of interest in recent rice and herbicide studies. One enzyme produced by *M. xanthus* is protoporphyrinogen oxidase (PPO). This is the target enzyme of many peroxidizing herbicides. The loss of PPO causes a build-up of singlet oxygen, which causes peroxidation of the membrane lipids, and eventually cell death. The gene for PPO (from *M. xanthus*) was inserted into rice, so that it would be over-expressed, and the transgenic rice tested was found to be resistant to peroxidizing herbicides. After exposure to the herbicides, the wild type rice died, but the transgenic rice was resistant (Lee et al, 2006). PPO was found in both mitochondria and chloroplasts in the cells and, under normal conditions, the wild type and transgenic rice grew at the same rate. After exposure to the herbicide, only the seeds from the transgenic plants were able to sprout (Jung et al, 2004).

2.2.3 Previous Research on Transformations

Anacystis nidulans (a cyanobacterium) is highly sensitive to oxidative stress. The cells are able to express Fe superoxide dismutase, but it does not provide enough protection when confronted with paraquat (an herbicide that promotes the formation of superoxide radicals). *Escherichia coli* cells contain two types of superoxide dismutase, Fe and Mn. While the Fe superoxide dismutase is constitutively expressed, the Mn superoxide dismutase is induced in the presence of paraquat. Using plasmid transformation, the Mn superoxide dismutase gene from *E. coli* was inserted into *A. nidulans* and into *E. coli*. There was a 6-7 fold increase of expression in *E. coli*, and a small increase in expression in *A. nidulans*. After exposure to paraquat, expression in *E. coli* increased 10-100 fold; however expression in *A. nidulans* did not change. This

was most likely due to a difference in induction mechanisms in the two bacteria. Although the gene was not induced by paraquat in *A. nidulans*, it was still produced in large enough quantities to noticeably increase the bacteria's resistance to paraquat (Gruber, 1990).

Another (independent) experiment was performed to determine if the resistance of *E. coli* to oxidative stress (caused by paraquat) could be increased by inserting the Cu/Zn superoxide dismutase gene from *Drosophila sechellia* into *E. coli* using a plasmid vector. The resistance to paraquat was increased in the *E. coli* transformants, however, none of the cells were able to continue growth after being exposed to paraquat in concentrations above 1 mM. It was speculated that this was due to the build-up of the H_2O_2 product formed in the reaction catalyzed by superoxide dismutase, a problem that may be solved by inserting the catalase gene, along with that of the superoxide dismutase (Goulielmos, 2003).

Geobacter is classified as an obligate anaerobe, though its genome contains the sequences for catalase and superoxide dismutase. Although the genes for these important enzymes are present, they are not expressed in high enough quantities to confer aerotolerance to *Geobacter*. By attaching these sequences to a constitutive promoter and a plasmid, then reinserting them into *Geobacter*, it may be possible to transform *Geobacter*, allowing the bacteria to be aerotolerant. This would make *Geobacter* easier to work with in laboratories, as well as in microbial fuel cells.

2.3 Microbial Fuel Cells

2.3.1 Overview

Microbial fuel cells (MFCs) offer a clean and renewable source of energy that could potentially be an effective alternative to current environmental power sources, such as solar, geothermal and wind (NRLAC, 2005). With the ability to function in a number of diverse environments on a wide array of fuels, MFCs are a potential power source for use in extreme conditions, such as environments that are anaerobic, have volatile temperatures and those with varied amounts of solar energy.

Microbial fuel cells (MFCs) convert chemical energy directly into electricity. Chemical energy can be defined as a biological substrate, such as glucose, acetate or wastewater. Bacteria act as the catalysts for this direct removal of electrons from the substrate. The electrons that result from this oxidation process are transferred to an anode, enter an electrical circuit and finally move to a cathode. A variety of mechanisms, such as electron mediators, direct membrane associated electron transfer or bacteria-produced nanowires, shuttle electrons to the anode. From the cathode, the electrons are then transferred to a high potential electron accepter, such as oxygen, to complete the oxidation process. As the current of electrons flows from the anode to the cathode, a potential difference is created and power is directly generated.

In general, bacteria are placed within an anoxic anode chamber to oxidize any organic matter that is available in the chamber. In the absence of oxygen, the electrons are unable to attach directly to oxygen as oxidation occurs. As a result the electrons attach directly to the anode.

Electrons, in combination with oxygen and protons, attach to the cathode and produce water as a final product. The aforementioned potential difference that is

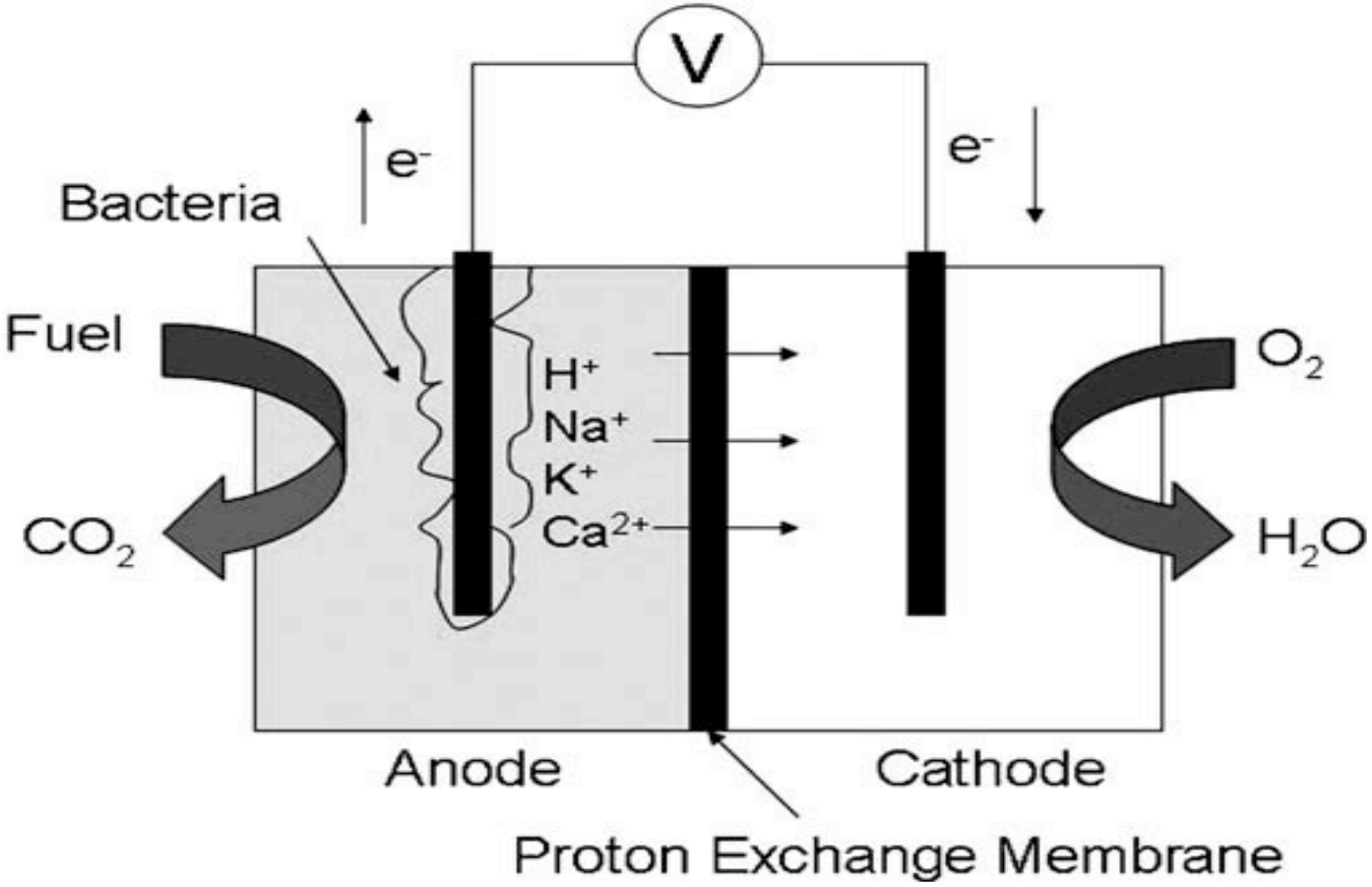
produced between the anode and cathode is about 0.5 V; it drives the fuel cell as long as the organic supply at the anode is continuously replaced (Logan et al., 2006).

2.3.2 Microbial Fuel Cell Design

Microbial fuel cells can be designed in a variety of ways. As *Figure 5* shows, one type of MFC is comprised of two chambers separated by a proton exchange membrane. This membrane functions to allow the flow of protons from the anode chamber into the cathode chamber while preventing oxygen from moving from the cathode chamber to the anode chamber. The anode is placed within one chamber, while the cathode occupies the second. Both sides of the anode remain sealed while opposing sides of the cathode are exposed to water and air.

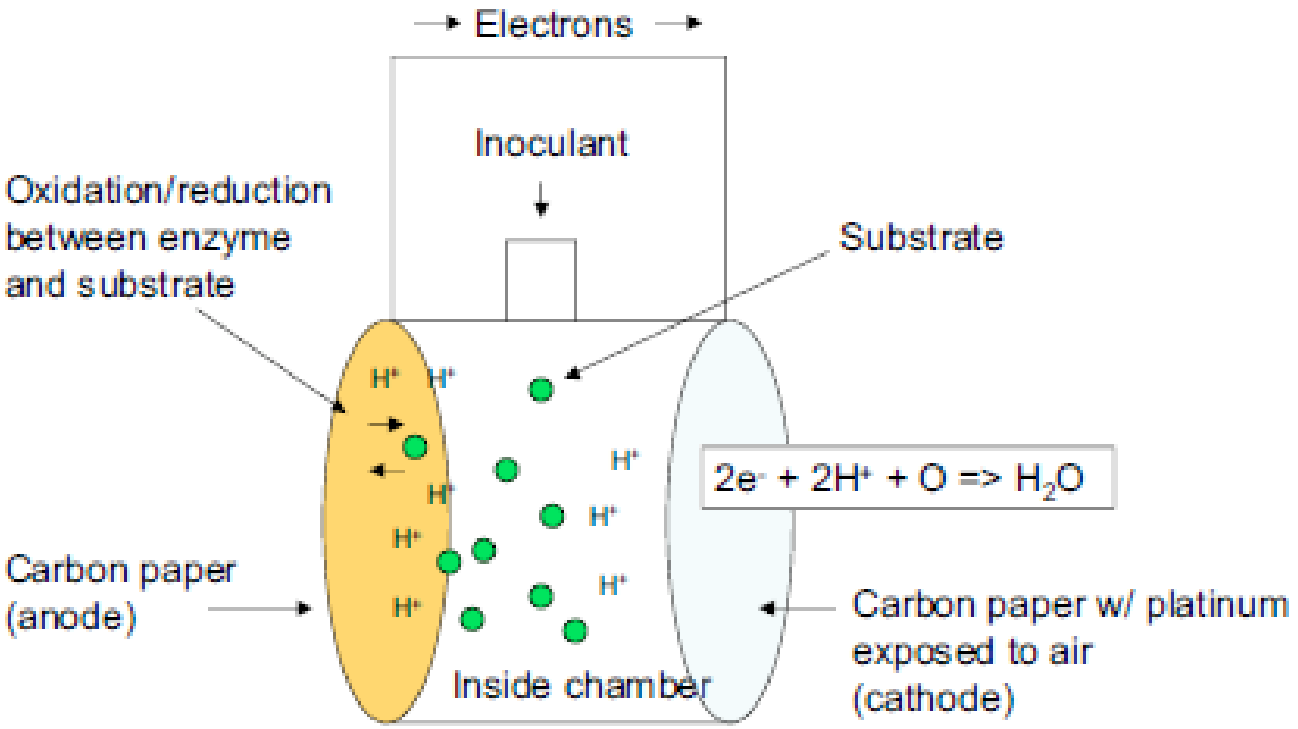
The MFC used for the purposes of our research was of a single-chambered design (*Figure 6*). This system creates a hydrogen gradient from the anode to the cathode, along which the protons diffuse. This diffusion balances out the pH of the solution within the chamber to create an ideal state for the *Geobacter*. As previously mentioned, the cathode is the aerobic portion of the MFC, meaning that oxygen can diffuse from the outside environment to the interior of the MFC. This process forms water in the presence of a proton gradient. A circuit connects the anode to the cathode, allowing the flow of electrons to complete the circuit.

Figure 5: Double chambered microbial fuel cell



Representation of a double chambered microbial fuel cell separated by a proton exchange membrane (Angenent et al, 2006).

Figure 6: Single chambered microbial fuel cell



Representation of a single chambered microbial fuel cell with an anaerobic anode and aerobic cathode (Liu et al, 2006).

The anode contains electrochemically-active microorganisms that function as biocatalysts and catabolize organic materials to electrons. Since the positively charged cathode is abiotic, it receives electrons from the anode and initiates the reduction of oxygen to water.

2.3.2.1 Microbial Components

Certain bacterial species and/or microbes can transfer electrons through a natural oxidation/reduction process. The primary bacteria that are capable of this process and that are used in MFCs are: *G. sulfurreducens, G. metallireducens, Shewanella putrefaciens* and *Rhodoferax ferrireducens* (Bond et al., 2003).

2.3.2.2 Anode Material

The anode and cathode materials must be conductive and chemically stable in solution. The most versatile material is carbon (graphite plates, rods, cloth, and carbon paper). Capacity can be increased with the addition of various chemicals such as Fe(III) and Mn(IV). It is unclear whether biofilm growth and/or particle build-up on the carbon surface has any negative long term effect on MFC operation (Logan et al., 2006).

2.3.2.3 Electron Acceptors

The cathode material must use a good electron acceptor that is able to support series of oxidation reactions. Ferricyanide ($K_3[Fe(CN)_6]$) is a good electron acceptor. The advantage of using it is that the cathode will have a potential close to that of an open circuit. However $K_3[Fe(CN)_6]$ can diffuse across the proton exchange membrane to the anode chamber. Oxygen is another ideal electron acceptor because it is easily available and sustainable (Logan et al., 2006). In addition, it has a high oxidation potential and produces water instead of chemical waste products.

Iron compounds are commonly used as transition metals for aerobic biocathodes because they are more readily available than other metals, such as manganese. MFC microbes oxidize Fe(II) to Fe(III) and release excess electrons to final acceptors, such as oxygen (He et al., 2006).

2.3.3 Limitations of Microbial Fuel Cells

Large-scale application of microbial fuel cells is primarily limited by "investment costs, [large scale] technical issues and factors limiting performance, both in terms of anodic and cathodic electron transfer" (Pham et al., 2006). Essentially, fuel cells are evaluated by the amount of electricity they produce. This power generation is affected by a number of different processes including the kinetics of electron transfer at the anode, electrical resistances and cathode potential losses (Reguera et al., 2006). In addition, specific limitations at the anode prevent the current density of the MFC from reaching maximum potential. This decreases the probability of making MFCs commercially feasible. It is possible that the "transfer of the electrons to the anode through electron shuttles and/or a biofilm matrix" may be why current density never reaches its maximum potential (Reguera et al., 2006). If this is the case, the processes by which the electron shuttles and biofilm matrices work could be modified to increase current density.

The diffusion of protons out of the biofilm limits current density as well. In addition to electrons, H^+ ions are produced from the oxidation reactions that occur within the MFC. The H^+ ions participate in the production of water at the cathode. As a result, H^+ ions must travel to the cathode chamber in order to participate in such reactions.

Research shows that poor H^+ transport to the cathode causes a "significant decrease in voltage efficiency" (Torres et al., 2008). In addition, the movement of other

ions as well as H^+ from the anode chamber causes a drop in pH. This pH drop "suppresses the microbial metabolism in the anode compartment, thereby reducing the efficiency with which organic compounds are organized" (Torres et al., 2008). As a result, it is thought that H^+ ion transport is the "primary limiting factor to reaching maximum current density" (Torres et al., 2008). Research suggests that the buffer within the MFC must be maintained at a higher concentration to improve H^+ transport and keep current density levels up.

Microbial fuel cells that utilize abiotic cathodes face limitations as well. Abiotic cathodes require a catalyst or electron mediator to achieve a high rate of electron transfer. These factors increase the cost and lower the operational sustainability of MFCs (He et al., 2006). One proposed solution to this issue is the use of aerobic biocathodes that use microorganisms to complete the cathode reactions. With oxygen as the terminal electron acceptor, electron mediators such as iron and manganese can be used. These mediators are directly reduced by the cathode and then re-oxidized by the bacteria (He et al., 2006). In addition, anaerobic biocathodes are an alternative solution because they directly reduce electron acceptors, such as nitrate and sulfate, through microbial metabolism (He et al., 2006).

In addition to the proposed solutions, future research should focus on refiguring "reactor configuration, power density and material costs" to make large-scale MFCs more economically feasible and realistic (Pham et al., 2006).

2.3.4 Current Research

2.3.4.1 Wastewater Treatment

Wastewater is recognized as a renewable resource that can produce electricity, fuels and chemicals. However, the aeration of sewage in wastewater management treatment facilities creates an energy demand of about 0.5 kWh/m^3. In addition, large amounts of excess sludge are produced, presenting problems for proper treatment and disposal. Microbial fuel cells can provide answers to several of the problems that traditional wastewater treatment facilities face. Essentially, MFCs enable the recovery of energy out of the wastewater in a more efficient process. Equally important, MFCs limit excess production of sludge (Wei et al., 2003).

Recent results show that current density and power production of MFCs may be approaching levels high enough to sustain full-scale implementation of bioelectrochemical wastewater treatments (Rozendal et al., 2008).

Bioelectrical wastewater treatment is "based on the use of electrochemically active microorganisms" such as those found in MFCs (Rozendal et al., 2008). Researchers aim to create a MFC that would be able to use a number of organic materials to treat real wastewater. However, application of MFCs to real wastewater instead of synthetic wastewater with known biodegradable substrates led to a decrease in power production.

This decreased power production suggested that the microbes in the MFC could not degrade the "complex materials within the wastewater" and/or that "competing processes consumed the [available] substrate," leaving the microbes with little organic matter to oxidize (Rozendal et al., 2008). In addition, the ion exchange membrane separating the MFC chambers could have contributed to the decrease in power

production. With the MFCs run on wastewater, ion species other than protons crossed the ion exchange membrane. This caused a "pH decrease in the anode chamber and a pH increase in the cathode chamber" that resulted in a "large potential loss of about 0.06 V per pH unit" (Rozendal et al., 2008).

Current research is focused on solving these issues in order to make MFCs an applicable and feasible solution to wastewater management treatment plans.

2.3.4.2 Implementation of Marine Microbial Fuel Cells

In addition to use within wastewater treatment facilities, MFCs have been implemented in oceanic environments to power sensors and machinery. The first successful marine MFC operated with a stainless steel electrode. Stainless steel was chosen based on the findings that biofilms formed in aerobic seawater "induce an efficient catalysis of oxygen reduction on stainless steels" (Dumas et al., 2007). Stainless steel is also a realistic material because it is commercially available in large quantities and is a practical and cost-effective solution.

Previous marine MFC designs utilized electrodes covered in graphite. However they were not practical to implement in oceanic environments because the graphite eroded from both the anode and cathode. In comparison, stainless steel MFCs was promising in experimental trials in terms of power production. In addition, the stainless steel electrodes did not erode and release waste products in the water. Researchers hope that the stainless steel marine MFC will be practical enough to "operate low-power consuming marine instrumentations" used by marine biologists (Dumas et al., 2007).

2.4 *G. sulfurreducens* Fuel Cells

2.4.1 Overview

Geobacter sulfurreducens has the ability to transfer electrons generated from metabolic oxidation of organic compounds directly onto solid anodes. It is this efficient transfer that considerably enhanced MFC power output (Shukla, 2004). Pure cultures of *G. sulfurreducens* use the anode as a unique electron acceptor without the assistance of any artificial electron mediators. In addition, the bacteria can catalyze electrochemical oxidation of a variety of organic compounds such as acetate, glucose, lactate, and certain alcohols. Many modern MFC designs "utilize strains of bacteria [such as *G. sulfurreducens*] that expire when exposed to aerobic environments for extended periods of time, eliminating their ability to donate electrons to the anode in the presence of oxygen" (Shukla, 2004).

Several problems with traditional microbial conversion of organic matter to electricity have arisen because "most microorganisms only partially oxidize their organic substrates and transfer a portion of these electrons to electrodes" (Bond et al., 2003). *G. sulfurreducens* electrodes, however, can "fully oxidize electron donors by using only an electrode as the electron acceptor" (Bond et al., 2003). *G. sulfurreducens* fuel cells are highly practical because electron transfer of cells at the electrode is direct and efficient.

When placed in anoxic marine sediments and connected to similar electrodes in oxic water, graphite and platinum electrodes conduct harvestable electrical power (Bond et al., 2003). It was based on this observation, that *G. sulfurreducens* were used within MFCs for the first time. In addition, *G. sulfurreducens* organisms were found to be highly enriched on MFC anodes. Since they "transfer electrons to other insoluble electron

acceptors" results of these preliminary studies suggested that the electrode surfaces served as terminal electron acceptors (Bond et al., 2003).

2.4.2 Current Research

2.4.2.1 Dimensionally Stable Anodes (DSA) for Industrial Scale MFCs

Many industrial processes that require oxygen and hydrogen use DSA electrodes particularly for their efficient electrocatalytic properties. DSA electrodes are useful in volatile environments because they are corrosion resistant and do not change shape throughout their lives. In addition, they are more compact than traditional, bulky graphite electrodes. *G. sulfurreducens* was used to determine if "dimensionally stable anodes [could] grow electrochemically active biofilms" (Dumas et al., 2008). If DSAs proved to be effective, they would be "excellent candidates for scaling up industrial MFC pilots" (Dumas et al., 2008).

Results showed that biofilms containing *G. sulfurreducens* developed on both graphite and DSA electrodes. The resulting biofilms produced on the DSA electrodes were able to "sustain current densities [that were] higher than previously reported" (Dumas et al., 2008). As a result, DSA electrodes used in *G. sulfurreducens* MFCs would be suitable for creating larger, more industrially effective MFCs.

2.4.2.2 Signaling Pathway Identification to Improve Power Production

Flagella and pili are two critical components of *G. sulfurreducens* that contribute to biofilm formation. These attributes also contribute significantly to electron flow in *G. sulfurreducens*-mediated MFCs. *G. sulfurreducens* was the bacterial species of interest in this research study because "it is often the predominant species in a variety of sedimentary environments where Fe(III) reduction is important" (Tran et al., 2008). In addition, the ability of *G. sulfurreducens* to "remediate contaminated environments and

produce electricity" sparked interest for further study (Tran et al., 2008). Therefore, researchers wished to explore the mechanisms that control the synthesis of the flagella and pili to potentially increase expression and thereby increase power production.

Specific signaling pathways are primarily responsible for flagella and pili synthesis. According to this study, a total of 70 homologs of chemotaxis genes were arranged in several clusters near and around the flagellar genes (Tran et al., 2008). However, unlike *E. coli*, not all of these clusters were located near the flagellar genes. Researchers concluded that "the numerous chemo-receptors and chemotaxis-like gene clusters of *G. sulfurreducens* appeared to be responsible for a diverse set of signaling functions" (Tran et al., 2008). Specifically, they reasoned that these gene clusters were connected with "gene regulation and biofilm formation through functionally and spatially distinct signaling pathways" (Tran et al., 2008). Further research proposed that modifications should be made to these signaling pathways to increase expression of flagella and pili and improve power production.

2.4.2.3 Increased Respiration Rates

Faster rates of respiration would cause *G. sulfurreducens* to more quickly convert organic compounds into electricity within MFCs. Therefore, many researchers focus their efforts on identifying which increasing respiration. A major strategy in this process is to study which gene deletions, if any, would increase respiration rates (Izallalen et al., 2008). This research would ultimately lead to strains that produce electricity faster than current strains.

Any gene deletions that resulted in an "increase in the cellular ATP demand, either by creating ATP-consuming futile cycles or decreasing the availability of reducing

equivalents" were thought to be successful at increasing respiration rates (Izallalen et al., 2008).

Researchers engineered a specific strain of *G. sulfurreducens* that showed increased rates of respiration. The specific genome of this new strain showed that "transcript levels were higher for genes involved in energy metabolism, especially in those encoding TCA cycle enzymes, subunits of the NADH dehydrogenase, and proteins involved in electron acceptor reduction" (Izallalen et al., 2008). Further efforts to target these specific isolates would contribute to permanent increased respiration rates and increased power output of *G. sulfurreducens* MFCs. While the current strains have only been proven to increase the rate of respiration it is predicted that they will also show a concurrent increase in electrical production.

2.5 In Summary

- As the world supply of fossil fuels is running low, world energy consumption is increasing, and the environment is an ever-growing concern, the need for clean, efficient energy sources is becoming stronger.
- There are many clean source options arising, but each one has a unique disadvantage.
- Microbial fuel cells are one example of a revolutionary power source, which may be viable in the future.
- The anaerobic bacteria *Geobacter sulfurreducens* can donate electrons to metals as part of its natural metabolism. This unique ability makes them almost perfect for use in microbial fuel cells.

- A disadvantage however, it that because the bacteria is classified as an obligate anaerobe and can not survive in atmospheric levels of oxygen.
- Superoxide dismutase and catalase are two enzymes aerobic bacteria produce, which confer resistance to toxic oxygen species, protecting the cell.
- Previous research has been done that shows the insertion of these genes and thus increased expression of these enzymes, increases bacterial tolerance to oxygen.
- By increasing the aerotolerance of *Geobacter sulfurreducens*, it will make these bacteria a more viable source of energy in a microbial fuel cell.

This leads to the hypothesis: overexpressing the anti-oxidative stress genes *catalase* and *superoxide dismutase* in *Geobacter sulfurreducens* will result in elevated tolerance of the bacteria to atmospheric oxygen.

Chapter 3: Methodology

3.1 Research Question

Are the catalase and superoxide dismutase genes in *Geobacter sulfurreducens* functional? If so, can over-expression of these genes lead to an increase in aerotolerance?

3.1.1 Research Objective

Team iGEM sought to investigate the functionality of oxidative stress enzymes in the anaerobic bacterium *G. sulfurreducens* and their effect on its aerotolerance. We chose to focus on catalase and superoxide dismutase due to their wide acceptance as two of the most important oxidative stress enzymes. We proposed to create a plasmid containing catalase and a plasmid containing superoxide dismutase then insert each separately into *G. sulfurreducens*.

This procedure began with cloning. We extracted the catalase and superoxide dismutase genes from *G. sulfurreducens* genomic DNA. This genomic DNA first had to be harvested from cultured cells. The genes of interest were then amplified using PCR and inserted into pCR2.1 using TA Cloning. These plasmids were transformed into *E. coli* and the orientation of the gene in the plasmid was checked using digests, followed by electrophoresis. Plasmid DNA was sequenced at this time.

A subcloning procedure allowed us to move our genes of interest from pCR2.1 to pRG5, a vector expressible in *G. sulfurreducens* and using a ptaclac promotor. This was accomplished by digesting pCR2.1 with our genes of interest with BamHI and XhoI and cutting out the genes of interest. pRG5 was also digested with BamHI and XhoI. The genes of interest were ligated into separate pRG5 plasmids and transformed into *E. coli*

for storage and amplification. The plasmid was then extracted from *E. coli* and transformed into *G. sulfurreducens*.

Data was collected to determine levels of superoxide dismutase and catalase activity before and after transformation with these genes. Gene expression levels, enzyme activity levels, and aerotolerance were measured in the wild type and compared to transformants.

3.2 Bacterial Species

3.2.1 G. sulfurreducens DL1

G. sulfurreducens DL1 is a wild type strain provided to us by Dr. Derek Lovley, University of Massachusetts, Amherst. It is the strain used in Lovley Labs for metabolic engineering research.

3.2.2 Escherichia coli Strain

E. coli DH10β is commonly used in microbiology labs for its uniquely useful properties (Durfee et al., 2008). It is known for its high transformation efficiency, ability to take up large plasmids, plasmid stability, and lack of methylation-dependent restriction enzymes. This strain was created using a series of genetic manipulations before the current era of molecular biology. It has become a ubiquitous choice for transformations in labs.

3.3 Growth Conditions

3.3.1 *G. sulfurreducens* Growth Conditions

Some of the growth conditions of *G. sulfurreducens* are unique to its genus and not found in most bacteria. Most unusual is its need for an added electron acceptor. In our

media, this need was met through either fumaric acid in our "Fumaric Acid Medium" (FAM) or through ferric citrate in our "Ferric Citrate Medium" (FCM). Both electron acceptors were 55 mM in their final (after inoculum) concentrations.

G. sulfurreducens was grown in almost neutral (pH 6.8-7.0) conditions. FAM and FCM were buffered with a bicarbonate/CO_2 buffer. *G. sulfurreducens'* production of hydrogen ions caused decreases in pH in media lacking a buffer. Growth temperature was held as close to 30°C as possible.

FAM consisted of 55 mM sodium fumarate, 30 mM $NaHCO_3$, 5 mM NH_4Cl, 1.5 mM KCl, 20 mM $NaCH_3CO_2$, and 5 mM NaH_2PO_4. A trace mineral solution from ATCC was added at a 100X dilution. The medium was then bubbled for one hour per liter with 70% nitrogen, 30% CO_2 to remove any dissolved oxygen. Following bubbling, the pH was adjusted to 6.8-7.0. A stock of 0.1% resazurin was added at 0.5 mL resazurin / L media as an oxygen indicator. L-cysteine was added at a 1 mM concentration; in an anoxic environment L-cysteine turns resazurin clear. In addition, it was important to check the pH of autoclaved media, as the pH often jumped to above 8.0. The pH was always readjusted to 6.8-7.0. This medium was modeled from a *G. metallireducens* growth medium used for bioremediation research (Piwkhow, 2008).

FAM was used as the primary growth medium for *G. sulfurreducens*. Anoxic conditions were easy to visually confirm due to the resazurin oxygen indicator. This medium turned from clear to pink in the presence of oxygen due to the color change upon oxidation of resazurin. The clear medium allowed visual inspection of turbidity for unambiguous detection of bacterial growth or lack of growth. Cell densities reached almost 10^9 cells/mL in stationary phase.

FAM-spec consisted of FAM with the addition of the antibiotic spectinomycin in order to select for transformed *G. sulfurreducens* cells during growth.

FCM consisted of 55 mM ferric citrate, 30 mM $NaHCO_3$, 5 mM NH_4Cl, 1.5 mM KCl, 20 mM $NaCH_3CO_2$, and 5 mM NaH_2PO_4. A trace mineral solution from ATCC was added at a 100X dilution. The medium was then bubbled for one hour per liter with 70% nitrogen, 30% CO_2 to remove any dissolved oxygen. The pH was then adjusted to 6.8-7.0. As before, it was important to check the pH of autoclaved media, as the pH often jumped to above 8.0. This medium is a *G. metallireducens* growth medium used for bioremediation research (Piwkhow, 2008).

FCM was used to confirm the presence of *G. sulfurreducens*. The medium was a rusty green-brown color before growth and light yellow after growth due to the reduction of ferric citrate to ferrous citrate by the bacteria. Because the ferric citrate can only be reduced by members of the *Geobacter* genus and a few other genera (none of which were used anywhere in the UMBI-CARB building), color change after inoculation is a simple way to confirm that the questionable inocula are actually *Geobacter*. This test was used to confirm the presence of *Geobacter* if contamination was suspected. *G. sulfurreducens* grew less densely in this medium than in FAM.

Growth plates consisted of FAM in a 1% agar. Prior to inoculation the growth plates were left overnight in an anaerobic chamber to equilibrate and allow all oxygen to diffuse out.

FAM-spec plates were FAM plates with spectinomycin added for selection of transformants.

ATCC Trace Mineral Solution consisted of 0.5g EDTA, 3.0g $MgSO_4 \cdot 7H_2O$, 0.5g

$MnSO_4 \cdot H_2O$, 1.0g NaCl, 0.1g $FeSO_4 \cdot 7H_2O$, 0.1g $Co(NO_3)_2 \cdot 6H_2O$, 0.1g $CaCl_2$ (anhydrous), 0.1g $ZnSO_4 \cdot 7H_2O$, 0.01g $CuSO_4 \cdot 5H_2O$, 0.01g $AlK(SO_4)_2$ (anhydrous), 0.01g H_3BO_3, 0.01g $Na_2MoO_4 \cdot 2H_2O$, 0.001g Na_2SeO_3 (anhydrous), 0.01g $Na_2WO_4 \cdot 2H_2O$, 0.02g $NiCl_2 \cdot 6H_2O$ per liter of distilled water (ATCC, 2002).

ATCC Vitamin Solution consisted of 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine hydrochloride, 5.0 mg thiamine•HCl, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg calcium D-(+)-pantothenate, 0.1 mg vitamin B12, 5.0 mg p-Aminobenzoic acid, thioctic acid per liter. While this solution was originally called for in both FCM and FAM protocols, we determined it had very little effect on the growth rate of *G. sulfurreducens* and thus left it out of our media (ATCC, 2002).

3.3.2 *E. coli* Growth Conditions

E. coli was used in pilot runs for aerotolerance and gene expression tests as well as to amplify plasmids. It was grown either on plates or in two separate liquid media: Luria-Bertani Broth (LB) and Terrific Broth (TB). Any liquid media were shaken during growth. All *E. coli* were grown at 37°C.

LB consisted of 1% tryptone, 0.5% yeast extract, and 0.05% NaCl in distilled water (Sambrook et al., 1989).

LB plates were made with LB in a 1% agar.

LB-spec plates were made with LB in a 1% agar. The antibiotic spectinomycin was added as the medium cooled. These plates were used for *E. coli* transformed with the plasmid pRG5.

LB-amp-Xgal plates were made with LB in a 1% agar. The antibiotic ampicillin was added as the medium cooled. These plates were used for *E. coli* transformed with the plasmid pCR2.1.

TB consisted of 1.2% tryptone, 2.4% yeast extract, 4 mL glycerol / L H_2O, 0.231% KH_2PO_4, 1.254% K_2HPO_4 in distilled H_2O (Sambrook et al., 1989). *E. coli* grew to much higher densities in this medium. For this reason, TB was used any time large amounts of *E. coli* were needed.

3.4 Anoxic Techniques

Due to its anaerobic nature, special procedures were required to keep conditions for *G. sulfurreducens* cells oxygen-free. It is important to realize that, although *G. sulfurreducens* is an anaerobe, it can survive in aerobic conditions for short periods of time. Conditions were kept anoxic for all work except the transformation of *G. sulfurreducens*, during which the cells were exposed to oxygen for less than one minute before being returned to anoxic conditions. Limited exposure to oxygen was detrimental to the *G. sulfurreducens* cultures in a small enough amount to allow successful transformation.

Cells were cultured in Bellco 20 mL glass anaerobic culture tubes. The tubes were covered with a gas impermeable rubber septum stopper and sealed with an aluminum ring. This system was designed for growth of methanogens intolerant of even minute amounts of oxygen. Provided that anaerobic technique was used to inoculate, this method proved sufficient for growth of *G. sulfurreducens.*

When inoculating cells from one rubber-topped tube to another, the syringes and needles used for the procedure had to be purged of oxygen before use. After assembly of

the needle and syringe, the syringe was filled and emptied of 100% carbon dioxide several times. Upon a third filling of the syringe, the carbon dioxide was not expelled into the air, but injected into the tube from which the growing cells were being transferred. This created a positive pressure, allowing for only outward flow of any gasses that might leak throughout the procedure. At least 1 mL of cells were withdrawn from the growing cells and injected into a fresh medium tube.

Twenty-three gauge needles were used for all transfers of *G. sulfurreducens*. This size needle was large enough to allow cells to pass from one environment to the next without shear stresses, but small enough to leave a negligible hole in the rubber top of tubes and bottles.

Cells grown on plates were kept in a Fluka Anaerobic jar and incubated at 30ºC. Any work on plates was done in a COY Vinyl Anaerobic Chamber. The anaerobic jar was sealed in the chamber and passed out through the airlock. Since the incubator used was a standard bench top incubator, the cells remained within the anaerobic jar even while in the incubator.

Frozen electrocompetent cells were stored in 0.6 mL plastic tubes. These tubes were stored in groups of three in each container in our normal 20 mL glass growth tubes. This allowed the stored cells to remain anoxic. When cells were removed to be transformed, only three tubes at a time were exposed to oxygen. This was a useful number of tubes, as we usually did three transformations at a time (a plasmid with a catalase gene, a plasmid with a superoxide dismutase gene, and a plasmid without an insert).

The *G. sulfurreducens* transformation procedure exposed the cells to oxygen, but only for a very short period of time. Electroporation could not have been performed within the anaerobic chamber because of logistical considerations. While exposure to oxygen was suboptimal, *G. sulfurreducens* has been shown to be quite tolerant of oxygen for even extended periods of time (Lin, Coppi, & Lovley, 2004). Bench top transformations undoubtedly reduced our transformation efficiency, but allowed for electroporation to be conducted.

3.5 Producing Electrocompetent Cells

In general, the production of electrocompetent cells involves re-suspending cells in a new salt-free solution at higher densities, portioning into 40μL aliquots, and freezing for future use. The procedure that we used to prepare *G. sulfurreducens* electrocompetent cells was very similar to this with only two major deviations. First, $MgCl_2$ was included in the electroporation buffer. Second, oxygen exposure had to be taken into consideration throughout the entire procedure (Kim et al., 2005).

An electroporation buffer was prepared consisting of 1 mM HEPES, 1 mM $MgCl_2$, and 175 mM sucrose. This buffer was bubbled with 70% CO_2 and 30% nitrogen for one hour before being sealed and autoclaved. It was then stored at 4°C for future use.

The $MgCl_2$ helped the cells survive the washing. It is believed that $MgCl_2$ stabilizes the cell membranes during the process.

The sucrose maintained a similar osmolarity to the FAM medium. *G. sulfurreducens* is very sensitive to changes in osmolarity.

Four hundred mL of mid-log-phase cultures (optical density at 600nm = 0.2 to 0.35; $9x10^7$ to $1.8x10^8$ cells/mL) was harvested by centrifugation at 4° C for 45 minutes

at 8,000 rcf (Coppi, Leang, Sandler, & Lovley, 2001). The supernatant was poured off and the cells were resuspended in 400 mL electroporation buffer. The cells were centrifuged and washed again before a final centrifugation. This final cell pellet was resuspended in about 1 mL electroporation buffer. Dimethyl sulfoxide (DMSO) was added to a final concentration of 10%. DMSO was necessary, as glycerol is not a strong enough cryoprotectant for the sensitivity of *G. sulfurreducens* to changes in osmolarity. Cells were then divided into 40 µL aliquots in 0.6 mL tubes, put three to a container in 20 mL glass vials, sealed shut, and frozen.

3.6 Microbial Fuel Cell

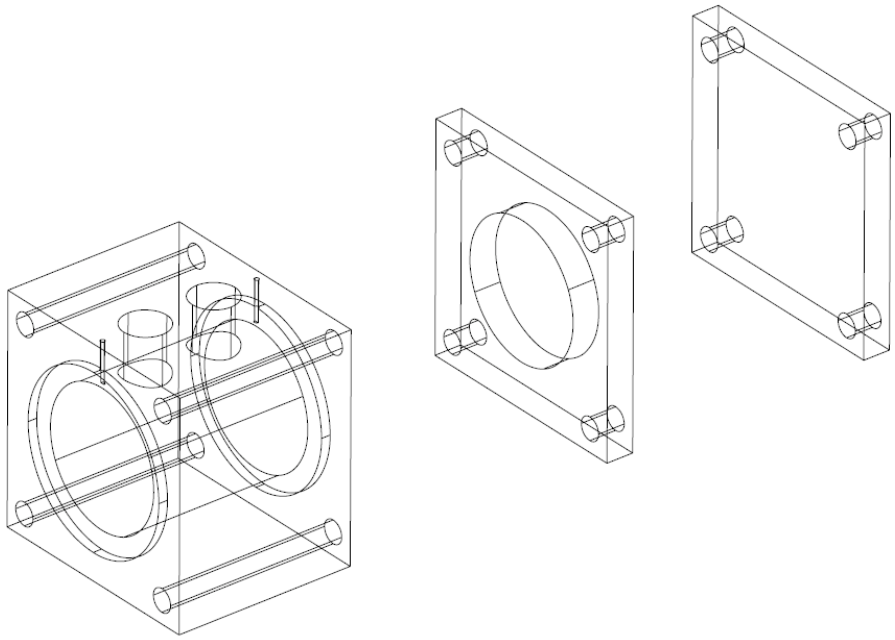
We built a single chamber microbial fuel cell based on designs published by microbial fuel cell researcher, Eric Zielke (Zielke, 2005). It consisted of a polycarbonate cylinder open at both ends. These ends were then covered in conductive material. The top of the fuel cell consisted of carbon paper coated on the liquid side with a platinum layer. The exterior portion of the carbon paper was left in open contact with the air; it was designed to act as the cathode. The platinum layer catalyzed the reduction of molecular oxygen (from outside the cell) to water with electrons produced at the anode:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O \text{ (Zhao et al., 2005)}$$

The other side of the fuel cell consisted of a carbon cloth anode exteriorly covered with polycarbonate to limit leakage of oxygen into the system. The entire cell was held together with four bolts. Bacteria were inoculated through a small rubber-covered port on

the side of the fuel cell after the cell was already anoxically sealed. A diagram of the fuel cell can be seen in *Figure 7*.

Figure 7: Polycarbonate microbial fuel cell design



This single chambered microbial fuel cell design consisted of a polycarbonate cylinder. One end was covered with carbon paper open to the air on one side and covered with platinum on the other. The opposite end was covered in carbon cloth layered with polycarbonate. Four bolts held together the entire fuel cell.

The fuel cell was operated with a special fuel cell medium. This medium was identical to the FCM or FAM mediums detailed above but it lacked both ferric citrate and fumaric acid as electron acceptors. This was intended to force the *G. sulfurreducens* to use the carbon cloth anode as an acceptor instead.

Before experimentation within the fuel cell was conducted the *G. sulfurreducens* were conditioned to an insoluble electron acceptor. This was done by inoculation into fuel cell media containing aluminum foil (Bond, & Lovley, 2003).

This fuel cell was used to compare electrical production before and after transformation. Specifically, the voltage across a 1 kΩ resistor was read 0.5, 1, 2, 5, 10, and 24 hours after inoculation using an Omega Engineering, Inc Digital Multimeter.

3.7 Cloning

The overall objective of this project required the construction of a plasmid containing catalase and superoxide dismutase genes from *G. sulfurreducens* downstream of promoters which would allow transcription to be easily turned on or off. This was intended to determine whether these genes were functional in *G. sulfurreducens* and if so, to determine how increased transcription affects aerotolerance and electrical production in the fuel cell described above. Thus, the first phase of the project was the creation of this plasmid, beginning with cloning.

Cloning consists of the insertion of a desired sequence of DNA into a gene expression vector, like a plasmid. The first step is to isolate the genes of interest. We accomplished this using polymerase chain reaction (PCR) with primers specific to our genes of interest. Next, these genes were inserted into a cloning vector.

PCR was used to acquire the genes for catalase and superoxide dismutase from *G. sulfurreducens*. The primers used for each are shown in *Figure 8*.

Figure 8: Specific primers for SOD and CAT

SOD forward: TCCAATCAAGACCTGTCAAGG
SOD reverse: CGGCACGCTCCCGATTGC
CAT forward: GCCGACGAAGTGAACCACTCC
CAT reverse: TTGATGCGCCGATCAACT

These primers were used with PCR to isolate the catalase and superoxide dismutase genes from G. sulfurreducens. The SOD forward and reverse primers targeted both ends of the DNA containing the SOD gene while the CAT forward and reverse primers did the same around the CAT gene. The genes of interest could then be amplified.

These primers were based on the complete sequence of *G. sulfurreducens* available through National Center for Biotechnology Information (NCBI) published by Lovley Labs (Methé et al., 2003). These are putative genes; they have not been proven to code for SOD or CAT but bear unmistakable genetic similarity to SOD and CAT in related species.

G. sulfurreducens genomic DNA had to be isolated and purified before PCR could amplify the CAT and SOD genes. To assure sufficient template DNA, 10 mL of *G. sulfurreducens* was grown to stationary phase. Cells were harvested from three cycles of repeated flash freezing followed by thawing. Cellular debris was cleared with 10 minutes of centrifugation at 12,800 rcf. The supernatant was pipetted into a new tube and the pelleted cells were discarded. PCR was conducted using this supernatant.

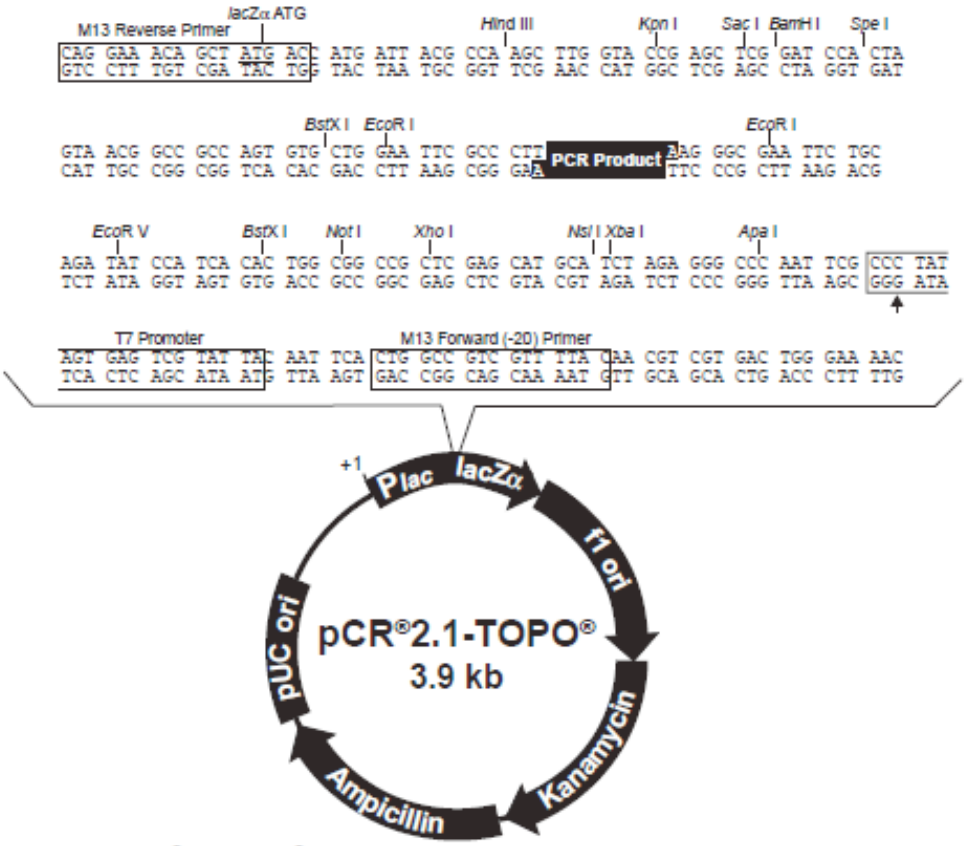
PCR was then used to amplify the genes of interest. Because annealing temperatures for these genes were unknown, we tried six different annealing temperatures: 52.0°C, 53.7°C, 56.3°C, 58.0°C, 60.5°C, and 62.0°C. The annealing temperature that showed the best results was 52.0°C, so all PCR reactions thereafter were run at that temperature.

PCR products were checked on an agarose gel and the correct size band (2183bp for CAT and 578bp for SOD) was cut out from the gel and isolated using a Qiagen QIAquick Gel Extraction Kit. To cut the band from the gel, the gel slice was dissolved in buffer and then spun at 12,800 rcf through a QIAquick column (Qiagen) to remove the agarose. The DNA was then washed with ethanol and spun again. Finally the DNA was eluted in 10 mM Tris·Cl in another spin cycle.

This DNA was inserted into a plasmid using a TA Cloning Kit from Invitrogen. This procedure took advantage of the quirks of the Taq polymerase in PCR. Taq leaves an adenine nucleotide overhanging each end of the genes it synthesizes. The kit-provided plasmid, pCR2.1 (shown in *Figure 9*) (Invitrogen), has overhanging thymine nucleotides. Since adenine and thymine are conjugate bases, they could be ligated together. The ligation reaction included 5.5 μL distilled H_2O, 1 μL 10x ligase buffer, 2 μL pCR2.1 vector, 1 μL PCR reaction, and 0.5 μL ligase. This reaction was left at 16°C overnight.

The products of this ligation were known as pCR2.1SOD and pCR2.1CAT for superoxide dismutase and catalase, respectively.

Figure 9: Plasmid pCR2.1



Comments for pCR®2.1-TOPO®
3931 nucleotides

LacZα fragment: bases 1-547
M13 reverse priming site: bases 205-221
Multiple cloning site: bases 234-357
T7 promoter/priming site: bases 364-383
M13 Forward (-20) priming site: bases 391-406
f1 origin: bases 548-985
Kanamycin resistance ORF: bases 1319-2113
Ampicillin resistance ORF: bases 2131-2991
pUC origin: bases 3136-3809

The ligation product was inserted into *E. coli* strain DH10ß using electroporation

The DNA products containing the CAT and SOD genes were inserted into plasmid pCR2.1. This plasmid was ideal because it had overhanging thymine nucleotides that could be ligated to the adenine nucleotides added by Taq polymerase during PCR.

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overnight in LB at 37°C. To extract plasmids, the transformants in broth were mini-prepped using a Qiagen Miniprep Kit (Qiagen).

For the plasmid extraction, transformed *E. coli* was grown overnight in 1.5 mL TB. The cells were then pelleted by centrifugation for one minute at 13,000 rpm and the supernatant was poured off. The cells were resuspended in a kit-provided alkaline buffer to cause cell lysis. The lysate was centrifuged for 10 minutes at 13,000 rpm and the supernatant was poured into a kit-provided affinity chromatography column. This column bound the DNA in the column and allowed all other solutes to pass through. Once the column was washed of unbound particles, everything but DNA, the plasmid DNA was eluted into a clean tube.

The plasmids were then digested with restriction endonucleases and electrophoresis was run on a 1% gel. The purpose of this was to determine the orientation of the genes. SOD was digested in two separate double digests. The first digest was with BamHI and NcoI. BamHI cuts once in pCR2.1 but not in SOD. NcoI cuts once in SOD but not in pCR2.1. Because of this, orientation of the gene was determinable from the size of the fragments on the gel. From this digest, we expected bands at 4500bp and 60bp (if the gene was in the orientation to insert properly into pRG5). Realistically, we expected the 60bp band to be too light to see due to a small amount of DNA. On the other hand, the incorrect orientation would have given us bands at 3932bp and 650bp. These two possibilities were easy to differentiate on a gel.

We also digested the SOD plasmid with BamHI and XhoI. This cut out the insert with different overhangs on either side. These incompatible overhangs meant when this gene inserted into pRG5 cut with the same enzymes, it could only ligate into the plasmid in the desired orientation.

The pCR2.1CAT plasmid was cut with BamHI and SphI to check for orientation. BamHI cut once in pCR2.1 but not at all in the CAT gene. SphI cut once in the CAT gene and once in pCR2.1. Our desired orientation gave us a band at 2103bp, a band at 1598bp, and a band at 2412bp. The incorrect orientation would have had a band at 585bp, 1598bp, and 3930bp. These two possibilities were also easy to differentiate on a gel.

As with SOD, to excise the CAT gene we cut with BamHI and XhoI. Neither enzyme cut in the CAT gene while BamHI and XhoI cut once each in the regions of pCR2.1 flanking the CAT gene.

3.8 Subcloning

3.8.1 Final Vector pRG5

The final vector for our genes was constructed from pCM66 *(Figure 10)*, pCD342 *(Figure 11)* and pSJS985 (Kim et al., 2005). The final pRG5 plasmid was provided by Lovley Labs at the University of Massachusetts, Amherst. The Ptaclac promoter, lac operon and multiple cloning site of pCD342 and antibiotic resistance of pSJS985 were inserted into pCM66. This resulting plasmid, designated pRG5 *(Figure 12)*, was unrelated to the ATCC official pRG5 plasmid.

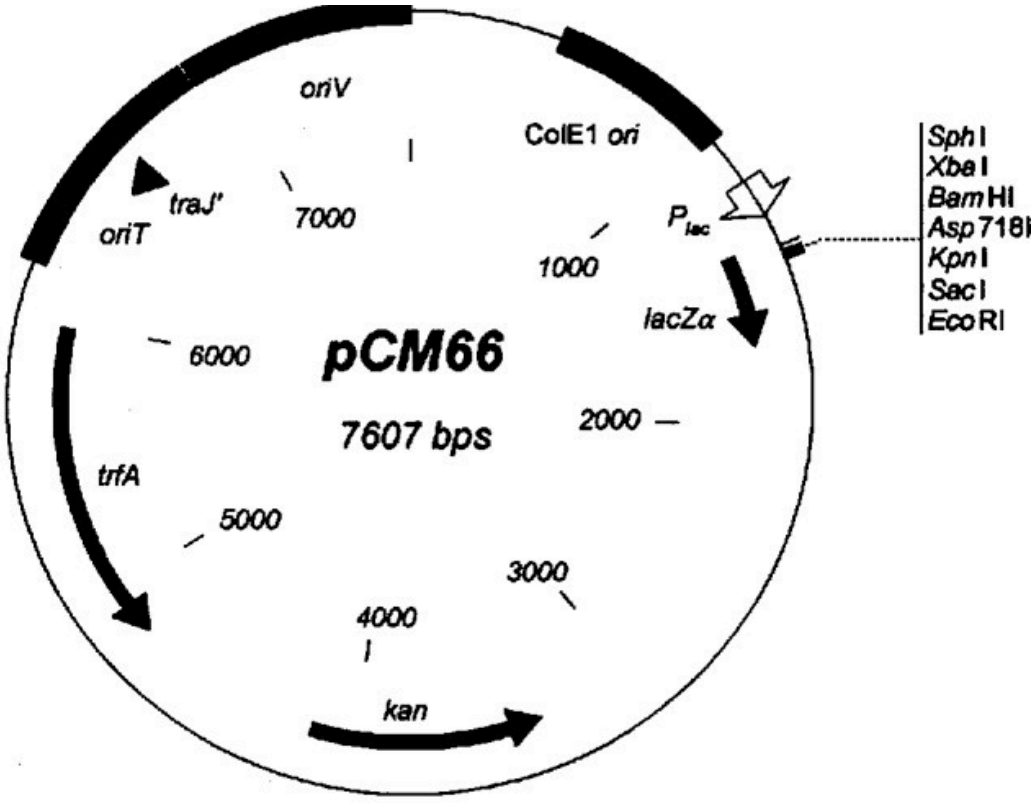
The portion of pCD342 inserted into pCM66 included Ptaclac, a lacZ-alpha operon, pCD342's multiple cloning site, and rrnB (Dehio, Knorre, Lanz, & Dehio, 1998),. The segment called "rrnB" is an operon which affects rRNA transcription (Bricker, 2000). Ptaclac is its promoter for the lac operon. It has exceptionally low expression in uninduced conditions (Dehio, Knorre, Lanz, & Dehio, 1998). The lacZ-alpha operon containing the genes of interest was induced by Isopropyl β-D-1-thiogalactopyranoside

(IPTG) (Simons, Houman, & Kleckner, 1987). IPTG (5 mM) was used in solution for activation.

The majority of the plasmid contained genes from pCM66. The origin of replication in pCM66 was ColE1, making pRG5 a high copy number type (Marx, & Lidstrom, 2001). The kanamycin resistance gene and multiple cloning site were excised from pCM66. pRG5 contained the trfA gene from pCM66. This gene code increases plasmid copy number and host range (Toukdarian, & Helsinki, 1998).

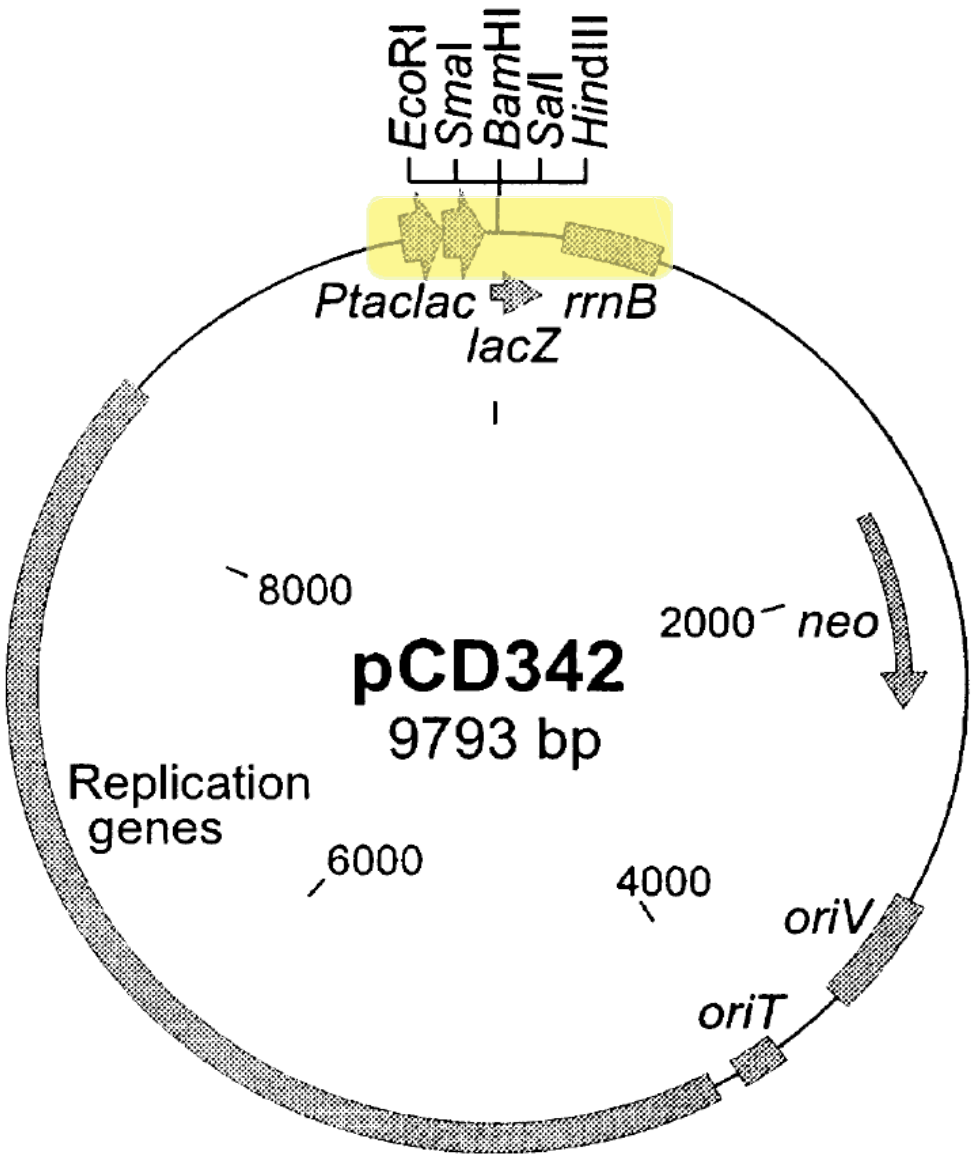
Antibiotic resistance to spectinomycin, coded by the gene aadA, was inserted into the plasmid. This was the gene excised from pSJS985 (Sandler, & Clark, 1994).

Figure 10: Plasmid pCM66



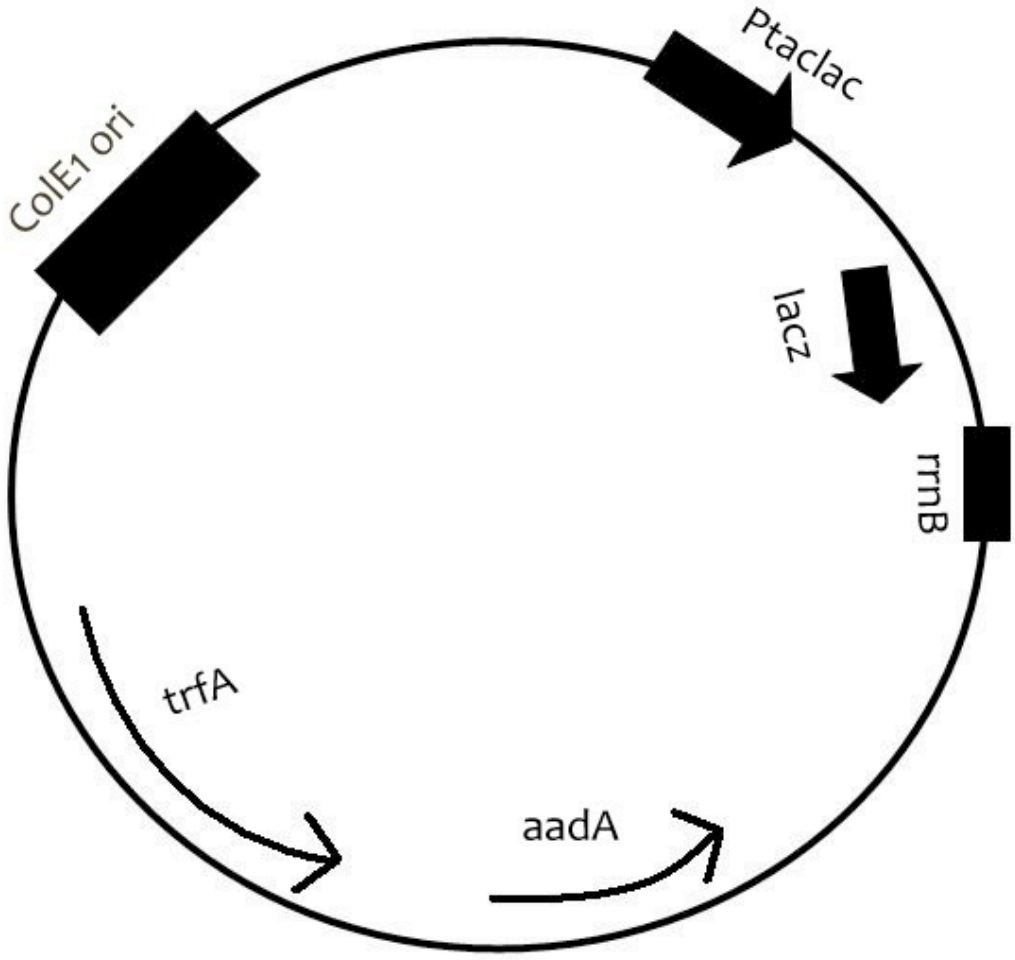
pCM66 consisted of the Ptaclac promoter, lac operon, multiple cloning site of pCD342 and antibiotic resistance of pSJS985.

Figure 11: Plasmid CD342



Portion of the pCD342 plasmid inserted into the final vector pCM666 highlighter above. It included a Ptaclac promoter, a lacZ-alpha operon, pCD342's multiple cloning site and operon rmB.

Figure 12: Final vector pRG5



Final plasmid pRG5 containing the components of plasmids pCM66, pCD342 and pSJS985. The majority of pRG5 contained genes from pCM66 including the trfA gene and origin of replication ColE1. The kanamycin resistance gene and multiple cloning site were excised from pCM66.

3.8.2 Subcloning Mechanism

Our goal in subcloning was to move the genes of interest from pCR2.1, the cloning vector, to pRG5 (*Figure 12*), a vector that is expressed in *G. sulfurreducens*. This was a six-step process. First, the gene-containing plasmids pCR2.1CAT and pCR2.1SOD were harvested using a QIAGEN Maxiprep Kit (Qiagen). Second, pRG5 and the genes of interest were digested with appropriate restriction enzymes. Third, the DNA fragments were run on gels, the gene bands cut out, and purified. Fourth, mixtures of the cut pRG5 and genes were ligated together. Fifth, these newly constructed plasmids were transformed into *E. coli* DH10β. Sixth, the plasmids were harvested from the transformed *E. coli* using a QIAGEN Miniprep Kit. These harvested plasmids were sent to Macrogen, Inc. for sequence verification.

In the first step, pCR2.1SOD and pCR2.1CAT plasmids were harvested using a QIAGEN Maxiprep Kit. This procedure required the *E. coli* cells containing the plasmids to be grown densely in liquid media. For this purpose, our *E. coli* cells were grown overnight in 4.5 mL TB. Cells were harvested by centrifugation for 15 minutes at 6000 rcf and 4°C. The pellet was resuspended in lysis buffers and vigorously inverted. Cellular debris was precipitated with kit-provided reagent followed by centrifugation at 20,000 rcf for 30 minutes at 4°C. The supernatant was transferred to a new tube and centrifuged again at 20,000 rcf for 15 minutes at 4°C. The supernatant was then put through a kit-provided affinity chromatography column, washed and the DNA eluted. After an ethanol wash, the plasmid DNA was stored at -20°C for future use.

The genes of interest must be cut out of this plasmid DNA and inserted into the final vector, pRG5. This was accomplished by adding 0.5 μL EcoRI and 0.5 μL BamHI

to 20 ng of each isolated plasmid. The same enzymes were added in the same concentration to 20 ng of a stock solution of pRG5 provided by Lovley Labs. These were incubated for 2 hours at 37ºC. After this time, the digestion was complete and the DNA was run on gel electrophoresis for purification. The appropriate bands were cut out and returned to solution in water.

CAT and SOD genes were then ligated into the cut pRG5 plasmids. The cut and purified DNA was combined into two separate tubes and ligated. One tube contained pRG5 and SOD, the other pRG5 and CAT. Five μL of ligase was added to each tube. The tubes were allowed to react at 16ºC overnight.

The now complete plasmids were transformed into plasmid-free *E. coli* DH10β. Frozen, electrocompetent DH10β cells were allowed to thaw on ice. Ten ng of plasmid DNA was added to 40 μL aliquots of DH10β cells. This solution was added to ice-cold electroporation cuvettes and immediately put into the electroporator. Voltage was set at 1.25 kv/cm and the resistance was set to 129 Ω. The cuvette was washed with 1 mL of 37ºC TB to wash out the cells. This solution was immediately withdrawn from the cuvette and added to 3 mL of 37ºC TB. This was incubated for 1 hour at 37ºC to allow the cells to recover. The cells were then spread on LB-amp-Xgal plates for colony selection.

The plasmids were harvested from the transformed *E. coli* using a QIAGEN Miniprep Kit as described above. The DNA was precipitated into an isopropanol solution.

The pRG5CAT and pRG5SOD plasmids were sent to Macrogen for sequencing. The sequence information from the selected colonies confirmed that, indeed, the

complete plasmid had been taken into the cells and one line was chosen with an exact match to published sequences for *G. sulfurreducens*' CAT and SOD.

3.9 Gene Expression Test

CAT and SOD gene expression levels were tested using reverse transcriptase polymerase chain-reaction (RT-PCR). This test uses reverse transcriptase, an enzyme that synthesizes DNA from an RNA template, to use conventional PCR techniques to semi-quantitatively display transcription levels of a gene.

RNA was extracted from transformed *E. coli* by using RNeasy plus Mini Kit (Qiagen), according to the manufacturer's instructions. DNA was synthesized by a reverse transcriptase reaction using the extracted RNA as a template.

PCR was run using the same protocol as in the original amplification of the CAT and SOD genes. The primers used were the same primers used in the original PCR (*Figure 8*).

This cDNA was run in a 1% agar gel. The presence of a band at the appropriate size (~700bp for SOD and ~2200bp for CAT) and its intensity told us whether and how strongly a gene was expressed.

3.10 Sequencing

DNA was miniprepped as described above and sent to Macrogen for all sequencing.

3.11 Transformation

3.11.1 *Geobacter sulfurreducens* Transformation

The plasmid pRG5 and pRG5 with inserts were the only vectors used in transformations performed on *G. sulfurreducens*. Only confirmed plasmids were transformed into *G. sulfurreducens* (not ligation products). This increased transformation efficiency.

During electroporation, a number of techniques were used to minimize exposure of *G. sulfurreducens* to atmospheric levels of oxygen. Because the anaerobic chamber was unavailable, the transformation had to occur in air. Several optimizations were necessary for successful transformation.

Since the electrocompetent *G. sulfurreducens* cells were stored at -80°C in separate, anoxic tubes, they could be warmed on ice while in anoxic conditions. The tubes holding the electrocompetent cells were only exposed to oxygen immediately before electroporation.

Preliminary set-up was instrumental in minimizing any exposure of *G. sulfurreducens* to oxygen. Syringes and needles were assembled and cleared of oxygen before electrocompetent cells were exposed to air. All growth tubes into which transformed cells would be inoculated were pre-warmed and the tops were sterilized (by dipping into ethanol and flaming) before any cells were exposed to air. Yeast extract (0.1% concentration) was added to these tubes. The electroporator was turned on and set to the proper settings (Voltage = 1.47 kv/cm; Resistance = 129 Ω). All growth tubes for the recovery time were labeled.

Once all preparations were made, 40 µL of cells were mixed with 20 ng DNA (another modification to prior protocol, this is four times the amount of DNA called for in

a publication from Kim et al., 2005) and injected into an ice-cold transformation cuvette. The cuvette was immediately electroporated and 1 mL of pre-warmed media was sprayed into the cuvette to wash out the cells. The cells and 1 mL of media were withdrawn into the same syringe and injected into a pre-labeled tube. This did introduce some amount of oxygen, but it was quickly removed by *G. sulfurreducens'* metabolic processes.

The cells were allowed to recover at 30°C for 4-6 hours before they were taken to the anaerobic chamber and spread onto FAM-spec plates. Colonies grew in about one week. All present colonies contained known plasmids by necessity; complete plasmids were inserted and untransformed cells could not grow in the presence of spectinomycin.

3.11.2 *Escherichia coli* Transformation and Selection of pCR2.1

Forty µL aliquots of electrocompetent *E. coli* DH10β were used in all *E. coli* transformations of pCR2.1. 1 µL of ~5 ng/µL plasmid DNA was added into this mixture.

After the transformation of *E. coli* with pCR2.1, transformants were selected by traditional microbiology practices described in more detail below. pCR2.1 contained genes for beta-lactamase and beta-galactosidase (Invitrogen).

The beta-lactamase broke down the antibiotic ampicillin to allow transformed DNA to grow on the ampicillin-containing LB-amp-Xgal plates. Since *E. coli* did not contain a plasmid, it could not grow on this antibiotic.

Beta-galactosidase breaks down Xgal which is a galactose linked to an indole, a compound that, when oxidized, becomes an indigo dye (Arakawa, Tsuji, & Maeda, 1998). *E. coli* with a plasmid would grow on the antibiotic, but this did not assure the presence of an insert in the plasmid. Plasmids that are intentionally inserted in the middle of the beta-galactosidase gene effectively inactivate it. When *E. coli* contains beta-

galactosidase, it breaks the galactose from the indole dye; this turns the colony blue. When the beta-galactosidase is broken (meaning a gene has probably been inserted) the colonies remain the white/yellow color indicative of *E. coli*.

Because of these conditions, non-blue, growing colonies were selected and sequenced. While there was no guarantee they contained an insert or an insert in the correct direction without a confirmed sequence, they were the most likely cells to have gene inserts.

Several desired colonies were swabbed from the LB-amp-XGal plates and inoculated into LB to grow up for assays and storage.

3.11.3 *Escherichia coli* Transformation and Selection of pRG5

E. coli transformations of pRG5 and pRG5 with inserts were carried out in the exact same manner as with pCR2.1. The difference comes in the selection.

Because the antibiotic resistance of pRG5 is only for spectinomycin, LB-spec plates were made. Transformations were spread on these plates. Colonies grew overnight.

Plates were not made with Xgal. This is because the plasmids were already confirmed to have the desired insert. All colonies which were able to grow on spectinomycin could be expected to have the desired plasmid.

3.12 Assays

3.12.1 Aerotolerance Tube Assay

The aerotolerance assay was a procedure used to test relative aerotolerance of bacteria. This procedure was modeled from a procedure published by Kikuchi and Suzuki in 1986. Bacteria were grown for two days and then optical densities were taken at 600

nm using a Thermo Scientific NanoDrop™ 1000 Spectrophotometer. The growth of the bacteria was in part dependent on the initial concentration provided. An appropriate concentration was established in baseline experiments. Inoculum size in our data-producing aerotolerance experiments was $2.97x10^6$ cells/mL.

FAM media was prepared with 1% agar and autoclaved. The medium was taken directly from the autoclave and cooled to 55°C on a silicon bead bath inside the anaerobic chamber. Fourteen mL of molten agar and 1 mL of bacteria were added to glass culture tubes. While the agar was still molten, the tubes were vortexed for approximately 10 seconds to evenly distribute bacteria through the media. The tubes were then allowed to cool inside the anaerobic chamber.

Before they were removed from the chamber half of the tubes were capped with rubber stoppers and half were left uncovered. Two tubes were set aside uninoculated as controls. These tubes contained only 15 mL of media (no bacteria). One of the controls was capped while the other was left uncovered.

These tubes were then removed from the glove box and placed in an incubator at 30° C. After 2 days the tubes were removed and pictures were taken. The distance between the top of the tube and the first signs of bacterial growth were measured. Normally there were distinct phases of growth and in some cases additional phase changes beyond the first were observed. In these cases the thickness of the individual layers of growth were also measured.

The final aerotolerance assay consisted of six data sets: SOD transformants with and without IPTG, CAT transformants with and without IPTG, and wild type *G. sulfurreducens* with and without IPTG.

3.12.2 Catalase Assay

Using a Catalase Assay Kit from Sigma-Aldrich Inc., CAT activity levels were measured in induced and uninduced wild type *E. coli*, wild type *G. sulfurreducens* and their transformants. This meant that peroxidase data was measured in twelve separate conditions: wild type *E. coli* and no IPTG inducer, *E. coli* with SOD insert and no IPTG inducer, *E. coli* with CAT insert and no IPTG inducer, wild type *E. coli* and 5 mM IPTG inducer, *E. coli* with SOD insert and 5 mM IPTG inducer, *E. coli* with CAT insert and 5 mM IPTG inducer, wild type *G. sulfurreducens* and no IPTG inducer, *G. sulfurreducens* with SOD insert and no IPTG inducer, *G. sulfurreducens* with CAT insert and no IPTG inducer, wild type *G. sulfurreducens* and 5 mM IPTG inducer, *G. sulfurreducens* with SOD insert and 5 mM IPTG inducer, and *G. sulfurreducens* with CAT insert and 5 mM IPTG inducer.

The Catalase Assay Kit protocol (Sigma-Aldrich) mixed a known concentration of hydrogen peroxide with cell lysate and measured the change in hydrogen peroxide concentration after five minutes. After the reaction, a Color Reagent containing 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminoantipyrine was added. 3,5-dichloro-2-hydroxybenzenesulfonic acid is a substituted phenol which couples oxidatively to 4-aminoantipyrine in the presence of hydrogen peroxide to give a red quinoneimine dye that absorbs at 520 nm. Hydrogen peroxide concentration was measured by reading the sample's absorbance at 520 nm on a spectrophotometer.

Before the Catalase Assay Kit could be used, cells had to be lysed in a manner that would not denature or deactivate the catalase. Repeated flash freezing and thawing either did not accomplish sufficient lysis or deactivated the endogenous peroxidase. Data

was taken from attempts with this protocol. While enzyme activity levels between strains (SOD+, CAT+ and wild type) proved to be accurate, overall activity was too low to produce reliable data.

Therefore, a new lysis procedure was developed in which cells were sonicated for 30 seconds on a 50% cycle at 20 kHz. Cell tubes were kept in an ice bath to help dissipate the heat created from sonication.

Before the assays could be done, stock materials from the kit had to be prepared. The included 10X Assay Buffer was diluted 10-fold. The prepared 1X Assay Buffer was 50 mM potassium phosphate buffer pH7.0. This was stored at room temperature.

The kit-provided solid peroxidase was dissolved in 1X Assay Buffer to make a 0.7% peroxidase solution.

A Color Reagent was prepared by mixing 60 mL 10X Assay Buffer with 140 mL water in a 250 mL beaker, then 10 mL of this dilute solution to the Chromogen Reagent vial. The solution of Chromogen Reagent was added back into the buffer-containing beaker, divided into small aliquots and frozen at -20°C. This reagent acted as a quantitative, colorimetric test for hydrogen peroxide. It is a 150 mM potassium phosphate buffer (3X Assay Buffer) containing 0.25 mM 4-aminoantipyrine and 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid.

It was necessary to calibrate the spectrophotometer readings to exact concentrations of hydrogen peroxide. The assay kit provided a protocol for this. Five 1000 µL solutions were prepared of H_2O_2 in 1X assay buffer. Tubes 1-5 contained 0 µmoles H_2O_2, 1.25 µmoles H_2O_2, 2.5 µmoles H_2O_2, 5.0 µmoles H_2O_2, and 7.5 µmoles H_2O_2 respectively. A 10 µL aliquot of each was transferred to tubes 6-10 respectively. 1

mL of Color Reagent was added to every tube 6-10. This was allowed to sit for 15 minutes while the Color Reagent reaction neared equilibrium. Spectrophotometer readings were taken at 520 nm. Concentration of H_2O_2 was plotted against absorbance and a curve was found that was used for experimental samples.

Once all setup was complete, the assay was ready to be performed. Because the reaction was to be performed at room temperature, all reagents were allowed to equilibrate to room temperature before proceeding. The reaction began when 50 µL of the prepared cell lysate and 25 µl of 1X Assay Buffer were mixed with 25 µl of a 200 mM H_2O_2 solution (called Colorimetric Assay Substrate Solution). Solutions were mixed by inversion and allowed to incubate for five minutes. At the end of five minutes, 900 µL Stop Solution (15 mM Sodium Azide) was added to each reaction tube and inverted to mix. Immediately afterward, 10 µL aliquots were removed from each reaction tube and were added to separate tubes each containing 1 mL Color Reagent. This solution was allowed to sit for 15 minutes at room temperature while the color developed. After 15 minutes, spectrophotometer readings were taken at 520 nm. Measured absorbances were compared to the standard curve generated earlier and hydrogen peroxide levels were found.

Enzyme activity was calculated in Microsoft Excel. First, hydrogen peroxide decrease was standardized based on the cell density going into the assay. The difference in micromoles of hydrogen peroxide was divided by the number of cells per mL in the assay. This number, while it did not directly measure either the total amount of catalase or the activity of an individual catalase unit, gave a holistic measure of catalase activity per cell.

3.12.3 Peroxidase Assay

As a redundancy for the Catalase Assay, we used a general peroxidase assay. Using a PeroxiDetect Kit from Sigma-Aldrich Inc., peroxidase activity levels were measured in induced and uninduced wild type *E. coli*, wild type *G. sulfurreducens* and their transformants. The same twelve conditions were tested in this assay as in the catalase assay.

The workings of the Peroxidetect Kit took advantage of peroxide's ability to convert Fe^{2+} to Fe^{3+} ions under acidic conditions. The Fe^{3+} ions then form a colored adduct with xylenol orange, which absorbs at 560 nm. The reaction can be described as:

$$Fe^{2+} + R\text{-}OOH \rightarrow Fe^{3+} + RO^{\bullet} + OH^{-}$$

$$Fe^{3+} + XO \rightarrow Fe^{3+}\text{-}XO \text{ (Colored adduct)}$$

Lysis procedure was exactly the same as our CAT assay. Originally we attempted repeated flash freezing and thawing, but significant data was produced using sonication. We sonicated for 30 seconds on a 50% cycle at 20 kHz, then centrifuged for 10 minutes at 13,200 rpm. The pellet was discarded and the supernatant was transferred to a clean tube.

After lysis, cells were centrifuged for 10 minutes at 12,800 rcf. The supernatant was pipetted to a clean tube and the pellet was discarded. All peroxidase assays were carried out on this supernatant.

Preparations for the reaction included preparing a 100 μM H_2O_2 solution, the Working Color Reagent, and a standard curve.

The 100 μM H_2O_2 solution was prepared from a kit-provided 30% (~9.8 M) H_2O_2. The kit protocol specified a 9.8 fold dilution to 1 M followed by two serial 10-fold dilutions to 10 mM. We then tested the level of H_2O_2 in solution by a spectrophotometer reading at 240 nm (the peak absorbance wavelength of H_2O_2). The kit specifies that the absorbance of H_2O_2 at 240 nm = 0.436 OD for a 10 mm pathlength. Using this known concentration, the solution was further diluted to 1 mM followed by a 10-fold dilution to 100 μM.

The Working Color Reagent was prepared by mixing 100 volumes of Aqueous Peroxide Color Reagent with one volume Ferrous Ammonium Sulfate Reagent.

The standard curve for was made by setting up a range of solutions from 0.0 to 8.0 nmoles per reaction volume. These were made by pipetting 0 μL, 10 μL, 20 μL, 40 μL, 60 μL, and 80 μL of 100 μM H_2O_2 into individual microcentrifuge tubes. The final volume in each tube was brought up to 100 μL with deionized water. 1 mL Working Color Reagent was added to each tube and allowed to incubate at room temperature for 30 minutes for color formation to be complete. The absorbance of each standard was read at 560 nm using water as a reference. A curve was plotted for use during experiments.

The procedure for performing the assay was similar to the standard curve reactions. 100 μL of each sample was added to its own tube. 1 mL Working Color Reagent was added to each tube and allowed to incubate at room temperature for 30 minutes for color formation to be complete. The absorbance of each sample was read at 560 nm using water as a reference. These readings were compared to the standard curve and normalized for cell density.

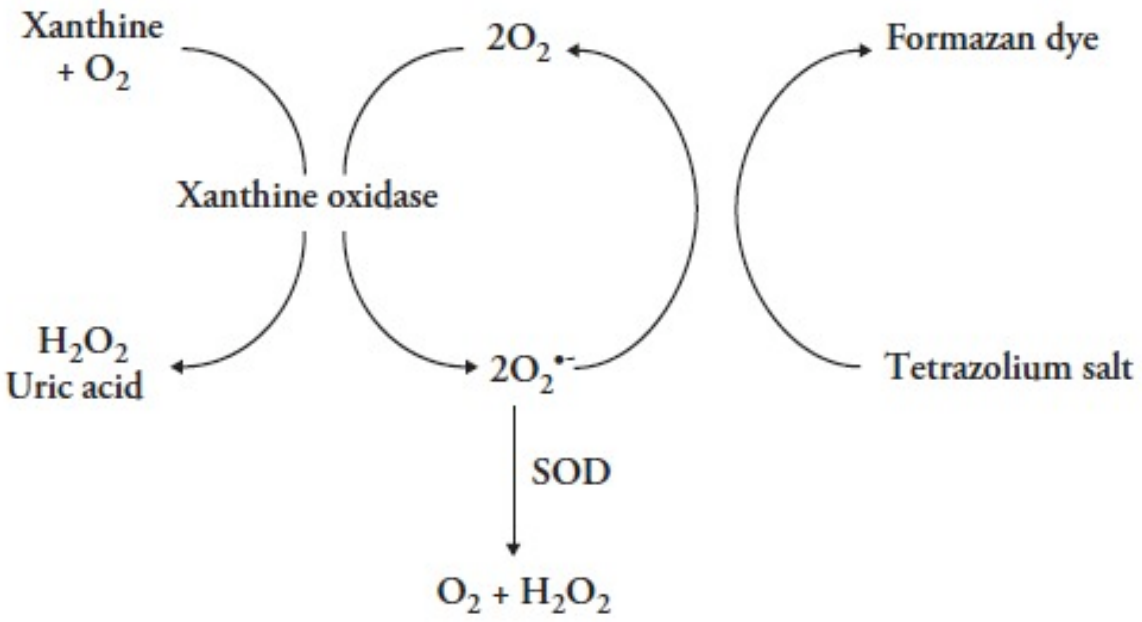
3.12.4 Superoxide Dismutase Assay

Using a Superoxide Dismutase Assay from Cayman Chemical Company (Cayman Chemical Company, 2008) we measured SOD activity in induced and uninduced wild type *E. coli*, wild type *G. sulfurreducens* and their transformants. The same twelve conditions were tested in this assay as in the catalase and peroxidase assays.

This assay uses tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (see *Figure 13*). When the superoxide breaks down the oxygen radicals produced by xanthine oxidase and hypoxanthine, it also converts the tetrazolium salt into formazan dye which absorbs at 450 nm.

Cells were prepared by sonication in the same manner as in the Catalase Assay Kit and the PeroxiDetect Kit.

Figure 13: Superoxide Dismutase Assay Net Reaction



The SOD assay measured activity in induced and uninduced E. coli, G. sulfurreducens and their transformants. The assay used tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. As the mechanism shows, the superoxide broke down the oxygen radicals produced by xanthine oxidase and hypoxanthine. It then converted the tetrazolium salt into formazan dye.

This kit, like the Catalase Assay Kit and the PeroxiDetect Kit, required prior setup. To be prepared were the Assay Buffer, Sample Buffer, Radical Detector, SOD Standard, and Xanthine Oxidase.

The final Assay Buffer consisted of 50 mM Tris-HCl, pH 8.0, containing 0.1 mM diethylenetriaminepentaacetic acid (DTPA) and 0.1 mM hypoxanthine. This is diluted from a 10X Assay Buffer provided in the kit. When stored at 4°C it is stable for up to two months.

The final Sample Buffer was 50 mM Tris-HCl, pH 8.0. This was diluted from a 10X Sample Buffer.

The Radical Detector was a solution of tetrazolium salt. Prior to use, 50 µL of the stock solution was pipetted into 19.95 ml of Assay Buffer and covered in tin foil. After dilution, this solution was stable for two hours.

The SOD Standard contains a solution of bovine erythrocyte SOD (Cu/Zn). This enzyme must be thawed slowly on ice. 20 µL of SOD Standard should be pipetted into 1.98 mL Sample Buffer.

The xanthine oxidase had to be thawed and diluted. Prior to use, 50 µL of the stock solution was pipetted into 19.95 ml of Assay Buffer and kept as cold as possible (though unfrozen). After dilution, this solution was stable for one hour.

Prior to use, 50 µL of the stock solution was pipetted into 19.95 ml of Assay Buffer and covered in tin foil. After dilution, this solution was stable for two hours.

To set up the standard curve, seven tubes were set up with diluted SOD Standard, Sample Buffer, and diluted Radical Detector. These tubes were filled with 0 µL, 20 µL, 40 µL, 80 µL, 120 µL, 160 µL, and 200 µL respectively. They were filled to 1000 µL total volume using Sample Buffer. These were run at the same time as the reactions of cell samples.

Experimental samples were run by adding 200 µL diluted Radical Detector and 10 µL cell sample to each tube.

All reactions (standard curve and samples) were initiated by adding 20 µL xanthine oxidase and shaken to mix. Reactions were incubated for 20 minutes and absorbance at 450 nm was read on a spectrophotometer.

Chapter 4: Results

4.1 Cloning

The first phase of the project consisted of construction of two plasmid vectors. This took place through a process of cloning followed by subcloning.

The first step of our cloning process was PCR. The DNA was extracted from wild-type *G. sulfurreducens* as well as from a cytochrome c knockout strain using DNA extraction procedures described as above. Each of these strains was grown with or without the addition of ATCC Vitamin Solution in the growth medium. This DNA was then run on a gel to ensure that DNA was correctly extracted (*Figure 14*).

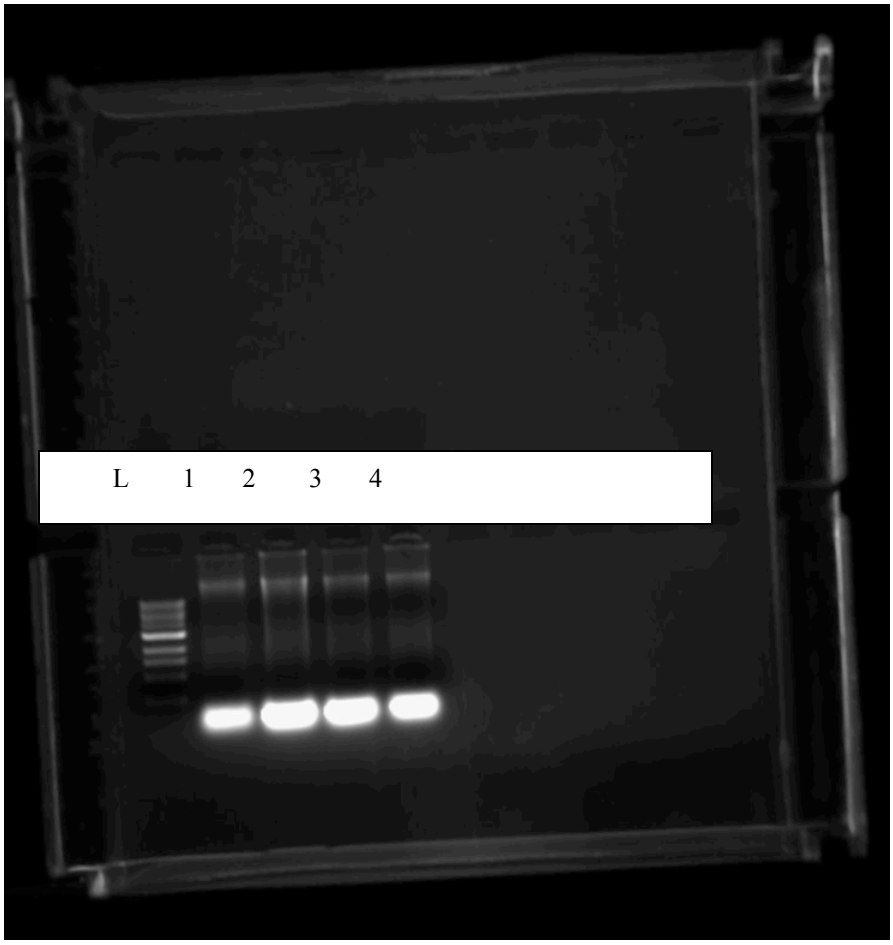
The first lane was a 1kb ladder; the second and third were wild type; and the fourth and fifth were cytochrome c knockout strains. The second and fifth lane contained strains grown with vitamins while the other two were grown without. All four samples had very similar patterns and quantities of DNA. They each showed a small quantity of very large slow moving DNA. This was likely a nicked circle of the bacterial chromosome. The much brighter faster moving band was likely super-coiled chromosomal DNA.

Despite previous recommendations that ATCC Vitamin Solution be included in growth media this data suggested relatively equal growth with or without its presence. More importantly it showed that the extraction procedure worked and did so with very little damage to the DNA. Only a small proportion of the DNA was even present as a nicked circle in the gel with far more present as super-coiled chromosomal DNA. This suggests that the DNA produced by this lysis would be well suited for PCR amplification of the CAT and SOD genes.

DNA from this lysis procedure was then used for PCR amplification as described above. Of particular concern at this step in the procedure was the annealing temperature for both CAT and SOD genes. Because this temperature was unknown, six different annealing temperatures were used for each amplification. These temperatures were 52.0°C, 53.7°C, 56.3°C, 58.0°C, 60.5°C, and 62.0°C.

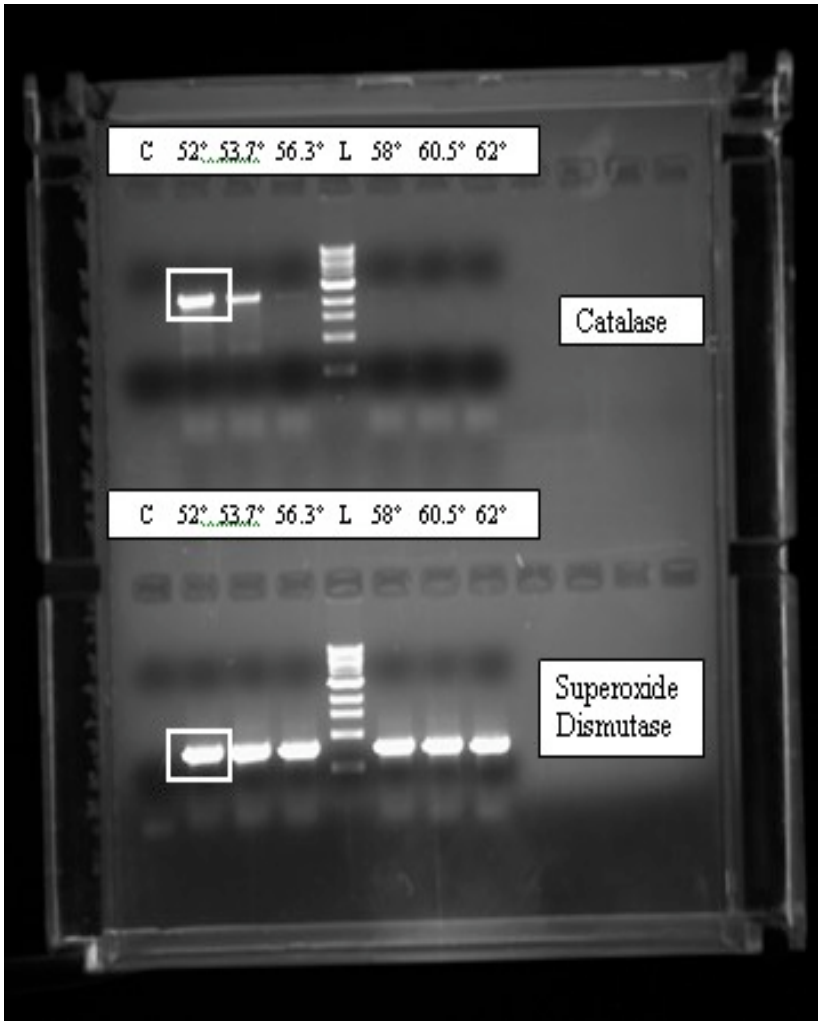
After the completion of PCR all of these samples were run on an agarose gel to identify PCR products. The results of this gel are shown in *Figure 15*. The first lane of each side of the gel was a control lacking genomic DNA. Lanes 2-4 and 6-8 were the temperatures listed above in corresponding order. The upper half of the gel was CAT and the lower half was SOD. The fifth lane contained a 1 kb ladder.

Figure 14: Genomic DNA



The first lane (lane L) contains a 1kb ladder. Lane 1 contains genomic DNA from wild type G. sulfurreducens grown with ATCC Vitamin Solution (VS) supplement. Lane 2 contains genomic DNA from wild type G. sulfurreducens grown without VS. Lane 3 contains genomic DNA from a Δcytochrome c G. sulfurreducens strain grown without VS. Lane 4 contains genomic DNA from Δcytochrome c G. sulfurreducens grown with VS. All four lanes contain significant amounts of DNA.

Figure 15: PCR products



The lanes marked C above were PCR reactions containing no genomic DNA. The rest of the lanes are labeled with the annealing temperature used in the PCR reaction. 52° C was clearly the best annealing temperature for Catalase while Superoxide Dismutase worked at any of the annealing temperatures tried. The bands chosen are boxed in above.

As can be seen in the gel the amplification was successful for CAT only with an annealing temperature between 52° and 56.3° C. Beyond this temperature there was no PCR product detectable through gel electrophoresis. However PCR was clearly most effective with an annealing temperature of 52°C. For this reason this was the gene product chosen for use for the rest of the cloning procedure.

The SOD genes were amplified very effectively regardless of the annealing temperature chosen. There was no noticeable difference in quantity in any of the annealing temperatures. Simply for consistency the same temperature was chosen as for the CAT gene and used for cloning. The bands chosen are boxed in white on *Figure 15*.

The need for a very specific annealing temperature for the CAT was likely the result of the 2.2kb CAT gene being significantly longer than the 578bp SOD gene. Because the SOD gene annealed much more quickly it did not require the same specificity of temperature in order for PCR to proceed correctly.

Following the PCR amplification the fragments chosen above were then ligated into the pCR2.1 plasmid using TA cloning. The plasmids were then transformed into *E. coli* and grown on LB-amp-Xgal to select for transformants. A number of white colonies were selected as transformants containing an insert for both SOD and CAT. These cells were grown overnight and miniprepped to remove the plasmid DNA.

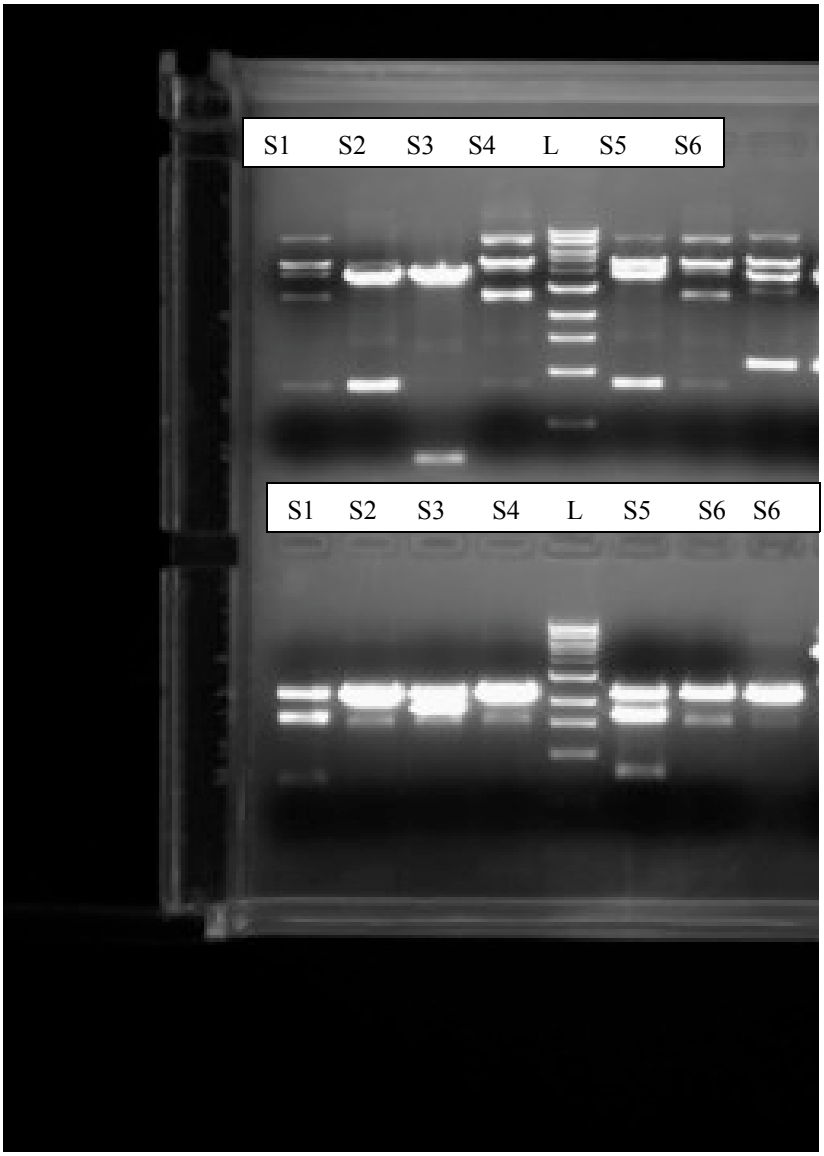
A double digest was then set up in order to check the orientation of the inserts. Because the restriction sites were not centrally located in the inserts these cuts produced different sized fragments depending on which orientation the insert had ligated into the plasmid. The SOD digest used BamHI and NcoI to cut. The products were then separated using agarose gel electrophoresis. If in the correct orientation then a 4.5kb fragment and a

60bp fragment would be seen. If in the wrong orientation but a complete insert then a 4kb fragment and a 650bp fragment would be produced. Any other fragment pattern indicated an incorrect insert. This digest is shown as the bottom of *Figure 16*.

In addition the SOD genes were both digested with BamHI and XhoI in order to remove the genes from the pCR2.1 plasmid so that they could be sent to Macrogen for sequencing. Also this DNA could potentially then be put into the pRG5 plasmid. These were run on the top of the gel in *Figure 16*. S2 through S6 were believed to have the correct size insert.

This was confirmed by the DNA sequencing. SOD 1 and SOD 5 were the correct insert but in reverse orientation. The BLAST searches and DNA sequencing data from these samples can be seen in the appendix. SOD 2, 4, and 6 had the correct insert in the correct orientation. Additionally SOD 2 had 100% identity with the published sequence. The BLAST search from this sequence is shown in *Figure 17*.

Figure 16: Orientation Check of pCR2.1 SOD



Top: *BamHI and XhoI double digest of putative SOD clones numbered S1-S6. In addition lane L contained a 1kb ladder. Bands of approximately the correct size (about 600 bp) can be seen in clones 1,2,4,5, and 6. They were most clear in clones 2 and 5.*

Bottom*: BamHI and NcOI double digest of putative SOD clones. As above of putative SOD clones were numbered S1-S6 and lane L contained a 1kb ladder. All 6 clones showed relatively uninformative banding patterns and so the digest shown on the top of the gel was used to have the clones sequenced.*

Figure 17: Comparison of SOD clones with published sequence

```
BLASTN 2.2.19+
Reference: Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000), "A greedy
algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.

RID: UPW8RS9E113
Query=  SOD2clone
Length=1239
                                                                      Score     E
Sequences producing significant alignments:                          (Bits)  Value

lcl|55621  LiteratureSOD                                              1070    0.0

ALIGNMENTS
>lcl|55621 LiteratureSOD
Length=579

 Score = 1070 bits (579),  Expect = 0.0
 Identities = 579/579 (100%), Gaps = 0/579 (0%)
 Strand=Plus/Minus

Query  213  TTATTTCAACCGTGCCTCGGCGGCCTTCCAGTCGATATTCTTGAAGAAGGCCTCGATGTA  272
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  579  TTATTTCAACCGTGCCTCGGCGGCCTTCCAGTCGATATTCTTGAAGAAGGCCTCGATGTA  520

Query  273  GTCGGGACGCTTGAGACCGTAATCGAGCATAAAGGCATGCTCGAAGACATCCATGATCAG  332
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  519  GTCGGGACGCTTGAGACCGTAATCGAGCATAAAGGCATGCTCGAAGACATCCATGATCAG  460

Query  333  GATGGGAGTGCAGCCGGCCGGATGAGCAACGTCGTGCTCGTTGACCCAGAAGTTAATGAG  392
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  459  GATGGGAGTGCAGCCGGCCGGATGAGCAACGTCGTGCTCGTTGACCCAGAAGTTAATGAG  400

Query  393  CTTGCCGGTGGCCGAATCCTGGTAGAGCACTGCCCAGCCGATACCCCGCATGGCGCCGGT  452
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  399  CTTGCCGGTGGCCGAATCCTGGTAGAGCACTGCCCAGCCGATACCCCGCATGGCGCCGGT  340

Query  453  GGCCCGGAAATCCTTCTCCCACGCCTCATAGCTGCCGAAGTCGGCAACAATTTTCGCGGC  512
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  339  GGCCCGGAAATCCTTCTCCCACGCCTCATAGCTGCCGAAGTCGGCAACAATTTTCGCGGC  280

Query  513  CAGCTTGCCGGCCTGGTCGATGGCACCGTTGCCGCCGAGATTCTCGAAATAGAACTCGTG  572
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  279  CAGCTTGCCGGCCTGGTCGATGGCACCGTTGCCGCCGAGATTCTCGAAATAGAACTCGTG  220

Query  573  GAGCCGCATGCCGTTGAACTCCCATCCCAGGCGCCGCTTCAGCTCCGCATACTCGGGAGT  632
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  219  GAGCCGCATGCCGTTGAACTCCCATCCCAGGCGCCGCTTCAGCTCCGCATACTCGGGAGT  160

Query  633  GGCAGTGGCACCGTCGGCGAGCATTTTGGCGAGGATGTCGAGCACCTTGTTCGTATTGGT  692
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  159  GGCAGTGGCACCGTCGGCGAGCATTTTGGCGAGGATGTCGAGCACCTTGTTCGTATTGGT  100

Query  693  CACATAACCCTGATAAAGGGTGAAATGGTTCTTCAGGAGAGCCTCGCTGAATCCCGCCAT  752
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  99   CACATAACCCTGATAAAGGGTGAAATGGTTCTTCAGGAGAGCCTCGCTGAATCCCGCCAT  40

Query  753  CCCGATCAGTTTCGAATAGTCTTTTGCTTCATAGGCCAT  791
            |||||||||||||||||||||||||||||||||||||||
Sbjct  39   CCCGATCAGTTTCGAATAGTCTTTTGCTTCATAGGCCAT  1
```

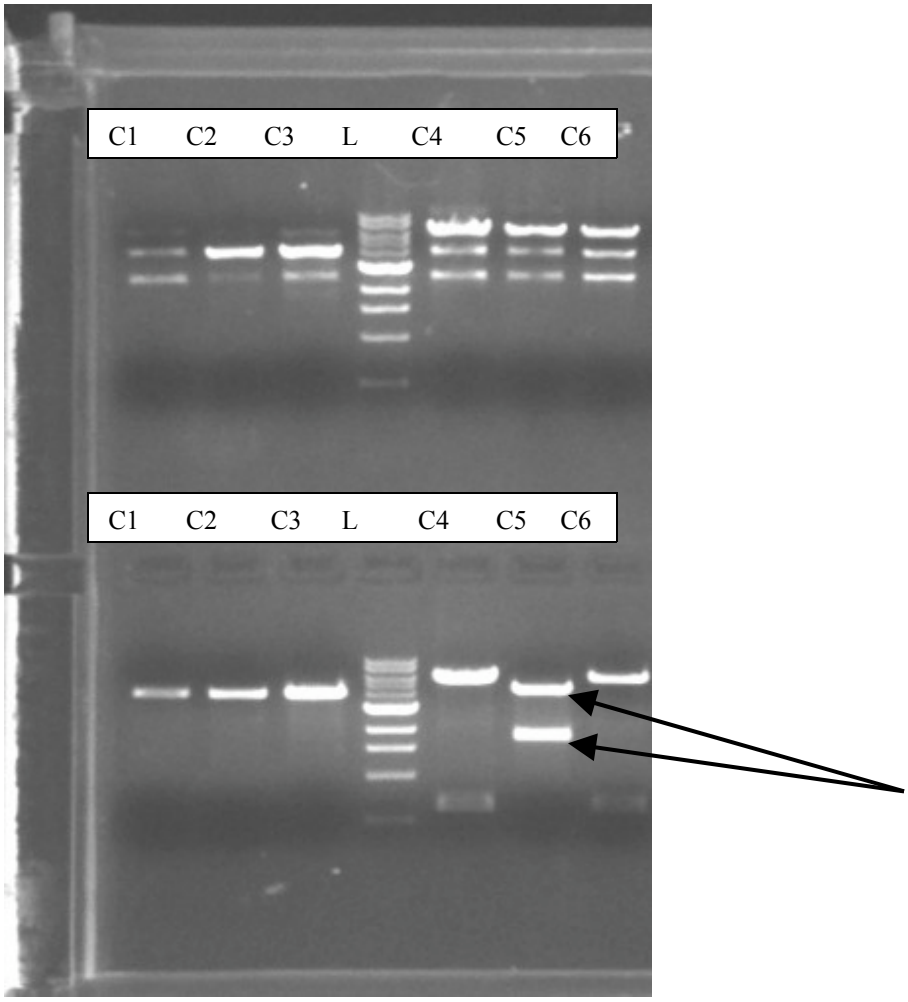
This is a BLAST search comparing the sequenced SOD clone 2 to the published sequence for Superoxide dismutase.

SOD2 was then transformed into *E. coli* and maxiprepped in order to maintain a stock of pCR2.1SOD.

Six putative catalase insert colonies were checked for orientation using a NotI digest. The correct orientation would have a 4.1kb and a 2kb fragment. The incorrect orientation would have a 5.5kb fragment and a 600 bp fragment. They were then run on an agarose gel. This can be seen in the bottom of *Figure 18*. CAT 5 was clearly cut in the correct orientation while the other five putative CAT inserts were not.

All the putative catalase insert colonies were also double digested with BamHI and XhoI to remove the insert for subcloning. These were also run on the top half of the gel shown in *Figure 18*. CAT5 was transformed into *E. coli* and maxiprepped in order to maintain a stock of pCR2.1CAT.

Figure 18: Orientation check of CAT clones



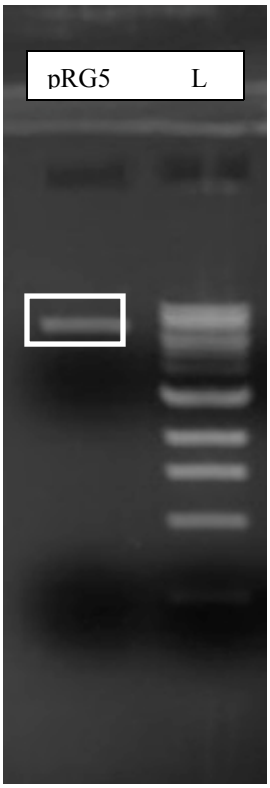
Top: *Putative CAT clones were digested with BamHI and XhoI. Six CAT clones were labeled with C1-6 and a 1 kb ladder was loaded in the lane labeled L. The CAT5 clone was cut out and purified.*
Bottom: *Putative CAT clones were digested with NotI. Lanes were labeled as above. CAT 5 showed clear bands at both 4.1 and 2 kb. The rest of the CAT clones lacked this pattern suggesting they were not correctly oriented clones.*

4.2 Sub-Cloning

The pRG5 vector was acquired from Derek Lovley. This plasmid was transformed into *E. coli* and maxiprepped to guarantee a lasting stock.

pRG5 was double digested with BamH1 and SalI in order to cut the plasmid open and create sticky ends for the CAT and SOD genes to be ligated in. The digested plasmid was run on a gel which is shown as *Figure 19*.

Figure 19: Digest of pRG5

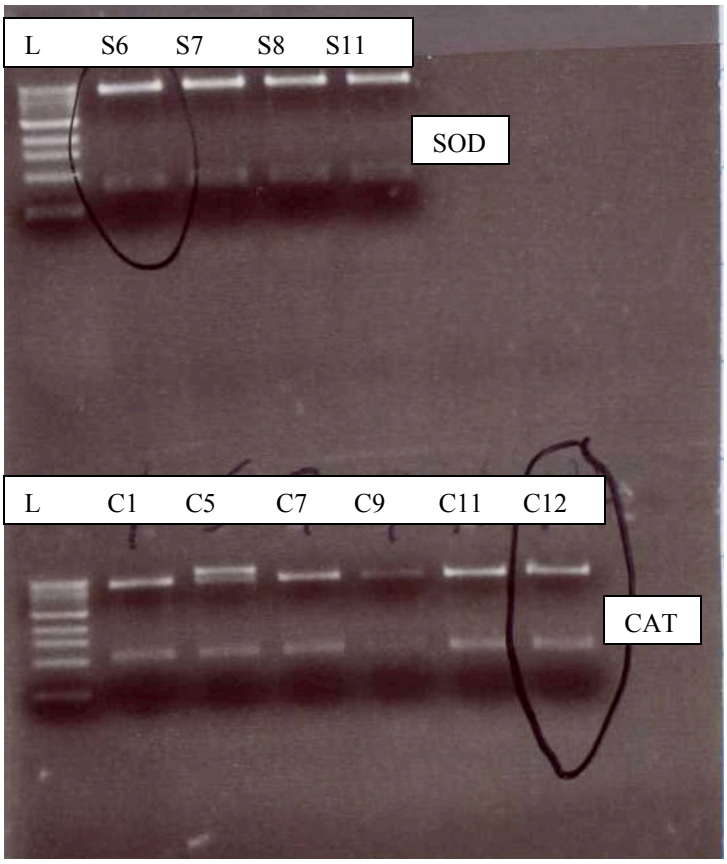


pRG5 was digested with BamH1 and SalI. The band shown is the opened vector, the small missing part of the multiple cloning site cut out does not show up on the gel. The ladder at right is a 1kb ladder.

The pRG5 was then cut out of the gel, purified and ligated separately with both CAT and SOD genes that had been cut with BamHI and XhoI. They were then transformed into *E. coli.* White colonies were selected as containing plasmids with inserts. There were six CAT colonies selected, 1, 5, 7, 9, 11, and 12 and four SOD

colonies selected. These colonies were miniprepped and digested using EcoRI. They were then run on an agarose gel which is included as *Figure 20*. All four of the SOD colonies appeared to contain the correct insert and so did 1, 7, 11, and 12 of the CAT. SOD 6 and CAT 12 were chosen and maxiprepped for later use in the transformation.

Figure 20: Insert check of pRG5



Putative pRG5 SOD and CAT transformants were digested with BamHI and XhoI. All of the transformants contained complete pRG5 plasmids with the correct insert.

4.3 Aerotolerance

Both an initial baseline measurement of the aerotolerance and a comparison of the wild type and transformed bacteria were conducted. As described above this test consisted of inoculating bacteria into sterile anoxic culture tubes and then allowing oxygen to diffuse into the system as the bacteria grew.

The baseline test was basically designed as a proof of principle. It confirmed that *G. sulfurreducens* growth was sensitive to the presence of oxygen (*Figure 21*).

This first baseline identified the importance of inoculum size. Larger inoculums, in terms of number of cells, appeared to be more aerotolerant. This is, however, caused by the cells growing to a high density more quickly and thus before inhibitory concentrations of oxygen have been established through diffusion.

In addition this problem was exacerbated by the ability of *G. sulfurreducens* to use oxygen as a terminal electron acceptor. It is possible that the phase lines in the tubes were the location where the concentration of bacteria was high enough to reduce oxygen as quickly as it diffused in, establishing a low enough level of oxygen to allow continued growth.

The aerotolerance assay was then conducted with the transformed bacteria. Wild-type, superoxide dismutase transformants, and catalase transformants were all pre-grown with or without the inducer IPTG. The concentration of bacteria was adjusted to the same level for each of the strains used. Transformants were grown with spectinomycin while wild-type were grown without.

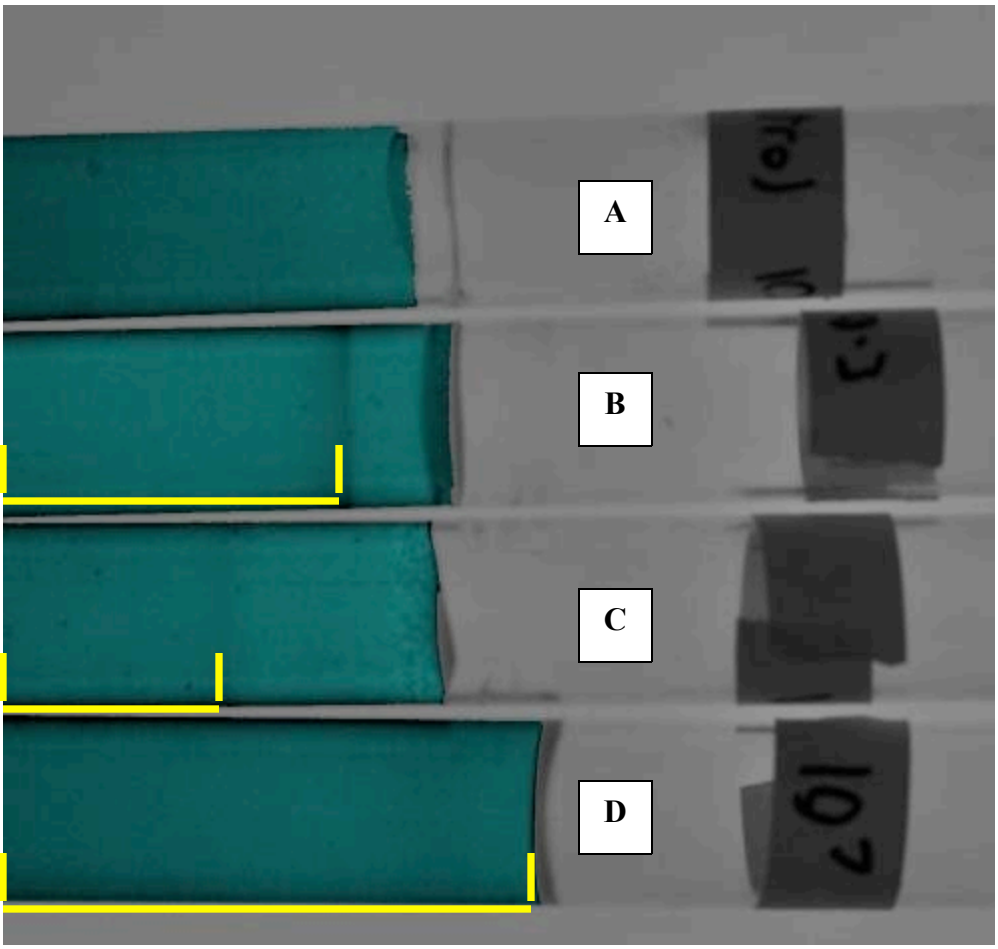
This assay was conducted in triplicate for both SOD and the wild type bacteria and in the figures all three tubes are pictured. CAT was only conducted in duplicate because of a lack of bacteria but both tubes are pictured for it as well. In addition a

positive control was conducted for each in which they were grown inside a closed tube with no oxygen. In each case the bacteria grew throughout the entire tube except for the induced wild-type. Negative controls were also conducted in which tubes were set up completely free of bacteria. One tube was capped and the other left open. Neither of these tubes showed any kind of bacterial growth, confirming that bacteria were not accidentally introduced from the environment.

One of the most obvious results was the small difference in aerotolerance between the uninduced and induced bacterial strains. In fact, the induced wild-type bacteria failed to grow at all regardless of the presence of oxygen. This can be seen in *Figure 22*. It seemed unlikely that this was the result of the IPTG and was instead probably the result of a problem with the original bacterial stock. In the transformed bacteria the IPTG had no effect. In both transformed strains the enzyme is behind the lac operon. This operon has a very low level of leakage when not induced and so the aerotolerance would have been expected to be very similar to that of the wild type bacteria. However it appears that neither the pRG5 nor the *G. sulfurreducens* contain the lacI gene. Thus induction had no effect.

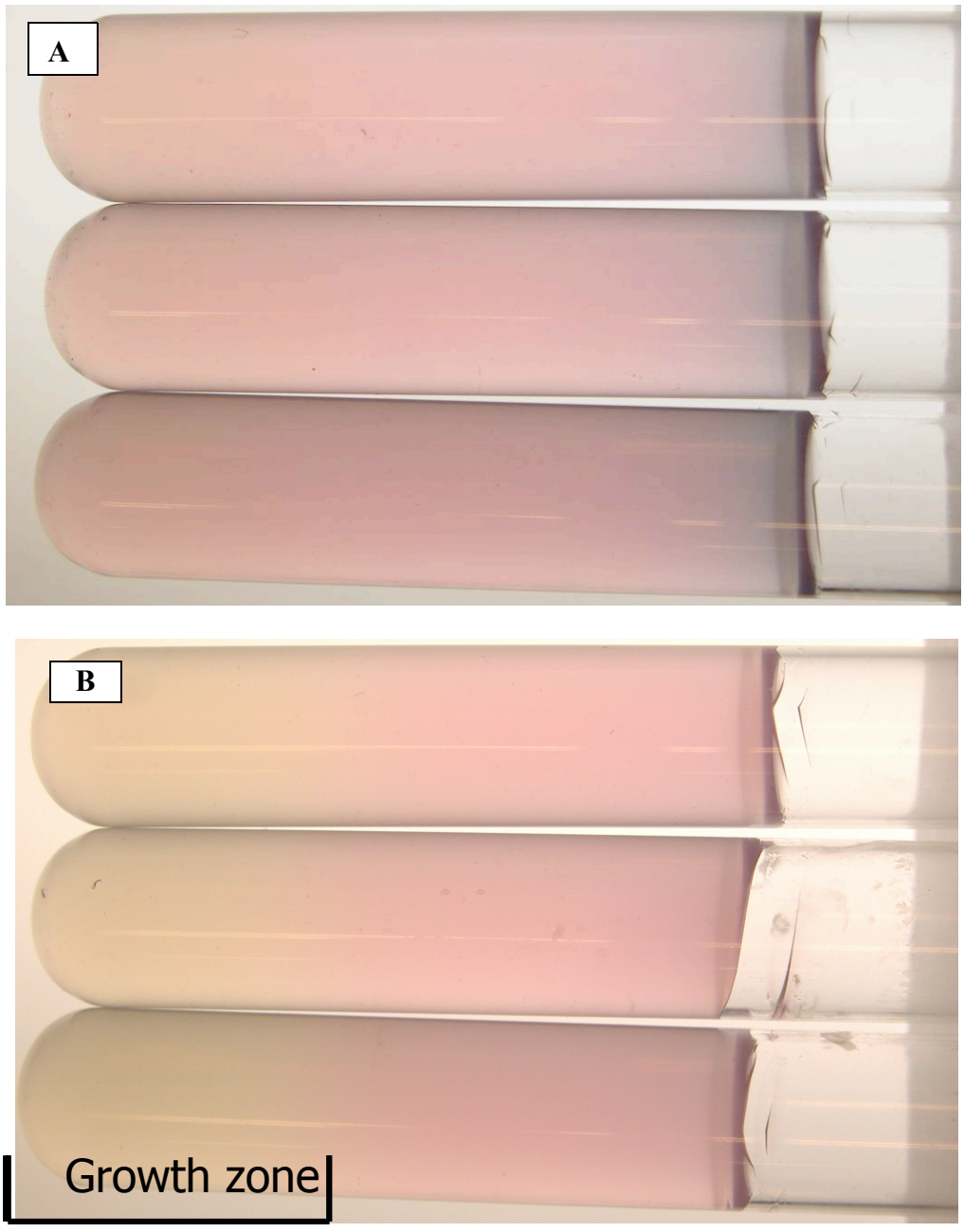
More interesting is the difference between the two transformed strains and the wild-type strain. The superoxide dismutase showed distinct increases in aerotolerance over the wild-type strains. This is shown in *Figure 23.* This increase in growth range is statistically significant for both the CAT and SOD strains. The t-test null hypothesis that the difference in means is zero was rejected with P-values of 4.18×10^{-5} and 0.007216, respectively.

Figure 21: Aerotolerance Baseline



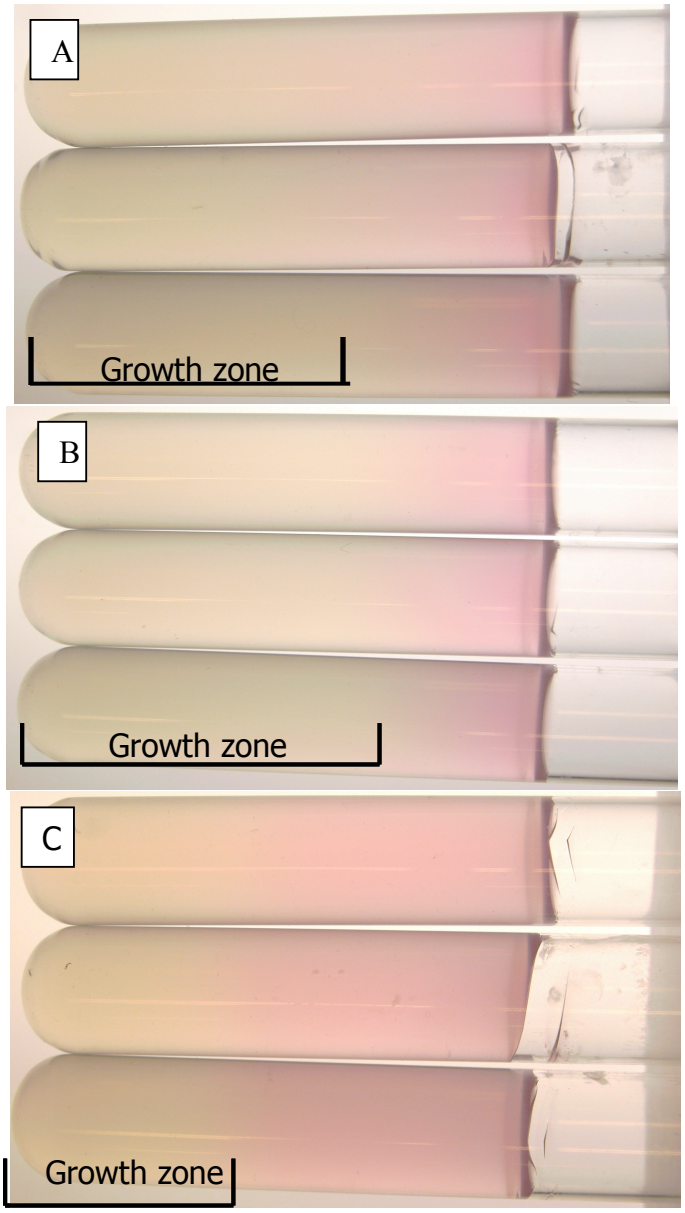
All four tubes have been colorized to help provide contrast
Yellow bars added to emphasize growth zone
***A**- No bacteria*
***B**- Geobacter*
***C**- 10x dilution of Geobacter*
***D**- Anoxic Geobacter*

Figure 22: Comparison of Induced and Uninduced Wild Type



Black bars added to emphasize growth zone
A: *wild-type induced G. sulfurreducens. The pink color is an oxygen indicator. All three replicates show the same pattern of complete oxygen diffusion and a complete lack of bacterial growth*
B: *wild-type uninduced G. sulfurreducens. This has less complete oxygen diffusion and has some bacterial growth at the bottom of the tube which is the yellow region.*

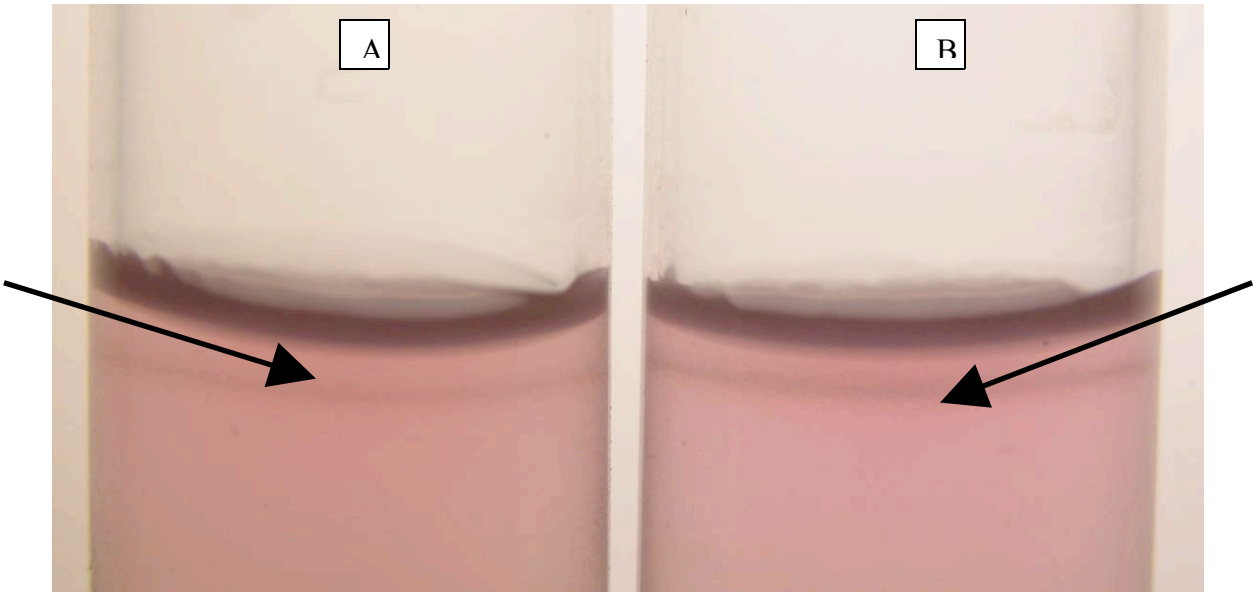
Figure 23: Comparison of Uninduced and Induced SOD transformants with uninduced wild type



Black bars added to emphasize growth zone
A: SOD uninduced- Shows significantly increased aerotolerance over wild type
B: SOD induced- Shows slightly increased aerotolerance over uninduced SOD
C: Wild-type uninduced- Low aerotolerance

In addition there was some growth at the top of the superoxide dismutase tubes. This was possibly transformed *G. sulfurreducens* but further tests are required to identify what species of bacteria. These tubes are shown in *Figure 24.*

Figure 24: Bacterial growth on top of SOD tube

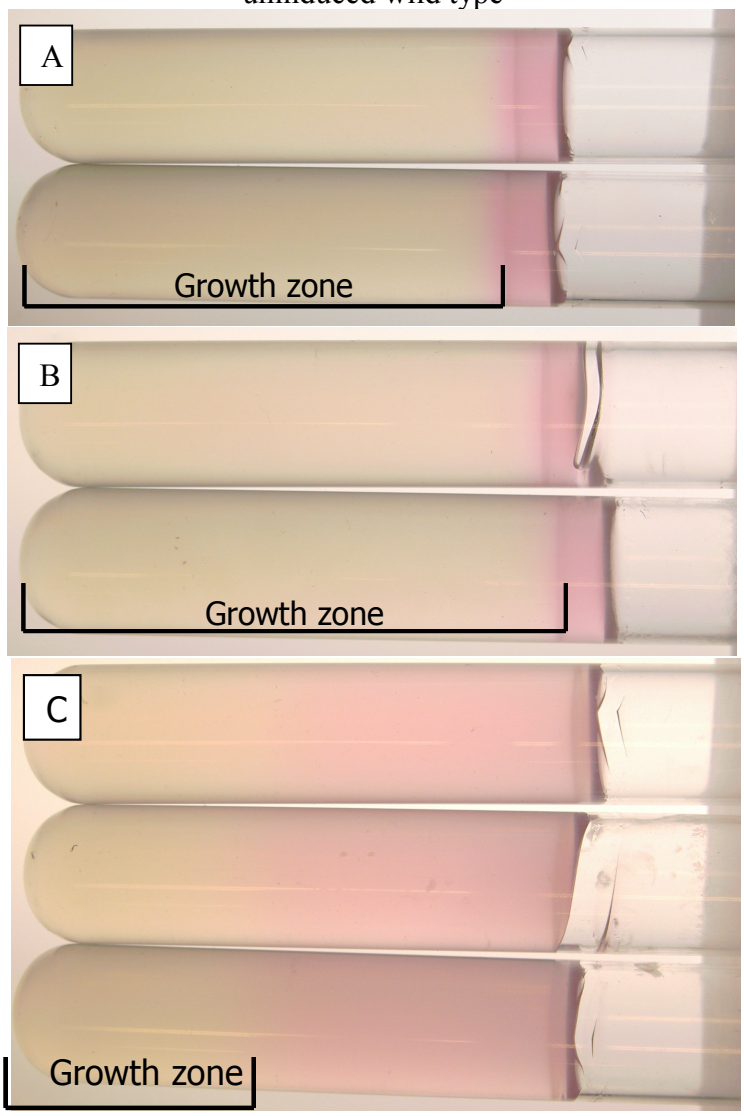


A: SOD
B: SOD + IPTG
There was a small layer of bacterial growth observed at the top of the agar in both tubes

Catalase conferred greater oxygen tolerance than superoxide dismutase. Both tubes showed much higher aerotolerance than in either superoxide dismutase or wild-type. The difference was large enough to reject the t-test null hypothesis with a P-value of 0.0089613. This was true of both the induced and uninduced catalase transformants. These assay tubes are pictured in *Figure 25*. The significant growth of catalase transformants was somewhat surprising considering that catalase

transformants had been rather slow growing compared to both superoxide dismutase transformants and wild type.

Figure 25: Comparison of Uninduced and Induced CAT transformants with uninduced wild type



Black bars added to emphasize growth zone
A: *CAT uninduced: grew very close to the surface*
B: *CAT induced: slightly closer to surface*
C: *Wild type uninduced: low tolerance*

Both of the transformed strains showed distinct increases in aerotolerance over wild-type *G. sulfurreducens*. It is important to note the consistency of the results in this assay. There is only a very small amount of deviation between each of the individual replicates shown in any of the figures above.

4.4 Catalase Activity

In addition to aerotolerance assays it was important to measure the actual levels of enzyme activity of the enzymes encoded by the plasmids. For this reason a catalase activity assay was performed.

The first of these entailed transforming *E. coli* with the pRG5 plasmids created above. The measurement of catalase activity was essential, as it has not been proven that *G. sulfurreducens* has a functional catalase gene. The catalase assay and peroxidase assays showed very similar data.

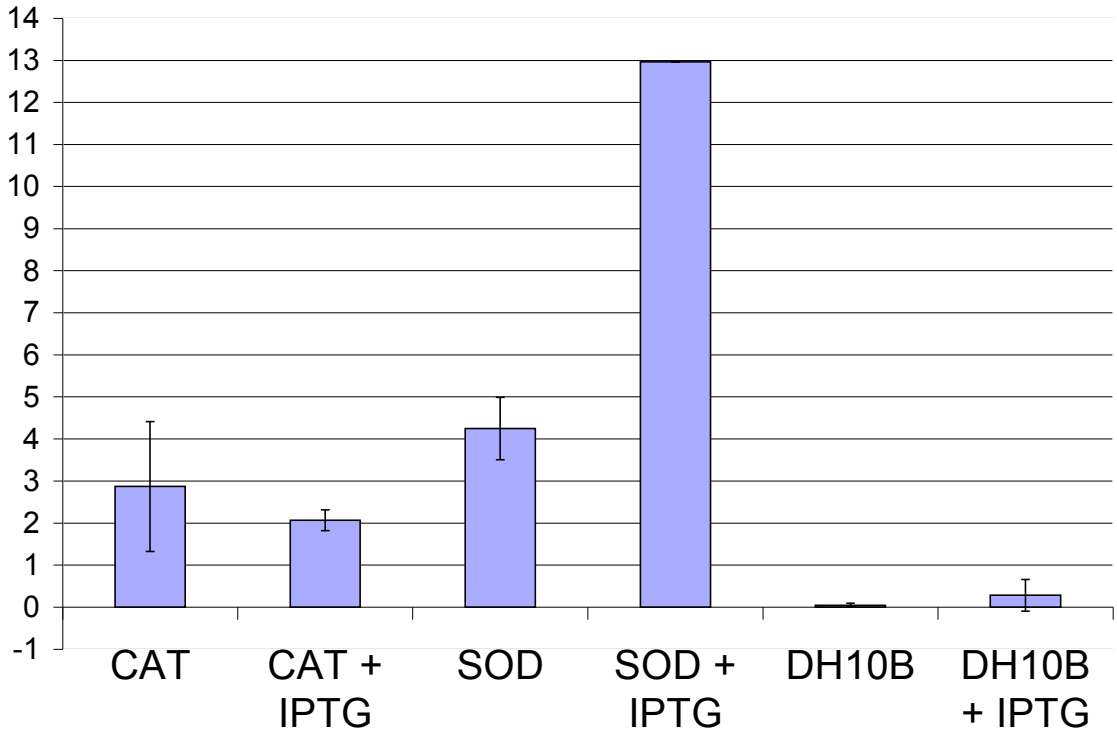
The catalase assay was performed twice. The first attempt showed almost no catalase activity in most samples. Interestingly, only the catalase transformant showed catalase activity, though activity was higher for uninduced than induced. This can possibly be explained by background transcription from a "leaky" operon and some inaccuracy in the data. It seems unlikely that this is the case, as Ptaclac, the promoter, is noted for showing a stark contrast in transcription levels between an induced and uninduced operon.

Most samples with the added (optional) bovine serum albumin (BSA) showed increases in hydrogen peroxide levels. While this is theoretically possible, it seems unlikely. Hydrogen peroxide is a reactive compound that degrades quickly even without catalase. In the presence of even minute levels of catalase, hydrogen peroxide

should break down extremely quickly. It seems more likely that BSA interfered with either the colorimetric reaction or with the spectrophotometer reading.

The second attempt at this assay was somewhat stranger than the first. This data is shown in *Figure 26*. While catalase showed elevated catalase activity, activity levels were again higher for uninduced catalase than for induced catalase. Even stranger is the SOD strain's greatly increased catalase activity. Induced SOD had by far the most notable increase in catalase activity.

Figure 26: Normalized Percent Decrease in Hydrogen Peroxide in 5 Minutes



Catalase activity was measured as a percentage decrease in hydrogen peroxide in a 5 minute period. Data was normalized by cell density. Bars shown are standard error.

It is important to note that the reading changes very little between wild type E. coli and wild type E. coli with IPTG. This means IPTG itself has very little effect on the absorbance at 240 nm.

Another point to note is that regardless of the enzyme expressed, all transformants showed higher catalase levels than wild type E. coli. This is possibly because the transformants grew to lower densities than the wild type. We normalized the hydrogen peroxide decrease by dividing by the density of cells in solution. This may not be an appropriate method to scale. It is possible that assuming this scaling is linear, led to a change in perceived enzyme activity levels, while actual levels were different.

Chapter 5: Discussion

Based on our results at the time of writing, it appears that our original hypothesis was correct. Over-expression of either catalase or superoxide dismutase in *G. sulfurreducens* resulted in drastically increased aerotolerance compared to wild-type.

These results mean that we have successfully constructed a plasmid that can affect the aerotolerance of *G. sulfurreducens*. While the transformants we created were not completely aerotolerant they did show a significant increase over the wild-type strain. This essentially acts as a proof of principle for the introduction of over-expressed oxidative stress protection enzymes in order to alter the aerotolerance of an organism. Further work could very well result in a strain of *G. sulfurreducens* that could survive in atmospheric oxygen.

Current work with *G. sulfurreducens* is made more difficult because of its lack of aerotolerance. The introduction of an aerotolerant strain would make lab work with this bacterium both less expensive and significantly easier than it currently is.

Aerotolerance might be essential to the successful application of *G. sulfurreducens*-based fuel cells in wastewater treatment. This is likely the first step in economically successful application of microbial fuel cells. The need to de-oxygenize the waste water before allowing it to come in contact with the bacteria would make wastewater treatment prohibitively difficult. It would probably necessitate the use of a batch process for electricity rather than a flow-through system. The introduction of aerotolerant *G. sulfurreducens*, like the strains we have created, would remove one of the major obstacles to the introduction of a flow-through design.

In addition, an aerotolerant strain could be used far more readily in industrial applications. Current fuel cells have to be purged of oxygen before inoculation for both the survival of the bacteria and to prevent the oxygen from scavenging electrons before they flow through the system. Because *G. sulfurreducens* has the ability to utilize oxygen as a terminal electron acceptor, an aerotolerant strain could be inoculated without purging the cell of oxygen. The bacteria would initially grow by reducing the oxygen until the supply is exhausted. At this point they would begin reducing the anode and producing electricity. This would completely eliminate the need for the labor and equipment that previously would have been required to prepare fuel cells.

Previous research has shown that *Geobacter* possess the ability to utilize oxygen as a final electron acceptor (Lin, Coppi, & Lovley, 2004). This mechanism is limited by the production of oxygen radicals, formed by metabolic reduction of oxygen. With the addition of the new CAT and SOD genes, this build-up of toxic metabolic byproducts is greatly lessened and this will likely decrease the current impediments to oxygen use by the bacteria. This could create a bias for the use of oxygen as the terminal electron acceptor in the electron transport chain because of the high reduction potential of oxygen. Using oxygen could allow the transformed *G sulfurreducens* strains to produce a significantly larger amount of oxygen from provided sugars. However, this energy would not be converted into electricity and so would be wasted in a fuel cell.

This problem can be avoided by growing *Geobacter* aerobically, but running the fuel cells under anaerobic conditions. Research could be conducted far more

efficiently than before. Researchers would not need to use anaerobic techniques and additionally the bacteria would likely grow more quickly using oxygen as the terminal electron acceptor. On the other hand, simply by placing the bacteria into a sealed fuel cell, the *Geobacter* would grow and consume the available oxygen and then switch to using the electrode as a terminal electron acceptor and therefore producing electricity.

Microbial fuel cells are by no means the solution to the approaching energy crisis but they could play an integral part of a multifaceted approach. An aerotolerant strain of *G. sulfurreducens* would drastically increase the viability of this form of energy production.

There are number of steps that need to be taken for this system to become completely viable. At the moment the genes for both catalase and superoxide dismutase are contained on plasmids. This has some limitations, the first of which is the need to maintain the plasmid. Currently it is selected for by growing transformants with spectinomycin. Oxygen stress could potentially be used to select for the aerotolerance plasmid instead. However, this would be problematic if the bacteria were ever placed into an anoxic medium because they could potentially lose the plasmid very quickly.

In addition it would prohibit the adding of additional plasmids. This would be a significant drawback for using this strain to do lab work as this tool would no longer be available for other researchers. Thus it could be worthwhile to attempt to add the genes under a more permanent method such as by knocking them into the genome.

Neither of the strains created were completely aerotolerant. Future research should utilize combinations of oxidative stress protection enzymes instead of simply one. This might allow the creation of completely aerotolerant strains. As above, this could be done using knock-ins instead of transformation.

There are a number of additional pieces of data that we would like to develop on this project as well. All of our current data on enzyme activity levels were based on expression in *E. coli* that had been transformed with our plasmids. While *E. coli* and *G. sulfurreducens* are of the same phylum and similar in a number of ways, gene expression in one cannot necessarily carry on to another. Our data do, however, lead to certain conclusions. Both superoxide dismutase and catalase show activity. This data on the surface suggests a higher catalase activity level for superoxide dismutase than for the catalase gene. This effect has been seen in every trial and assay performed.

This is possibly due to the SOD enzyme working in reverse due to the large excess of its normal product. Le Chatelier's principle says than any reversible reaction in this situation will be pushed toward substrate. In this assay, any superoxides created will quickly react and change forms, no longer considered a substrate for SOD. Therefore, during these assays, there will always be much more product than substrate for the normal SOD reaction. While this is theoretically very difficult because of the thermodynamics, the only other possible explanation we could identify was mislabeling of the plasmids which was ruled out through a miniprep and digest.

We saw surprisingly small differences in aerotolerance between the induced and non-induced transformants. It would be informative to see the difference in activity levels based on catalase and supeoxide dismutase assays in transformed *G. sulfurreducens*. The lac operon is supposed to have very low levels of leakage when it is not induced, but our results indicated otherwise. There was a much larger difference in aerotolerance between wild-type and uninduced than between uninduced and induced. However, this is explained by the lack of lacI in the pRG5CAT and pRG5SOD plasmids as well as a lack of lacI in *G. sulfurreducens.* Our "inducible" promoter did have a high level of expression, but it was at this high level of expression by default.

Another point that requires increased research is the actual electrical production of the transformed bacteria. It is entirely possible that the overproduction of these enzymes could slow the growth rate significantly and stop it from producing electricity as efficiently. On the other hand, it is also possible that under low levels of oxygen, like those that would be present in continual flow wastewater, the transformed bacteria might produce electricity more efficiently. Either way this is information that would be extremely useful.

Overall our research confirmed our initial hypothesis. We succeeded in transforming a strain of *G. sulfurreducens* and increasing the aerotolerance. This alteration could eventually lead to the creation of a completely aerotolerant strain and also serves as a model for transforming future microaerotolerant organisms. This strain could potentially drastically decrease the cost and effort required in research, as well as have some commercial applications.

The rising demand for energy, climate change, and dwindling fossil fuel supplies make the state of renewable energy research an important and exciting prospect. An "impending energy crisis" is only that if we let it be. Successfully developing the next thirty years' energy technology could turn this crisis into an energy revolution, changing not only the way we look at energy production, but how we interact with the world around us. Cheap, renewable, reliable energy is one of humanity's major upcoming goals.

Geobacter-based fuel cells and sewage treatment can be an element in transforming this problem into an opportunity. While its prospects as a viable energy technology are yet to be seen, we believe the introduction of an aerotolerant strain would move microbial fuel cell technology towards producing electricity on an everyday basis.

This project is a part of making *Geobacter*-fuel cells viable. Its engineering goals were to push *Geobacter*-based fuel cells a step closer to development, facilitate future research in this area, and decrease the cost of *Geobacter*-fuel cells. The scientific goals were to test the functionality of the superoxide dismutase and catalase genes in *G. sulfurreducens* and the effect their over expression would have on aerotolerance. The successful accomplishment of these goals is a step towards a realistic energy solution.

Appendices

CAT1

NCBI **Blast 2 Sequences results**

PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2

x_dropoff: 0 expect: 10.00(wordsize: 11 Filter ☑ View option

Standard

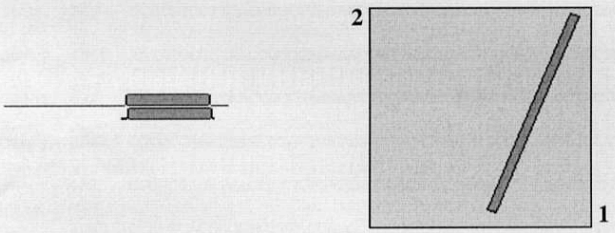
Masking character option X for protein, n for nucleotide Masking color option

Black

☐ Show CDS translation Align

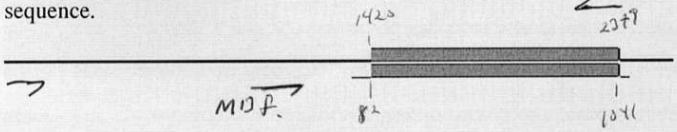
Sequence 1: CATgenbank
Length = 2584 (1 .. 2584)

Sequence 2: CATclone1
Length = 1082 (1 .. 1082)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

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Identities = 951/961 (98%), Gaps = 0/961 (0%)
Strand=Plus/Plus

Query  1420  TGGGGCCCCGCTCGCGCTACCTCGGCCCGGACGTGCCCGCGGAAGAACTCATCTGGCAGG  1479
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  82    TGGGGCCCCGCTCGCGCTACCTCGGCCCGGACGTGCCCGCGGAAGAACTCATCTGGCAGG  141

Query  1480  ACCCGGTCCCGGCGGTCACTCACCAGCTGATCGACCGACAGGACATCGCCGCCCTCAAGG  1539
```

CAT2

NCBI **Blast 2 Sequences results**

PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2

x_dropoff: 0 expect: 10.00 wordsize: 11 Filter ☑ View option

Standard

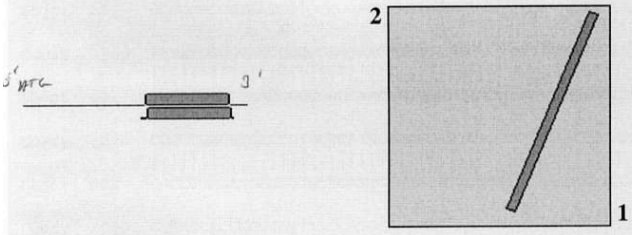
Masking character option: X for protein, n for nucleotide Masking color option

Black

☐ Show CDS translation Align

Sequence 1: CATgenbank
Length = 2584 (1 .. 2584)

Sequence 2: CATclone2
Length = 1084 (1 .. 1084)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

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Strand=Plus/Plus

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              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  82    TGGGGCCCCGCTCGCGCTACCTCGGCCCGGACGTGCCCGCGGAAGAACTCATCTGGCAGG  141

Query  1480  ACCCGGTCCCGGCGGTCACTCACCAGCTGATCGACCGACAGGACATCGCCGCCCTCAAGG  1539
```

CAT5 - correct orient

NCBI **Blast 2 Sequences results**

PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2

x_dropoff: 0 expect: 10.00(wordsize: 11 Filter ☑ View option

Standard

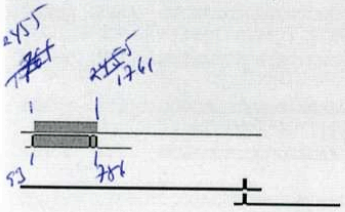
Masking character option X for protein, n for nucleotide Masking color option

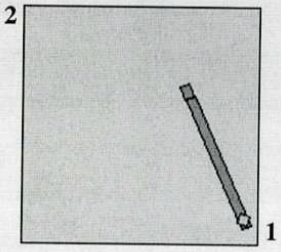
Black

☐ Show CDS translation Align

Sequence 1: Geobacter Catalase
Length = 2584 (1 .. 2584)

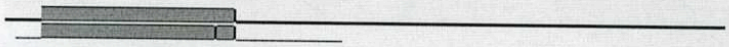
Sequence 2: CAT5clone with M13forward
Length = 1168 (1 .. 1168)





NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.



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 Strand=Plus/Minus

Query  1761  GGCAAGCAGGTCTCGCTGGCCGACCTGATCGTGCTGGGCGGCTGCGCGGCGGTCGAACAG  1820
             |||| ||||||||     |||| ||||||||  || |||||  || |||||  || ||||||
Sbjct  786   GGCAGGCAGGTCTTTTGGGCCTACCTGATCTAGCGGGGCGCNTGNGCGGCTGTGGAACAG  727

Query  1821  GCCGCGAAGAAAGCCGGCCACGATGTGACCGTCCCCTTCACCCCGGGCCGCGCCGATGCG  1880
```

CAT 5 - correct orientat

Blast 2 Sequences results

NCBI | PubMed | Entrez | BLAST | OMIM | Taxonomy | Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 0 expect: 10.00(wordsize: 11 Filter ☑ View option
Standard
Masking character option: X for protein, n for nucleotide Masking color option
Black
☐ Show CDS translation Align

Sequence 1: Geobacter Catalase
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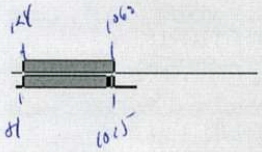
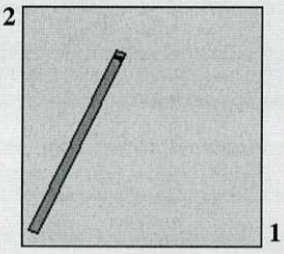
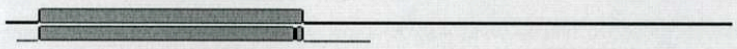
Sequence 2: CAT5clone with M13reverse
Length = 1253 (1 .. 1253)





NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.



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Strand=Plus/Plus

Query  124   CGAGTGACAACCGAAAGGCAAAATCGCCGACGAAGTGAACCACTCCACTATCCCAGGACC  183
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  81    CGAGTGACAACCGAAAGGCAAAATCGCCGACGAAGTGAACCACTCCACTATCCCAGGACC  140

Query  184   ATGAAAGGAGCCGATGCATGAGTGCTGACAACGAGAGCAGAACAGGCAGGCGGGCAGCCG  243
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SOD1

NCBI **Blast 2 Sequences results**

PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2

x_dropoff: 0 expect: 10.00 wordsize: 11 Filter View option

Standard

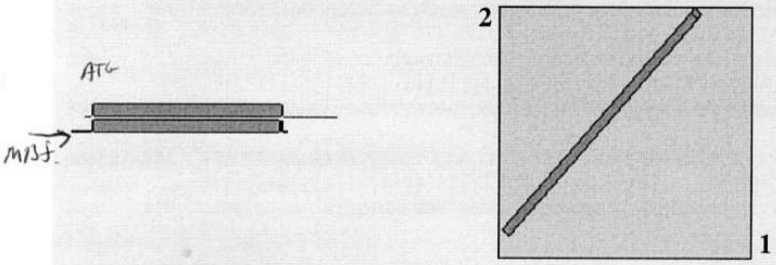
Masking character option: X for protein, n for nucleotide Masking color option

Black

Show CDS translation Align

Sequence 1: SODgenbank
Length = 879 (1 .. 879)

Sequence 2: SOD1clone
Length = 749 (1 .. 749)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

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 Strand=Plus/Plus

Query  31   TTCATGGTAAGGTTGAGACCGTGCCCGTTGGCATGTGATCCAATCAAGACCTGTCAAGGA  90
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  78   TTCATGGTAAGGTTGAGACCGTGCCCGTTGGCATGTGATCCAATCAAGACCTGTCAAGGA  137

Query  91   GGAAGCAACCATGGCCTATGAAGCAAAAGACTATTCGAAACTGATCGGGATGGCGGGATT  150
```

SOD3

Blast 2 Sequences results

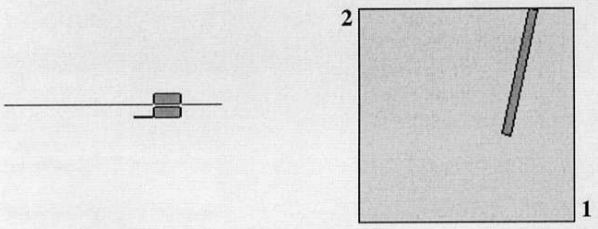
PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 0 expect: 10.00 wordsize: 11 Filter View option
Standard
Masking character option: X for protein, n for nucleotide Masking color option
Black
Show CDS translation Align

Sequence 1: SODgenbank
Length = 879 (1 .. 879)

Sequence 2: SOD3clone
Length = 192 (1 .. 192)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

```
Score =  216 bits (112),  Expect = 2e-52
Identities = 112/112 (100%), Gaps = 0/112 (0%)
Strand=Plus/Plus

Query  604  TCTCAAGCGTCCCGACTACATCGAGGCCTTCTTCAAGAATATCGACTGGAAGGCCGCCGA  663
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  81   TCTCAAGCGTCCCGACTACATCGAGGCCTTCTTCAAGAATATCGACTGGAAGGCCGCCGA  140

Query  664  GGCACGGTTGAAATAACCCAGTTCAGATCGGACACAGGGGGCGCTTACGGCG  715
```

SOD4

Blast 2 Sequences results

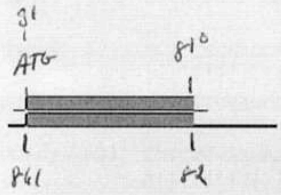
PubMed Entrez BLAST OMIM Taxonomy Structure

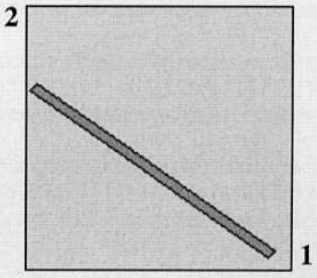
BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 0 expect: 10.00 wordsize: 11 Filter ☑ View option
Standard
Masking character option: X for protein, n for nucleotide Masking color option
Black
☐ Show CDS translation Align

Sequence 1: SODgenbank
Length = 879 (1 .. 879)

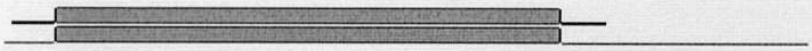
Sequence 2: SOD4clone
Length = 1242 (1 .. 1242)





NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.



```
 Score = 1494 bits (777),  Expect = 0.0
 Identities = 779/780 (99%), Gaps = 0/780 (0%)
 Strand=Plus/Minus

Query  31   TTCATGGTAAGGTTGAGACCGTGCCCGTTGGCATGTGATCCAATCAAGACCTGTCAAGGA  90
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  861  TTCATGGTAAGGTTGAGACCGTGCCCGTTGGCATGTGATCCAATCAAGACCTGTCAAGGA  802
                      ATG
Query  91   GGAAGCAACCATGGCCTATGAAGCAAAAGACTATTCGAAACTGATCGGGATGGCGGGATT  150
```

SOD5

Blast 2 Sequences results

PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2

x_dropoff: 0 expect: 10.00 wordsize: 11 Filter ☑ View option

Standard

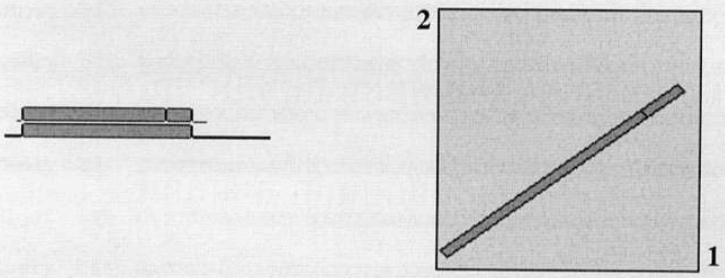
Masking character option: X for protein, n for nucleotide Masking color option

Black

☐ Show CDS translation Align

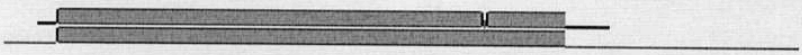
Sequence 1: SODgenbank
Length = 879 (1 .. 879)

Sequence 2: SOD5clone
Length = 1225 (1 .. 1225)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.



```
Score = 1436 bits (747),  Expect = 0.0
Identities = 772/782 (98%), Gaps = 2/782 (0%)
Strand=Plus/Plus

Query  31   TTCATGGTAAGGTTGAGACCGTGCCCGTTGGCATGTGATCCAATCAAGACCTGTCAAGGA  90
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  82   TTCATGGTAAGGTTGAGACCGTGCCCGTTGGCATGTGATCCAATCAAGACCTGTCAAGGA  141

Query  91   GGAAGCAACCATGGCCTATGAAGCAAAAGACTATTCGAAACTGATCGGGATGGCGGGATT  150
```

SOD6

NCBI **Blast 2 Sequences results**

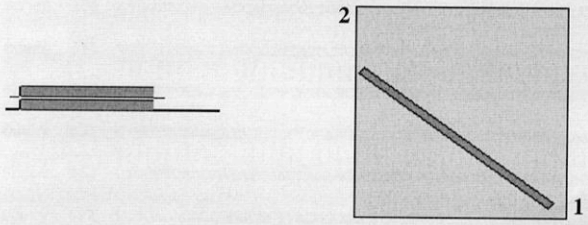
PubMed Entrez BLAST OMIM Taxonomy Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.2.17 [Aug-26-2007]

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 0 expect: 10.00 wordsize: 11 Filter View option
Standard
Masking character option X for protein, n for nucleotide Masking color option
Black
Show CDS translation Align

Sequence 1: SODgenbank
Length = 879 (1 .. 879)

Sequence 2: SOD6clone
Length = 1240 (1 .. 1240)



NOTE:Bitscore and expect value are calculated based on the size of the nr database.

NOTE:If protein translation is reversed, please repeat the search with reverse strand of the query sequence.

```
 Score = 1494 bits (777),  Expect = 0.0
 Identities = 779/780 (99%), Gaps = 0/780 (0%)
 Strand=Plus/Minus

Query  31   TTCATGGTAAGGTTGAGACCGTGCCCGTTGGCATGTGATCCAATCAAGACCTGTCAAGGA  90
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  862  TTCATGGTAAGGTTGAGACCGTGCCCGTTGGCATGTGATCCAATCAAGACCTGTCAAGGA  803

Query  91   GGAAGCAACCATGGCCTATGAAGCAAAAGACTATTCGAAACTGATCGGGATGGCGGGATT  150
```

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