ENGINEERED VESICLES FOR PERCHLORATE DEGRADATION

BY

SEAN KIRK POUST

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Environmental Engineering in Civil Engineering
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

Adviser:

Research Assistant Professor Julie L. Zilles

ABSTRACT

This study demonstrates the use of engineered vesicles to reduce perchlorate. Specifically, cell-free extracts containing perchlorate reductase and chlorite dismutase enzymes were encapsulated in a triblock copolymer vesicle functionalized with the outer membrane porin OmpF. The porin allows for perchlorate transport into the vesicles, inside which the encapsulated enzymes transform perchlorate to chloride. Perchlorate reduction was quantified using a methyl viologen colorimetric technique. The vesicle solutions had perchlorate-reducing activities ranging from 35-45 units per liter. This work shows that vesicles can provide a mechanism to utilize environmentally-relevant biological enzymes. When incorporated into a vesicle, the enzymes could be used outside of environmental conditions where they would normally be expressed by natural bacteria.

ACKNOWLEDGEMENTS

I would like to thank my adviser, Julie Zilles, for supporting me in this work, putting up with my stubbornness and guiding me to do my best work, even when I made it quite difficult. I also want to express my gratitude to Manish Kumar for thinking of this project, writing a proposal to make it possible, and asking me to come back to Illinois to make it a reality. Manish has also been an invaluable guide through the peril-filled halls of academia and has helped me to think of and get real research done. Don Cropek, Irene MacAllister and Steve Grimme at CERL have been excellent resources. I would also like to thank the Army 6.1 Basic Research program for my research assistantship during this work. The Salyers lab has been very gracious in letting me use their ultracentrifuge and the faculty of the Environmental Engineering Program have generously allowed me to use much of their equipment. Michelle Marincel has been a wonderful lab mate and I've greatly enjoyed developing our skills together. The undergraduate students involved in this project, Arun Paniker and Emily Bozek, have supported me and allowed me to do much of this work. Most of all, I wish to thank my wife, Gail Poust, for supporting me and allowing me to pursue yet another hairbrained idea requiring a cross-country move. Her patience and love for me seems to be infinite, for which I am very grateful.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Many environmental contaminants can be transformed to innocuous compounds by biological enzymes. These contaminants are often poor substrates for growth; to remove them cells require that a narrow range of environmental conditions be maintained. Maintaining appropriate conditions represents a significant amount of engineering effort for many biological treatment systems [1]. For example, engineered systems that utilize biomass for the treatment of perchlorate must add excess electron donor to reduce preferred electron acceptors such as nitrate and oxygen before perchlorate is reduced [2]. To remove biological oxygen demand (BOD), activated sludge treatment requires pumping of large amounts of oxygen to maintain aerobic conditions. Activated sludge also creates large amounts of excess biomass which requires disposal through incineration, land application, or landfilling [3,4]. To remove more recalcitrant compounds than BOD, some have suggested that genetic modifications could be used to create new metabolic pathways to remove these compounds. These techniques could also be used to expand the range of conditions in which cells can grow [5,6]. However, current regulations generally do not allow the release of genetically modified bacteria into the environment, as the modified genetic material could be spread in an uncontrolled manner by bacterial cells [7]. Due to their versatility, biological cells have applications to many environmental engineering systems. Nevertheless, they have disadvantages that make them less than ideal in certain cases.

Cells provide cheap, versatile and abundant sources of catalysts to increase the rate of reaction of a plethora of reactions. However, the goals of an engineer and the goals of the cell are quite different. Whereas the engineer's goal is to catalyze a reaction, the cell's goal is to replicate itself and pass on its genes. While most of the time both parties can be satisfied, they do conflict in some systems, as illustrated above. One solution to these problems is to create a simplified version of the cell that performs only one task of interest, regardless of the ambient conditions. For an engineer, the ideal biological cell would be a catalyst that regenerates itself and does not foul, something like the packet of enzymes shown in Figure 1. Enzymes could be encapsulated in some sort of shell and a transmembrane pore would allow transport into and out of the vesicle. Several methods have been proposed to create these simplified cells, or as they will be known in this work, engineered vesicles. These vesicles can still utilize the potency of biomolecules, but as the system is much more defined from an engineering perspective than a biological cell, vesicles can obviate many of the shortcomings of strictly biological systems.

In essence, an engineered vesicle encapsulates specific proteins inside a membrane and incorporates transport proteins into that same membrane, as illustrated in Figure 1. The combinations of these components can be tailored to serve a specific purpose. Proteins can be functionally incorporated into the membrane itself to permeabilize the membrane, similar to a cellular membrane. The *Escherichia coli* outer membrane protein OmpF has been used in the past for this purpose [8]. Additionally, specific proteins can be encapsulated in the interior of the vesicle. Superoxide dismutase and β-lactamase have been successfully encapsulated in the core of an engineered vesicle [9,10]. Proteases have been shown to be ineffective against encapsulated enzymes [10,9]. The membrane can thus protect these encapsulated enzymes from environmental conditions and proteolytic degradation. As these vesicles are incapable of reproducing, genetically engineered proteins can be incorporated without the concern that the altered genetic information could move into the environment as it could from a genetically modified organism.

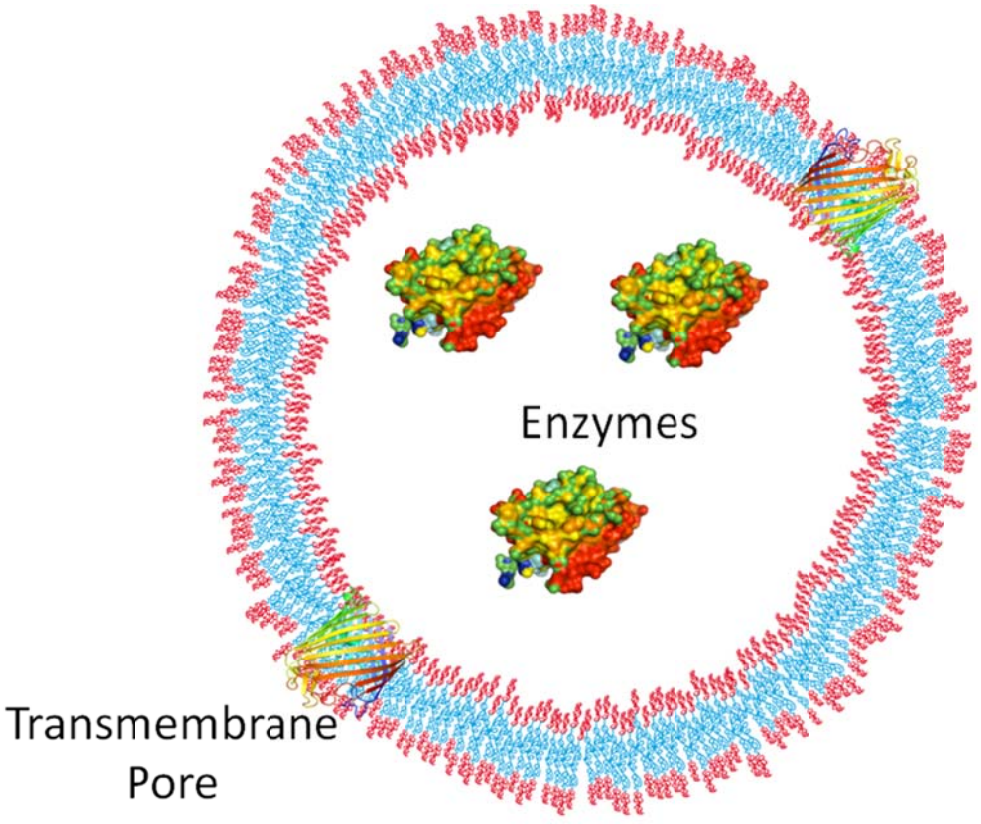


Figure 1 – Idealized Biological Catalyst. For many applications, a simplified cell would be an ideal method of utilizing the potency of biomolecules, without the complications of biological cells.

Many studies have used vesicles formed from lipid membranes (liposomes) to investigate how this kind of system could be tailored for different functionalities. However, lipids are subject to oxidation over time and liposomes have a limited number of applications after 30 or more years of research [11,12]. Unlike lipid membranes, vesicles created from polymer-based membranes are stable for long periods of time and are resistant to oxidation, making them more suitable for engineering applications [13]. Vesicles constructed from biomimetic triblock copolymers have been used to create hybrid abiotic-biotic systems with several applications [13,14,10]. Triblock copolymer membranes mimic the structure of lipid membranes with hydrophobic groups in the interior of the polymer and exterior hydrophilic groups [15]. This lipid bilayer-like structure allows for the functional insertion of membrane proteins, as demonstrated with several proteins [16,17]. These membranes are generally thicker than their lipid counterparts: a polymer membrane is approximately 10 nm whereas a lipid membrane is generally 5 nm. Despite this significant increase in thickness, membrane proteins have been shown to functionally insert in the polymer. It is theorized that the polymer surrounding the membrane protein compresses to match the size of the protein [18]. The polymer generally forms vesicle-like structures with diameters ranging from 50 to 500 nm [9]. Methacrylate end groups can be incorporated into the triblock structure, and can be polymerized under UV light to give additional stability to the vesicles [15]. Applying these polymer vesicles to environmental applications is the focus of this work.

This work explores the advantages of engineered vesicles as compared to traditional biological treatment. Specifically, my work attempts to create engineered vesicles with encapsulated perchlorate-reducing enzymes and incorporated membrane proteins that allow perchlorate transport. To understand how engineered vesicles might be advantageous when treating perchlorate, one must first understand issues surrounding current perchlorate treatment, bacterial perchlorate enzymes/metabolism and the design of this proposed engineered vesicle. These issues are addressed in each of the subsequent sections.

Perchlorate Toxicity, Regulation and Current Treatment Technologies

Perchlorate is an environmental contaminant arising from the historical disposal of munitions and rocket fuel into the environment, before it was understood that this compound had human health effects. It is a thermodynamically strong oxidant, with many uses in the aerospace and defense industries. However, at ambient temperatures and pressures, perchlorate is very stable in aqueous solution and is almost kinetically unreactive. Perchlorate affects iodide uptake by the thyroid gland and can subsequently affect hormone production—especially in sensitive subpopulations. There has been significant debate as to

the levels at which health effects arise. There is a general consensus that infants and the fetus of a pregnant woman are the most sensitive subpopulations for thyroid toxicity, but no studies have directly measured the toxic effects of perchlorate on those populations [19].

As a result of this lack of data, proxies must be used and assumptions made to determine an appropriate drinking water standard. To protect sensitive subpopulations, the EPA in 2002 suggested that perchlorate should be treated to below 1 µg/L in drinking water, based on a rat model [20]. Other studies have reported that concentrations as high as 180 and 220 µg/L in drinking water would be required to have an effect on iodide uptake in adult humans, and no effect on hormone production is observed until even higher concentrations of perchlorate are reached [21]. In 2005 the National Academy of Science used the data in [21] and an uncertainty factor of 10 (to account for sensitive subpopulations) when proposing an equivalent drinking water standard of 24.5 µg/L [22,19]. Many states have also proposed limits for perchlorate in drinking water, varying from 1-18 ppb [19]. The wide variety of proposed drinking water standards is a reflection of the wide variety of methodologies used to support them. At present, no national standard exists [19]. Nevertheless, perchlorate is detectable in the drinking water of 16 million Americans [23] and may constitute a significant health concern [24,25].

Over 15.9 million kg of perchlorate have been released into the environment due to rocket and missile use [26]. Before 1997, it was difficult to detect the environmental fate of these 15.9 million kg, as perchlorate could not be reliably detected below 400 ug/L. In 1997, a new analytical method became available with a detection limit of 4 ug/L, and perchlorate contamination was quickly determined to be widespread. Subsequently, the EPA published a draft risk assessment concerning perchlorate. In 2002 the EPA released a revised risk assessment for public comment and peer review, and in 2003 the EPA, Department of Defense, NASA and Department of Energy asked the National Academy of Science (NAS) to review the draft risk assessment after disagreement about the proposed reference dose. In January 2005, an NAS report concluded that existing studies did not support a link between perchlorate exposure and developmental effects and proposed raising the perchlorate reference dose and drinking water standard from 1 ug/l to 24.5 ug/L [19]. The EPA adopted the National Academy of Science's proposed reference dose in January 2005 [22], but in October 2008 made a preliminary decision not to establish a national drinking water standard. The EPA stated that regulating perchlorate would not provide a "a meaningful opportunity for health risk reduction for persons served by public water systems [27]." At the same time, the agency proposed a interim health advisory value of 15 ug/L [27]. After the transition from

the Bush Administration to the Obama Administration, the EPA asked the NAS to reevaluate the interim health advisory value. As the agency is reconsidering its previous positions, a national drinking water standard may be possible in the future [28,29]. If a national standard were enacted, demand for perchlorate treatment technologies would increase greatly. However, many water treatment utilities are treating perchlorate voluntarily due to perceived health effects and public demand. As health concerns grow, even in the absence of a national standard, demand for perchlorate treatment technologies may increase.

Viable abotic and biotic technologies exist for perchlorate treatment to meet proposed limits, but all have shortcomings that would hinder their widespread implementation. Example abotic systems include: ion exchange [30], reverse osmosis [31], electrodiaylsis removal (EDR) [32], and granular activated carbon (GAC) [33]. These abiotic technologies utilize adsorption or diffusion-limited filtration to remove perchlorate from bulk solution. These technologies concentrate perchlorate, but do not remove it from the environment. This creates a brine solution with elevated salinity and perchlorate levels. This brine must be further treated either chemically or biologically. Biotic processes require an exogenous electron donor to reduce perchlorate to chloride and oxygen. However, microbes will utilize oxygen and nitrate as electron acceptors before perchlorate is utilized and thus biotic processes must remove alternative electron acceptors before perchlorate is utilized [2].

The most commonly used process for ex situ perchlorate removal is ion exchange. Ion exchange is a physical-chemical process in which ions on a solid surface are exchanged for ions of similar size and charge. In the case of perchlorate, chloride is often exchanged for perchlorate using specialized media. Ion exchange media has a limited capacity and must be regenerated when exhausted. This is achieved using a backwash solution with high salt concentrations, which replaces the perchlorate with another anion. This creates a salty solution highly enriched in perchlorate. Membrane technologies are another common method of treating perchlorate. Electrodialysis reversal uses electric current to drive perchlorate and its accompanying cation across selective membranes and into a brine solution. Reverse osmosis uses high osmotic pressures to drive water across a semipermeable membrane, creating a permeate solution mostly free of perchlorate, and a brine solution enriched in perchlorate and other electrolytes that were present in the source water. All of the brine solutions created by these processes require further treatment [34].

Ex situ treatment using a bioreactor places perchlorate-laden water in contact with microbes capable of degrading perchlorate to chloride. The reduction of perchlorate to chloride requires the addition of electron donor, usually in the form of acetic acid, ethanol,

methanol or hydrogen. Other nutrients such as ammonia or phosphorous may be added if the source water alone cannot support the growth of organisms. Common bioreactor configurations are fluidized-bed reactors and fixed-bed reactors. In both configurations, microbes grow on some kind of media: either sand, granular activated carbon, or plastic media. Fluidized beds pump water through at a high enough rate to suspend the media, whereas a fixed-bed utilizes static media. Fluidized beds can provide greater surface area for microbes and thus a smaller reactor volume, but this requires higher pumping rates. If alternative electron acceptors like nitrate or oxygen are present, the microbes will preferentially utilize those and not degrade perchlorate. Thus, control of the influent nitrate and dissolved oxygen concentrations is vital for successfully operation of perchlorate-reducing bioreactors. To prevent clogging of the reactor from microbial growth, backwashing at high flow rates is used to remove excess biomass. This prevents flow channeling, short-circuiting and high headloss across the reactor [34].

An interesting union of these technologies exists in the BIOBROx^{TM} (**Bio**destruction of **B**lended **R**esidual **Ox**idants) process. In this process, drinking water from a contaminated source is treated with ion exchange, electrodialysis reversal, or reverse osmosis and the brine is then treated with a bioreactor. However, the source of electron donor for the brine is municipal wastewater. Instead of using an expensive consumable like acetic acid for an electron donor, a waste product is utilized and treated. Additionally, enough wastewater is blended to reduce the salinity of the brine to a level that non-halophilic bacteria can tolerate. The process has been implemented at full-scale in a 3.75 million gallon per day facility and is operated by Magna Water District in Magna, Utah. The effluent from this process can then be safely discharged to the sewer or put into a reuse system [35].

Bacterial Perchlorate Metabolism

Two principle enzymes have been implicated in the reduction of perchlorate to chloride: perchlorate reductase and chlorite dismutase. Perchlorate reductase (Pcr) catalyzes the reduction of perchlorate to chlorate and from chlorate to chlorite as illustrated in Figure 2. Both of these reactions require an external source of reducing power, usually in the form of an exogenous electron donor [25]. Pcr is a 420 kDa protein believed to be a trimer of heterodimers (a_3b_3) containing iron, molybdenum and selenium [36]. The Pcr protein is found in the periplasm of perchlorate-reducing bacteria, but it is a soluble protein [25]. Chlorite dismutase (Cld) then catalyzes the dismutation of chlorite into chloride and O_2 (Figure 2). As this is a dismutation reaction, no external electron donor is required in this step. Cld is a heme-containing 120 kDa protein and is the only known enzymatic process which catalyzes

O-O bond formation beyond photosystem II [37]. The Cld protein is necessary to alleviate the buildup of toxic chlorite during the respiration of perchlorate [38]. This protein is found on the outer membrane of perchlorate reducing bacteria [25].

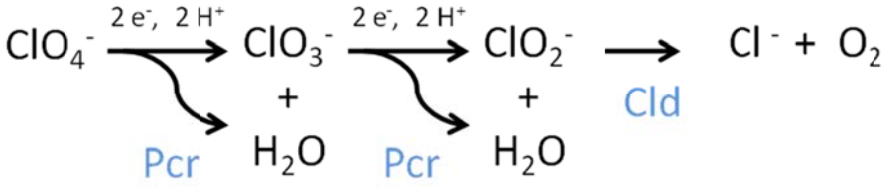


Figure 2 – Reactions catalyzed by perchlorate reducing enzymes, perchlorate reductase (Pcr) and chlorite dismutase (Cld). Enzymes are shown in blue, chemicals are shown in black.

Perchlorate-reducing bacteria only reduce perchlorate under anaerobic conditions when alternative electron acceptors are not present [39,40], because perchlorate reductase (Pcr) is only expressed under anaerobic conditions in the presence of perchlorate [41]. Chlorite dismutase (Cld) is expressed constitutively in some perchlorate reducing strains, such as the bacterium *Dechlorosoma* sp. KJ [42]. The requirement of anaerobicity for Pcr expression is a major limiting factor and design consideration for the use of bacteria to treat perchlorate contamination [2]. If this requirement could be removed, it would be much easier to treat perchlorate. Although some authors reported that perchlorate-reducing enzymes are oxygen sensitive [36], others have found that anaerobically-stored perchlorate-reducing extracts decrease in activity at the same rate as aerobically-stored extracts [43]. If the oxygen sensitivity of perchlorate reduction is purely at the level of transcription, these enzymes could be utilized outside of the cell in aerobic conditions to reduce perchlorate, but few studies have investigated this. Outside of the cell however, these enzymes—like any enzyme—will be subject to proteolysis and loss of activity over time.

Creating a Perchlorate-Reducing Engineered Vesicle

To protect and encapsulate these perchlorate-reducing enzymes, a polymer vesicle was used in this study. As illustrated in Figure 3, this engineered vesicle has four principle components: a transmembrane pore capable of transporting perchlorate, a biomimetic polymer membrane, encapsulated perchlorate-reducing enzymes, and a source of reducing power. In these engineered vesicles, perchlorate enters the vesicle through the pore embedded in the polymer membrane, and is reduced to perchlorate by the perchlorate-reducing enzymes. The pore utilized is the *Escherichia coli* outer membrane protein F (OmpF), which

is described in more detail below. The membrane is a biomimetic ABA triblock copolymer with hydrophobic interior groups and hydrophilic exterior groups, which can support inserted membrane proteins such as OmpF. In this work, the perchlorate-reducing enzymes were supplied in a cell-free extract and encapsulated in the interior of the vesicle. The reducing power for these reactions was provided by methyl viologen.

OmpF has been used in previous encapsulation studies to transport ions across a triblock copolymer membrane [9,14]. This protein is a homotrimer of 37 kDa subunits proteins, and each protein subunit contains a 600 dalton pore that allows small solutes in and out of the membrane via passive transport/diffusion. The pore has a slight cation specificity, but anions such as perchlorate can be transported through the pore [44]. OmpF was chosen as it has been used before in similar studies and is large enough to allow the passage of the materials of interest. Enzymes targeted to a specific compound (perchlorate in this case) can be encapsulated inside a vesicle and coupled to transport proteins (such as OmpF) that allow the influx of that compound and the efflux of break-down products. This system thus represents a novel means of utilizing environmentally-relevant enzymes, protecting them in a polymer shell and coupling them to transport processes. Unlike a cell, this system does not adapt to a preferred substrate and so will reduce perchlorate whenever the reaction is thermodynamically favorable.

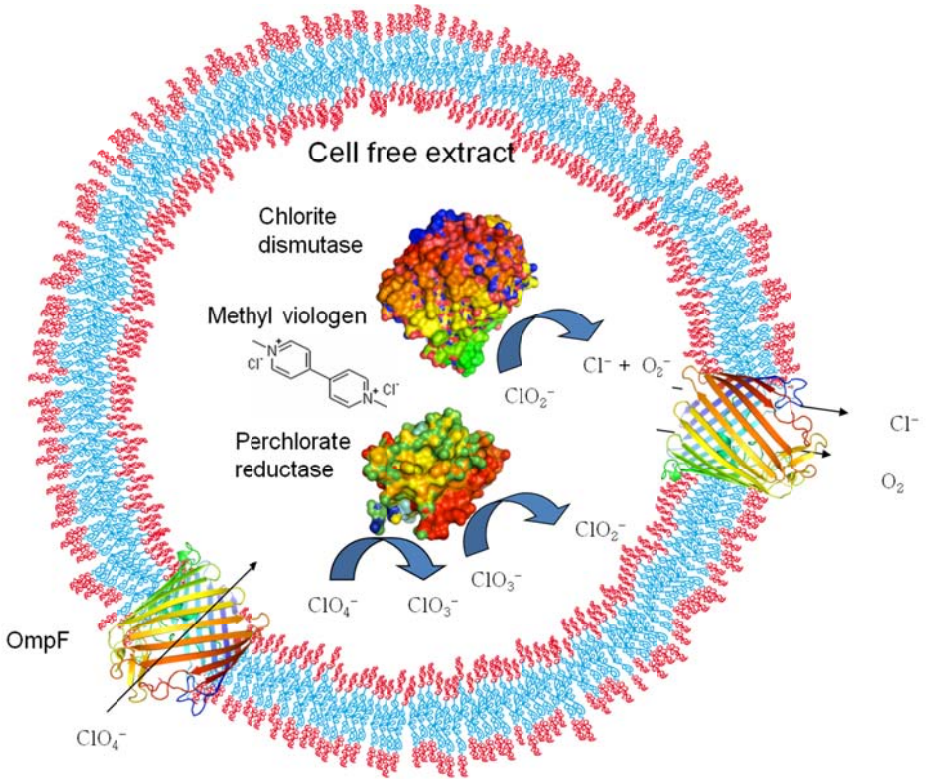


Figure 3 – Schematic of perchlorate reducing engineered vesicle. Methyl viologen and perchlorate enter the vesicle through the OmpF pore. Once inside, the encapsulated enzymes catalyze the reduction of perchlorate to chloride. The oxidized methyl viologen and chloride can then exit the cell through the OmpF pore.

One interesting extension of this idea is to utilize a specific transporter instead of OmpF to move a contaminant inside the vesicle. The sodium iodide symporter could be used instead of OmpF for this purpose in these engineered vesicles. The sodium iodide symporter (NIS) is the membrane protein that normally transports iodide into the thyroid for hormone synthesis. Perchlorate is a very potent competitive inhibitor of iodide transport, as the NIS protein will preferentially transport perchlorate into the thyroid; this is the primary mechanism for perchlorate toxicity in humans [45,46]. Thus, if purified and reconstituted into a polymer membrane, this system could couple the NIS membrane protein (eukaryotic) to the bacterial enzymes (prokaryotic) that could degrade a contaminant. This would not only represent a novel means of treating perchlorate, but also the creation of a new metabolism combining proteins from different domains of life in a synthetic context. Even more importantly, this system would be able to concentrate perchlorate, making the treatment of low concentrations of perchlorate possible (possibly far below current detection limits).

Utilizing NIS has not been attempted in this work due to the difficulty of purifying eukaryotic membrane proteins and incorporating them into a polymer membrane. In addition to the difficult task of purifying and incorporating NIS, a source of reducing power must be provided for the encapsulated perchlorate-reducing enzymes to function. Unlike the OmpF-based vesicles, which used methyl viologen as a source of reducing power, NIS-based vesicles will not be permeable to methyl viologen. The incorporation of an additional protein into the membrane or encapsulation of a system for generating reducing power may be necessary to allow for electrons/reducing power to be shuttled inside the vesicle.

Results of this Study

My results show that polymer vesicles with encapsulated perchlorate-reducing enzymes and membrane-incorporated OmpF can reduce perchlorate. Using these engineered vesicles, perchlorate will be reduced at enzymatic rates whenever conditions are thermodynamically favorable, rather than being subject to the protein expression patterns of the cell.

These vesicles circumvent many of the shortcomings of biological treatment, while retaining its advantage of completely reducing perchlorate to chloride at enzymatic rates. This approach could also be applied to other enzymes and other contaminants. Conceivably any process for which biological treatment is difficult could be distilled into an engineered vesicle approach. The relevant enzymes could be purified, placed inside a vesicle with membrane proteins and then used to treat recalcitrant compounds. At first, these vesicles will have a limited number of high-value applications, as the recombinant enzymes, proteins and polymer are difficult to produce, whereas many kinds of cells are simple to grow and incorporate into engineered systems. Although this approach would be used in high value applications initially, it could become much more widespread as the components become easier to produce.

MATERIALS AND METHODS

Chemicals and Reagents: The ABA3 was a symmetric poly-(2-methyloxazoline)-block-poly-(dimethylsiloxane)-block-poly-(2-methyloxazoline) ($PMOXA_{15}$-$PDMS_{110}$-$PMOXA_{15}$) block copolymer and was a generous gift of the Meier lab at the University of Basel, Switzerland. This polymer was used for all vesicle studies. A description of the synthesis of a similar polymer is described in reference [47]. All other chemicals used were of reagent grade.

Strains and Growth Media: Strain BAA-33 *Azospira oryzae* and *Dechloromonas agitata 700666* were obtained from ATCC (ATCC, Manassas, VA). *Azospira oryzae* was grown in mineral media as described [39]. *Dechloromonas agitata* was grown in mineral media as described [48]. Actively growing cultures of both strains were used to create 30% glycerol stocks which were stored at -80˚C.

Growth Curves of *Azospira oryzae* and *Dechloromonas agitata*: *Azospira oryzae* and *Dechloromonas agitata* were grown as described before [39,48]. The strains were maintained as glycerol stocks, streaked to nutrient agar and grown at 35˚C for 2 – 3 days. 10 mL starter cultures were prepared from streak plate colonies and incubated at 35˚C on a shaker at 100 rpm. After the starter cultures reached an optical density (at 600 nm) of 0.3, growth cultures (10 mL) were inoculated in triplicate with 1 mL of the starter culture for growth curves and incubated at 35˚C on a shaker at 100 rpm. Samples were taken from each triplicate culture for later chlorate or perchlorate analysis and optical density measurements were taken at 600 nm over time.

Preparation of Cell-Free Extract Containing Perchlorate-Reducing Enzymes: Cell extract containing perchlorate reducing enzymes was obtained as described before [49]. *Azospira oryzae* was maintained as a 30 % glycerol stock, streaked to nutrient agar and grown at 30˚C for 2 – 3 days. 100 mL starter cultures in mineral media [39] were prepared from *Azospira* streak plates. Upon reaching an optical density (at 600 nm) of ~ 0.6 cultures were transferred to 5 L mineral media. After reaching an optical density (at 600 nm) of ~ 0.6, the 5 L cultures were pelleted, resuspended in 10 mL 50 mM potassium phosphate buffer (pH 6.0), and broken using a sonic dismembrator (Fisher Scientific, Waltham, MA). Three five minute lysis cycles (3 sec on 2 sec off) were used to break the cells. The lysate was subsequently centrifuged at 5,000 x g for 15 min. The resulting supernatant was then centrifuged at 140,000 x g for one hour using a Beckman preparative ultracentrifuge

(Beckman-Coulter, Fullerton, CA) to obtain a red supernatant enriched in perchlorate reductase and chlorite dismutase.

Expression and Purification of OmpF: Porin proteins were obtained by overexpressing and purifying the *Escherichia coli* outer membrane protein, OmpF from the strain BL21(DE3) omp8 [50]. Protein purification was performed as described [14] and briefly summarized here. Cultures were inoculated 1:200 from overnight cultures into 1 L Luria-Bertani broth and grown to an optical density (at 600 nm) of ~ 0.6. Cultures were then induced with IPTG at 0.4 mM for 6 hours. Cells were then pelleted (10,000 x g, 10 min). Cells were resuspended in 20 mM Tris-Cl pH 8.0, 2 % SDS and broken using a sonic dismembrator (Fisher Scientific, Waltham, MA). Three five minute lysis cycles (3 sec on 2 sec off) were used to break the cells. The OmpF-containing peptidoglycan layer was pelleted by centrifugation (60 min, 40,000 x g). The resulting small black pellet was resuspeneded in prextraction buffer (0.125 % octyl-POE, 20 mM phosphate buffer, pH 7.4) and centrifuged to remove the membrane fraction (140,000 x g, 40 min). The membrane fraction was resuspeneded in extraction buffer (3 % octyl-POE, 20 mM phosphate buffer, pH 7.4) to solubilize the OmpF. The remaining membrane fraction was removed by centrifugation (140,000 x g, 40 min). OmpF purity was verified using SDS-PAGE. A detailed protocol for this procedure can be found in Appendix A.

Preparation of ABA Vesicles, OmpF Incorporation and Cell-Free Extract Incorporation: Block copolymer vesicles were prepared by using the film rehydration method. Twelve milligrams of polymer was first dissolved in chloroform (2 ml), and the chloroform was evaporated slowly in a rotary vacuum evaporator at room temperature using a vacuum of 100 mbar. This formed an even film on the inside of round-bottomed flasks. This film was then further dried under a high vacuum (5 mbar) for at least 4 h. 1.5 mL of cell extract was then added to the film with alternating vigorous vortexing and periodic sonication (of duration 30 s) for several minutes. This mixture was then left stirring for at least 8 h. The resulting suspension was extruded once through a 1-um track-etched filter (Nucleopore, Whatman), followed by extrusion ten times through a 0.4 or 0.6-um track-etched filter (Nucleopore, Whatman) to obtain monodispersed unilamellar vesicles. To incorporate OmpF into ABA3 vesicles, varying amounts of an OmpF stock solution (in 20 mM phosphate buffer, 3% octyl-POE, pH 7.4) were added during the formation of the polymer vesicles. The resulting protein-containing vesicles were purified chromatographically by using a column packed with Sepharose 2B (Sigma, St. Louis, MO) or Sephacryl 500-HR (GE Healthcare, Waukesha, WI) to remove nonincorporated protein and trace detergent [14]. To quantify any

contaminating protein outside the vesicles, the BCA protein assay was used. A more detailed protocol for this procedure can be found in Appendix A.

OmpF Transport Experiments: Two batches of vesicles hydrated in HEPES buffer with 2.5 mM carboxyfluorescein were made for OmpF transport experiments. One film was hydrated in the presence of OmpF at a 1:200 molar ratio of protein to polymer and one film was hydrated in HEPES alone with carboxyfluorescein. These vesicles were extruded and size excluded using standard methods as above. A fluorescence plate reader was used to measure the fluorescence at an excitation wavelength of 494 nm and an emission wavelength of 519 nm of both sets of vesicles in triplicate.

Perchlorate-Reducing Enzyme Activity Assays: Perchlorate-reducing activity was assayed using methyl viologen, as described before [49]. Activity levels were measured by monitoring the oxidation of reduced methyl viologen (MV) at 578 nm in anaerobic cuvettes (Helma, Müllheim, Germany). The assay mixture (50 mM Tris, pH 7.5, 0.5 mM MV) was combined with vesicles and prereduced by a small amount of dithonite (0.2 M). The reaction was then started by the addition of 20 uL of perchlorate (.1 M). A more detailed protocol for this procedure can be found in Appendix A.

Analytical Techniques for Chlorate, Perchlorate and Protein Concentrations:

Chlorate Measurements Using Ion Chromatography: Chlorate concentrations were measured using ion chromatography using an Ion Pac AS-18 Hydroxide-Selective Anion Exchange column from Dionex on a Dionex ICS-2000 system (Dionex, Sunnyvale, CA) using 65 mM KOH eluent and a 1.2 mL/min eluent flow rate. Information on this method can be found in reference [51].

Perchlorate Measurements Using HPLC MS: Perchlorate concentrations were measured using high performance liquid chromatography in tandem with mass spectrometry. A Waters IC-Pak Anion HR (4.6 x 75 mm) column preceded by a Waters IC-PAK Anion guard column (Waters, Milford, MA) was used to prepare samples for injection onto the mass spectrometer. The eluent was 50 % 100 mM ammoium formate and 50% acetonitrile at a flow rate of 0.5 mL/min on an anionc column. Perchlorate was detected at an m/z ratio of 99.5 on the mass spectrometer. Additional details about the method can be found in reference [52].

Protein Measurements: Protein concentrations were measured using the BCA kit (Pierce, Rockford, IL).

Construction of pSPOmpF and Expression: The ompF gene was PCR amplified from *Escherichia coli* strain ME9062 using the primers OmpF1 and OmpR1 (Table 2). Both

primers were constructed using a segment of the ompF sequence with an NdeI site engineered into the upstream primer and the XhoI site engineered into the downstream primer with 3 base pairs at the end to allow for restriction digestion. The pET 28b(+) vector and the resulting ompF were double digested with NdeI and XhoI. A 3:1 molar ratio of insert to vector was used to insert the amplicon into the vector, which was then transformed into XL-10 for plasmid maintenance. The plasmid was sequenced and shown to be free of errors (data not shown). Protein expression was achieved by transforming the resultant vector into BL21 (DE3) and inducing with 1 mM IPTG.

Construction of pSPNIS and Expression: The rNIS plasmid was a gift from the Carrasco group. The rNIS plasmid contains the sequence of the sodium iodide symporter from *Rattus norvegicus*. NIS was PCR amplified from this template. Similar to ompF, primers were designed to add NdeI and XhoI sites to NIS for insertion into pET 28b(+) (Table 2). The plasmid and amplicon were double digested with NdeI and XhoI. Ligation was performed with a 3:1 molar ratio of insert to vector, which was then transformed into XL-10 for plasmid maintenance. The plasmid was sequenced and shown to be free of errors. Protein expression was achieved by transforming the resultant vector into BL21 (DE3). Cultures were inoculated 1:200-1:1000 from overnight cultures into Luria-Bertani broth and grown to an optical density (at 600 nm) of ~ 0.6. Cultures were then induced with IPTG at 1 mM. Cells were then pelleted at various time points (10,000 x g, 10 min).

Histidine-Tagged Western Blot: Standard techniques were used for running SDS PAGE gels [53]. Western blots were performed by transferring from a SDS PAGE gel to either a nitrocellulose or PVDF membrane overnight at 17V at 4°C. Membranes were blocked for 1 hour in 30 mL of Tris-Buffered Saline Tween-20 (TBST) with 1.5% dry milk. Membranes were then incubated at a ratio of 1:4000 Anti His_{6+}-Perxoidase (2) (Roche, Basel, Switzerland) to TBST with 0.5% dry milk solution for 1.5 hour. Membranes were subsequently washed three times for 5 minutes each with TBST. Membranes were developed with BCIP/NBT substrate (Promega, Madison, WI) for 1-5 minutes.

Protease Experiments for Cell-Extract and Vesicles: Cell extract was digested with proteinase K by adding proteinase at 2% of total protein to cell extract and incubating overnight at 37°C. Perchlorate-reducing activity was measured before and after digestion. Vesicles were digested with proteinase K by adding 5% (v:v) 20 mg/mL proteinase K solution (Roche). Vesicles were then incubated for 4 hours at room temperature and size excluded.

Transmission Electron Microscopy: These measurements were conducted on vesicle samples by using a Philips 400 microscope (Philips). The samples were prepared by dilution up to 1,000 times and then stained with 2% uranyl acetate on plasma-treated copper grids.

Table 1 – Strains and Plasmids Used

Strains:	**Description:**	**Reference:**
Escherichia coli:		
Omp8	OmpF overexpression strain, Amp^R	[50]
ME9062	ompF PCR template	[54] [55]
XL10	Ultracompetent cells	Stratagene
BL21 (DE3)	pET system overexpression Strain	Stratagene
BL21 (DE3) pSPOmpF	OmpF overexpression strain, Kan^R	This study
BL21 (DE3) pSPNIS	NIS overexpression strain, Kan^R	This study
Other bacteria:		
Dechloromonas agitata strain CKB	Perchlorate reducing bacterium	[48]
Azospira oryzae strain PS	Perchlorate reducing bacterium	[39]
Plasmids:		
pET 28 b(+)	Protein overexpression plasmid, Kan^R	Novagen
pSPOmpF	His-tagged OmpF overexpression plasmid, Kan^R	This Study
pSPNIS	His-tagged NIS overexpression plasmid, Kan^R	This Study

Table 2 – Primers Used in this Study

Name:	**Purpose:**	**Sequence:**
OmpF1	OmpF Sequencing Primer	5' - CTACCTATCGTAACTCCAACTTCT - 3'
OmpR1	OmpF Sequencing Primer	5' - CCAAAGCCTTCGTATTCG - 3'
NISF1	NIS Sequencing Primer	5' - TCATCCTGAACCAAGTGACC - 3'
NISF2	NIS Sequencing Primer	5' - AGCTGTGACTGTGGAAGACC - 3'
NISF3	NIS Sequencing Primer	5' - CCAAAGGAAGACACTGCCAC - 3'
NISR1	NIS Sequencing Primer	5' - TTGGTCACAGCAGGGATGTC - 3'
NISR2	NIS Sequencing Primer	5' - CGTAGATGAATGAGAGCCC - 3'
NISR3	NIS Sequencing Primer	5' - CCTTCATACCACCCACGGT - 3'
T7 pro	Sequencing	5' - TAATACGACTCACTATAG - 3'
T7 term	Sequencing	5' - GCTAGTTATTGCTCAGCGG - 3'
NIS Amp 1	NIS amplification primer	5’- CATATGGAGGGTGCGGA - 3’
NIS Amp 2	NIS amplification primer	5’ - CTCGAGGTTGGTCTCCACA - 3’
OmpF Amp 1	OmpF amplification primer*	5' - CAGCATATGATGAAGCGCAATATTCTG - 3'
OmpF Amp 2	OmpF amplification primer*	5' - TATCTCGAGGAACTGGTAAACGATACC - 3'

*See Appendix B for more detail on the development of these primers

RESULTS AND DISCUSSION

Production of Perchlorate Reducing Cell-Free Extracts:

Perchlorate reductase and chlorite dismutase are not commercially available. Several expression systems have been used to attempt to express perchlorate reductase from recombinant sources, but without success [43]. Chlorite dismutase has been overexpressed recombinantly, purified and shown to be active [37]. We are currently attempting to replicate this and obtain high concentrations of chlorite dismutase. In the meantime, cell-free extracts from perchlorate-reducing bacteria (PRB) were used as the source of these two proteins. Cell-free extracts have been shown to have high levels of perchlorate-reducing activity [49].

The perchlorate-reducing bacterium *Azospira oryzae* was grown as described in the materials and methods. Initially, the media in reference [39] was prepared as described in reference [49]. However, the resazurin used in [49] as a redox indicator seemed to hinder the growth of the organisms and it was difficult to get the strain to grow well and consistently. A comparison of growth in media with and without resazurin is shown in Figure 4. After many failed attempts, resazurin (included in reference [49], but not in reference [39]) was omitted from the media and the strain began to grow much better. Cultures were then inoculated in triplicate and optical density (at 600 nm) and perchlorate concentrations were measured over several days. Results are shown in Figure 5 b). As the cells grew, the perchlorate in the media was consumed, indicating that the cells were utilizing perchlorate. After a lag phase, the cultures grew to a maximum optical density measurement of approximately 0.65 Au and then exited log phase. The exit from the logarithmic phase seemed to correspond with the exhaustion of perchlorate, as measured by HPLC MS. DAPI stained images of the *A.oryzae* pure culture are also shown in Figure 5 a).

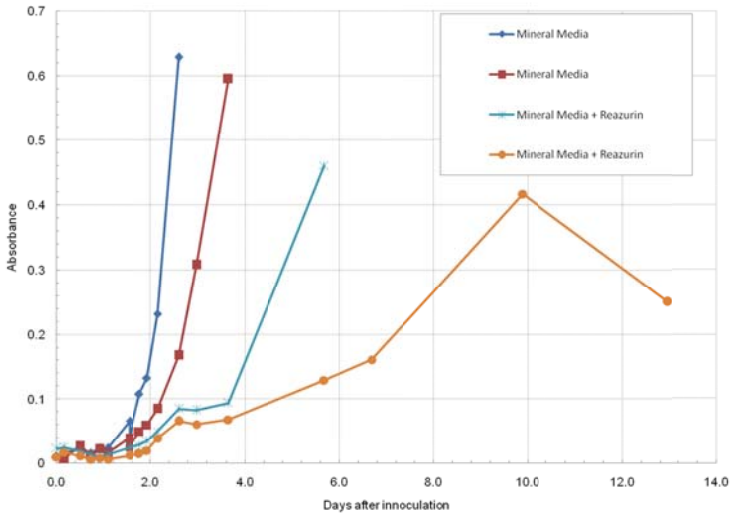
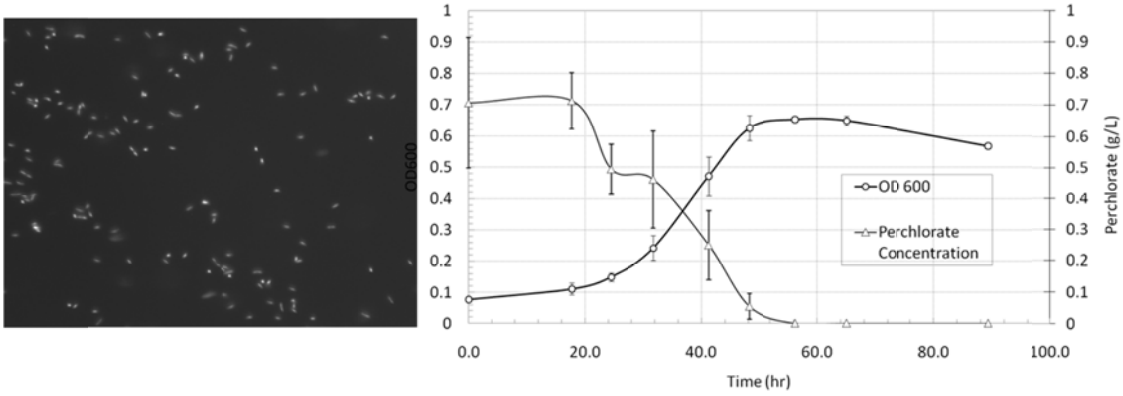


Figure 4 – Growth of *A. oryzae* in Mineral Media with and without Reazurin. *A. oryzae* grew better in the absence of resazurin.



a) b)

Figure 5 – a) DAPI Stained Image of *A. oryzae* Cells b) *A. oryzae* Growth Curve. As the cells grew, the perchlorate in the media was consumed, indicating that the cells were utilizing perchlorate as their terminal electron acceptor.

The growth curve in small batch cultures showed that the cells were growing using perchlorate, meaning they must be expressing perchlorate reductase and chlorite dismutase. Larger volumes (5L) of *A. oryzae* cells were grown to prepare sources of these enzymes. Cell pellets were collected when OD_{600} measurements were approximately 0.6, and cell-free extracts were prepared as described in the materials and methods. The activities of these cell extracts were measured using enzyme assays as described in the materials and methods.

Typical raw data results for a methyl viologen activity assay are shown in Figure 6. The baseline was recorded for 100 seconds and then perchlorate was injected into the cuvette to start the reaction (in Figure 6 note the sudden drop in absorbance around 100 seconds as the cuvette is removed to inject the perchlorate). The absorbance in the cuvette is then measured over time.

The slope of the decline can be used to calculate the activity per Equation 1 [56]. One unit is defined here as: 1 μmol MV^+ oxidized / min (note: reference [49] defines 1 U as 2 μmol MV^+ oxidized / min). Activities of these extracts were quite variable, ranging from 4 U/mL to 40 U/mL. The variability of the extract activities may have been due to differences in preparation method: slight differences in the initial oxygen concentrations in the media, the timing of pelleting in the growth curve, or freezing of the cells prior to making cell extract. The activity measured previously was 3.45 U/mL [49], which is slightly lower than the activities measured here. This is due to the greater dilution of extracts used in their preparation method. Overall, the extract activities and preparation methods reproduced in my work were similar to the work in [49]; these extracts were used for further encapsulation studies.

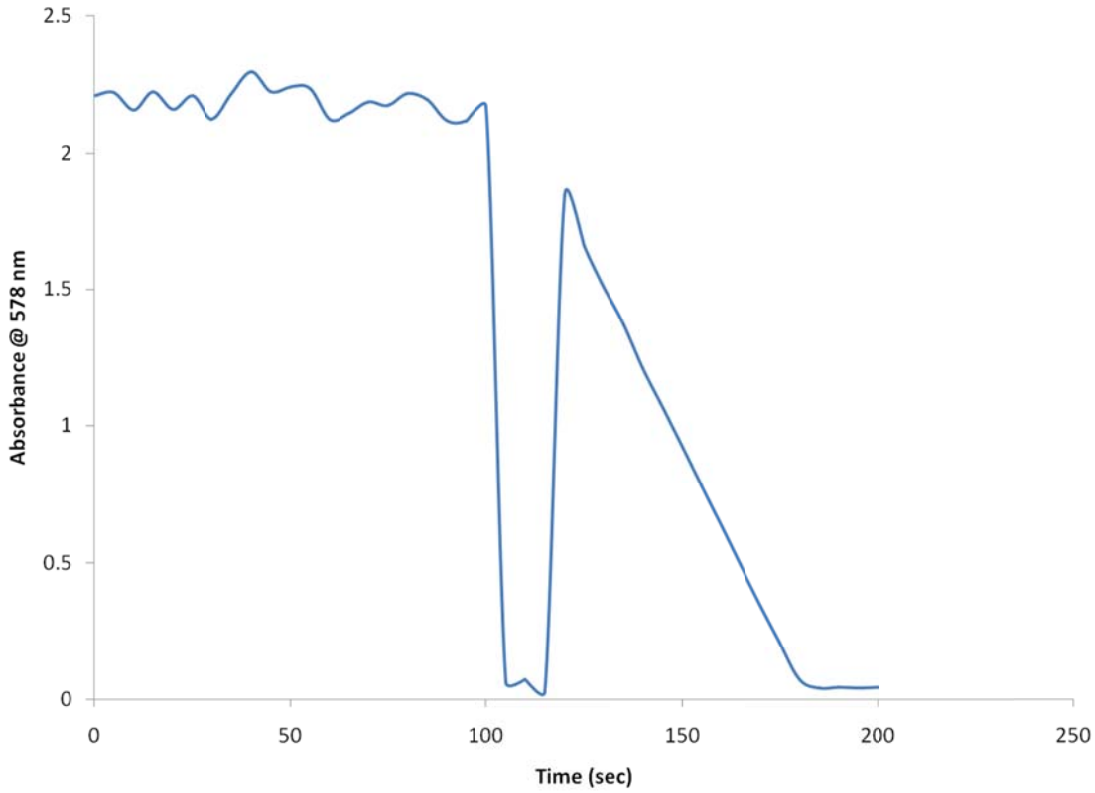


Figure 6 – Typical Perchlorate-Reducing Activity Assay. After a baseline of 100 seconds is recorded, perchlorate is injected into the sample and the reduction of perchlorate can be monitored by the decline in absorbance over time.

$$\boldsymbol{activity}\left(\frac{\boldsymbol{U}}{\boldsymbol{L}}\right) = \frac{\frac{\boldsymbol{AU}}{\boldsymbol{min}} \times \boldsymbol{TV}}{\boldsymbol{\varepsilon} \times \mathbf{b} \times \mathbf{SV}} \times \mathbf{1000}$$

Equation 1 – Activity of Extracts AU/min = slope from UV data, TV = Total volume of assay mixture, ε = micromolar absorptivity (liters per milimoles x centimeters, for methyl viologen 13.1 [57] or 9.7 [49] L/(mMol*cm)) SV = Sample Volume, b = path length (in centimeters). 13.1 L/(mMol*cm) was used as the absorptivity in my calculations.

To explore the use of another strain as a source of perchlorate reductase and chlorite dismutase the perchlorate reducing bacterium *Dechloromonas agitata* was cultivated anaerobically. The published media for *D. agitata* used chlorate as a substrate [48], whereas the media for *A. oryzae* uses perchlorate [39]. As perchlorate reductase is required to reduce

both perchlorate and chlorate, it will be expressed in the presence of either compound. A growth curve for the organism is shown in Figure 7. As with *A. oryzae*, as the optical density of the culture increased, the chlorate levels decreased, indicating that the cells were growing using chlorate and not oxygen as their electron acceptor. Growing larger volumes of this culture was tried but was unsuccessful, and thus only the *A. oryzae* cells were used as a source for perchlorate reductase and chlorite dismutase.

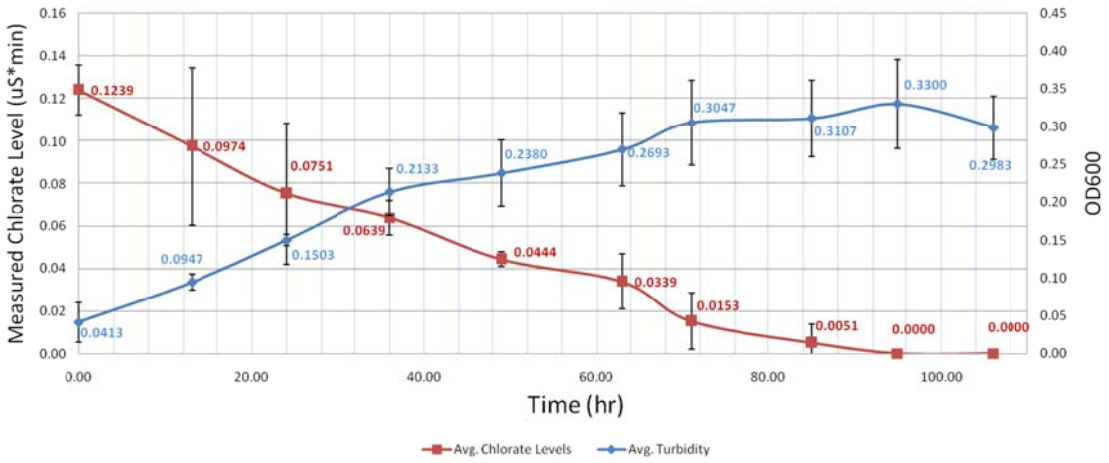


Figure 7 – *Dechloromonas agitata* Growth Curve. Similar to the *A. oryzae*, as the cells increased in number, the concentration of chlorate decreased. This indicated that *D. agitata* was using chlorate as its termnial electron acceptor.

Purification of OmpF:

The next component of the system to be purified was the OmpF pore. Outer membrane porins are associated with the pepitdoglycan layer in the cell wall. The peptiodoglycan layer is SDS-insoluble, whereas the majority of cellular proteins are soluble in SDS. Outer membrane porins are associated with the peptidoglycan layer and this difference in solubility is utilized to enrich for porins. In the procedure, broken cells are exposed to SDS to solubilize most of the cellular protein, and then the peptidoglycan layer (SDS-insoluble) can be pelleted away from the remaining cellular protein. The porins in the pellet can then be solubilized using the detergent Octyl-POE and the remaining peptidoglycan can be pelleted away from the now solubilized protein [14]. The strain I used, Omp8 [50], had the genes for its outer membrane porins (besides *ompF*) deleted, and thus the only major protein that could be solubilized from the peptidoglycan layer was OmpF.

Some difficulty was encountered when attempting to purify OmpF, as the cells must be well lysed before the addition of SDS. In my experience, sonication worked better than a

French press for this purpose. Additionally, the use of freshly prepared SDS solutions was important. After these issues were resolved, the purifications worked fairly consistently and yielded good amounts of protein. A gel from a representative purification is shown in Figure 8. After induction, a large band around 38 kDa developed, corresponding with the monomer of the OmpF protein. Purified OmpF is stable as a trimer in 2% SDS up to 70˚C and will run as a trimer when unheated [44], as can be seen on the gel. When heated at 95˚C for 5 minutes, the trimer form of the protein breaks down into a monomer form and the protein will run at approximately 38 kDa, as does the induction band in the induced cells. This trimer/monomer behavior helps to confirm that OmpF is indeed what is present in the purified fraction.

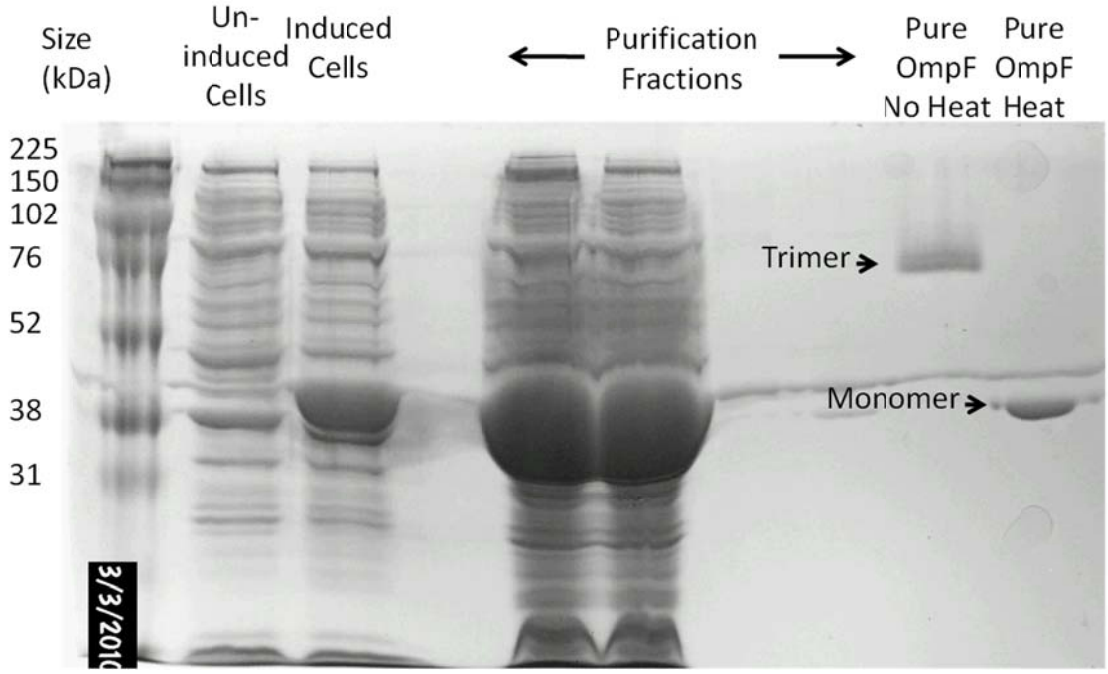


Figure 8 – Purification of OmpF. All samples besides the pure OmpF "no heat" lane and the 2nd and 4th purification fraction lanes were heated at 95°C for 5 min. The purification fractions are the supernatants from the first (lanes 5 and 6) and second (lanes 7 and 8) centrifugations of the procedure. As OmpF is stable in SDS, when unheated it will run as a trimer. When heated in breaks down into its monomer form and will run at approximately 38 kDa. This electrophoretic behavior confirms that OmpF was purified.

As an alternative method to purify OmpF, a Histidine-tagged OmpF vector was created as shown in Figure 9. At the beginning of this study, access to an ultracentrifuge was not available and the procedure used for the Omp8 strain would have been difficult. To circumvent this problem, we created a Histidine-tagged OmpF plasmid to purify OmpF using affinity chromatography. OmpF was amplified as described in the materials and methods and ligated into the NdeI and XhoI restriction sites of the pET 28b(+) vector. This plasmid was sequenced and shown to be free of any errors (data not shown). Pilot expressions using this vector in BL21 (DE3) cells showed good overexpression of OmpF, as can be observed in Figure 10. This OmpF is slightly larger than the OmpF derived from Omp8, as two histidine tags and a thrombin site are incorporated from the pET 28 b (+) vector.

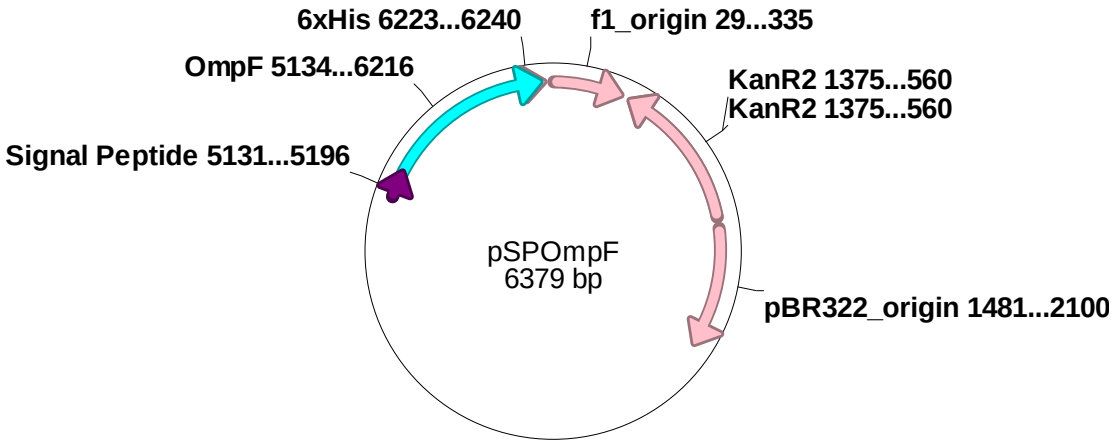


Figure 9 – Construction of Histidine-Tagged OmpF Plasmid. The OmpF gene was PCR amplified and ligated into the pET 28 b(+) vector.

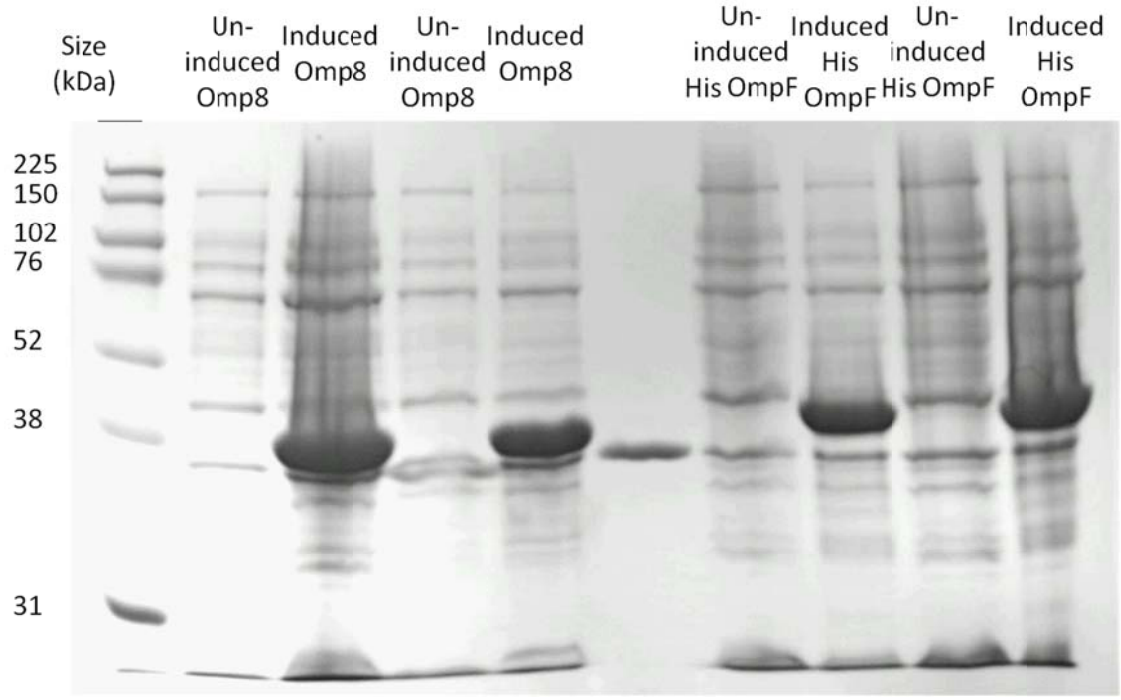


Figure 10 – Histidine-tagged OmpF Expression. The second and fourth lanes are uninduced samples of Omp8 cultures, and the third and fifth lanes are the corresponding induced cultures. The sixth lane is not relevant in this context. The seventh and ninth lanes are uninduced samples of BL21(DE3) pSPOmpF cultures, and the eighth and tenth lanes are the corresponding induced cultures. Expression of OmpF can be seen by the strong bands developing at approximately 38 kDa in both the Omp8 strain and the BL21(DE3) pSPOmpF post induction. As the sequence for OmpF in pSPOmpF contains two Histidine tags and a thrombin site, it runs slightly larger than the OmpF from the Omp8 strain.

As this plasmid was able to yield good overexpression of Histidine-tagged OmpF, we attempted to purify OmpF from the BL21 (DE3) pSPOmpF. The first steps of the purification were performed exactly as was done before with the Omp8 strain, i.e. the SDS properties of the peptioglycan layer were utilized to purify OmpF. The Omp8 strain has many outer membrane proteins deleted from its chromosomal DNA, thus when proteins are extracted from the outer membrane, only OmpF will be present. The BL21 (DE3) pSPOmpF strain does not have the same chromosomal deletions as the Omp8 strain, and thus we were expecting more contamination present when the protein was solubilized. We planned on utilizing an affinity tag purification after the SDS purification, but as this Histidine-tagged OmpF was rather pure after solubilization, this was not necessary. The purity of the

Histidine-tagged OmpF can be seen in Figure 11. The presence of a Histidine tag was confirmed by Western blotting (data not shown). As the Omp8 purification has been used for similar vesicle studies, we decided to use the OmpF derived from Omp8 rather than a new purification method for OmpF.

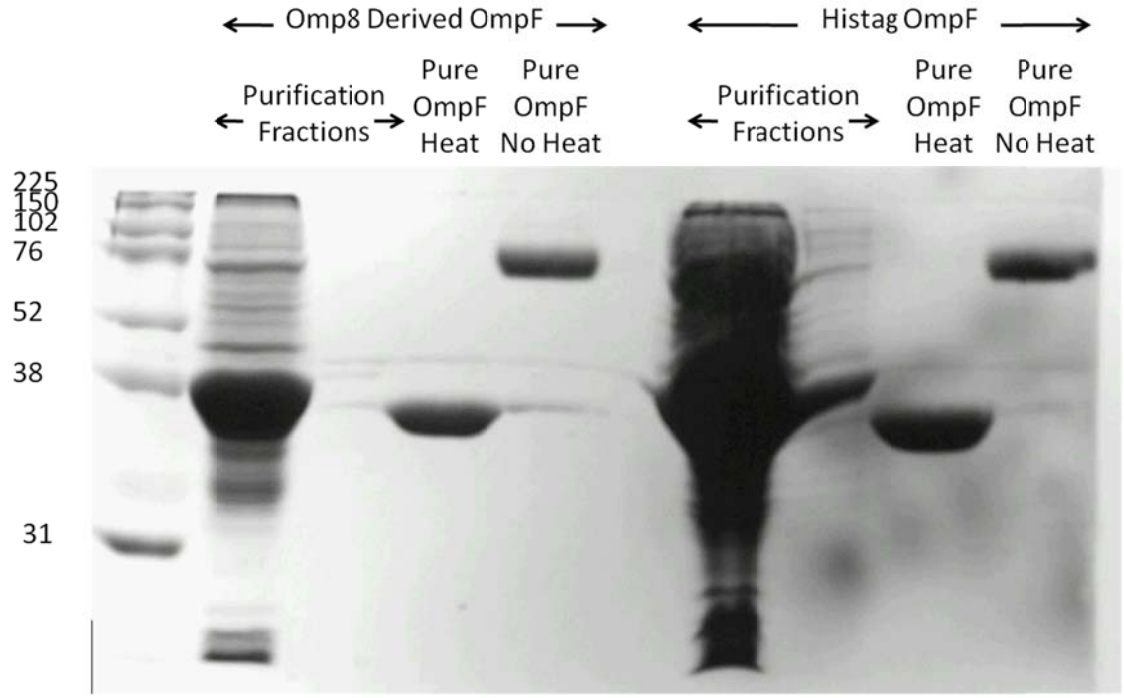
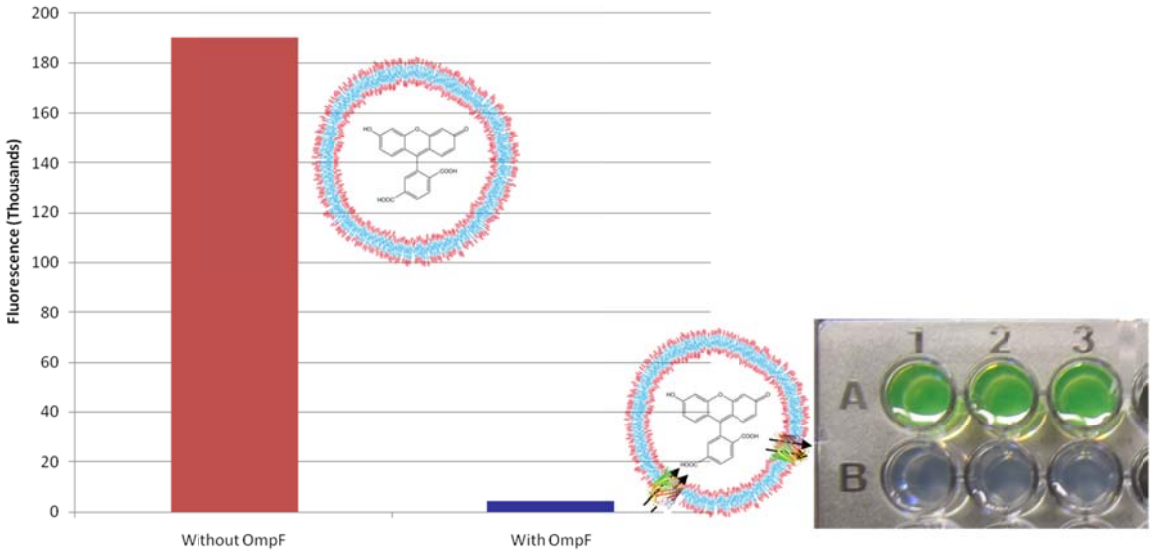


Figure 11 – Histidine-tagged OmpF Purification. Using a procedure similar to the Omp8 purification method, a Histidine-tagged OmpF was also purified from BL(21) DE3 pSPOmpF.

Transport of Small Solutes Through OmpF:

To measure if transport of small solutes (such as perchlorate and electron donor) was possible through reconstituted OmpF pore, fluorescent dye experiments were carried out as described in the materials and methods. These experiments were carried out using OmpF provided by our collaborators from the University of Basel, as I had not yet attempted the purification procedure myself. Vesicles with OmpF pores should allow molecules smaller than 600 daltons to diffuse out of the pore and into bulk solution. To test this hypothesis, two batches of vesicles were hydrated with 2.5 mM carboxyfluorescein, one batch with OmpF and one without. During the size exclusion separating vesicles from unencapsulated dye, the carboxyfluorescein was able to flow out of the OmpF vesicles, but it was retained by the vesicles without pores. Thus, the vesicles without OmpF had an almost 50 fold greater

fluorescence than then vesicles with OmpF (florescence of vesicles was measured in triplicate), as illustrated in Figure 12. The difference in the amount of encapsulated dye can even be seen visually in Figure 12 b). The vesicles without OmpF are in row 'A' and have a very distinct green color (from the dye), whereas the vesicles with OmpF simply look turbid (row 'B'), as the dye has flowed out through the pore leaving only the turbidity from the vesicle. Based on these results, the OmpF purified using the protocol in the materials and methods transports small solutes.



a) b)

Figure 12 – OmpF Transport Experiments, a) Fluorescence of Vesicle Batches b) Image of Vesicle Batches. The dye was retained by the vesicles without pores. Vesicles without pores thus had much greater fluorescence than vesicles with pores. The error bars are too small to be seen on the graph.

Initial Encapsulation Experiments and Problems with Aggregation in Cell-Free Extracts:

After the two principal components of the engineered vesicle were prepared, perchlorate-reducing enzymes and a transport protein, the next step was to assemble the components into a complete perchlorate-reducing vesicle. The formation of vesicles is a self-assembly process in which a polymer film is hydrated with solutions of the cell-free extract and membrane proteins. Over the course of a few days, polymer vesicles are formed, encapsulating soluble proteins, and the membrane proteins insert into the biomimetic polymer. The vesicles are small and only encapsulate a small portion of the total bulk solution. To separate the vesicles from the unencapsulated protein, a size exclusion column is

used. Large materials such as vesicles do not fit inside the pores of the size exclusion media, and thus go through the column quickly, in what is termed the void volume. Smaller materials like proteins can enter the pores in the media and will thus go through the size exclusion column much more slowly. In these experiments, two sets of vesicles are made, one with OmpF and one without. As methyl viologen cannot enter the no OmpF vesicles, they should not have detectable activity.

After incubation for a few days (the time needed to form vesicles), the cell-free extracts began to aggregate into larger material. The effect of this can be observed in Figure 13. The "fresh" cell-free extract (run on a column immediately after ultracentrifugation) has only a single peak with a shoulder, whereas the cell extract after storage has an additional, earlier peak, representing aggregates that have formed and come off the column in the void volume. This presented a problem for the purification of perchlorate-reducing engineered vesicles, as these aggregates could not be resolved from the polymer vesicles using size exclusion. The presence of these aggregates created the potential for enzymatic activity in the vesicle fractions from sources other than the vesicles. For example, in an encapsulation experiment on 5/6/2010, 7.6 U/L of activity was detected in vesicles without OmpF, similar to the amount of activity detected in the OmpF vesicles (15.69 U/L). The source of this contaminating activity was thought to be the aggregates. Subsequent experiments, detailed below, focused on eliminating these aggregates.

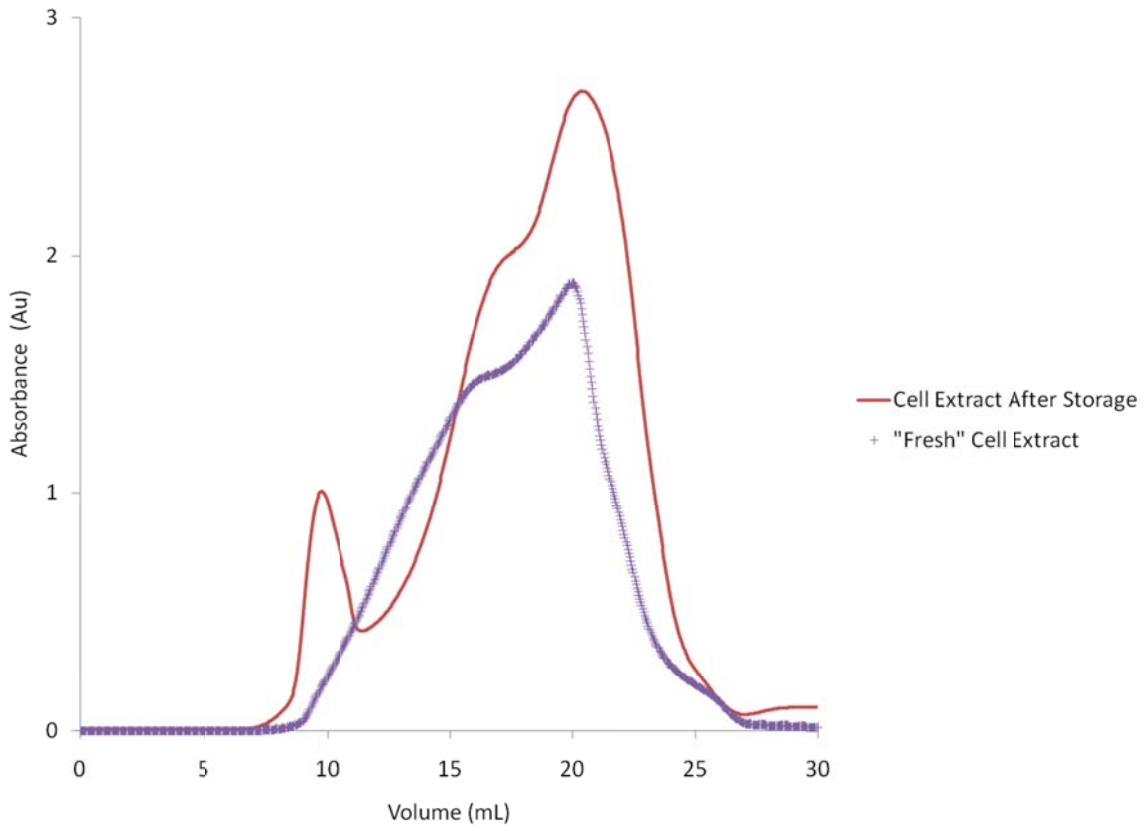


Figure 13 – Cell Extract Size Exclusion after Storage. After time, materials in the cell extract aggregate into larger material. This larger material comes off the size exclusion column in the void volume, making it difficult to resolve this material from vesicles.

In previous experiments, before I was aware of the aggregate problem, I had treated vesicles with proteinase K. This treatment seemed to greatly reduce the turbidity of the samples, possibly because it was breaking up the aggregated material. Proteinase K digestion was therefore attempted after extrusion and immediately prior to size exclusion to break up the aggregates and reduce them to the size range expected for proteins in the cell extract. Thus, the contamination of the aggregates in the void volume should be removed when performing size exclusion after digestion. As illustrated in Figure 14, proteinase K treatment removed the void volume peak present in the stored cell extract. This allowed the vesicles in the void volume to be separated from the unencapsulated protein.

To confirm that the protease was indeed breaking up the aggregates present in the void volume, vesicles contaminated with aggregates were split into two fractions, one treated with protease and one not treated with protease. These samples were then size excluded again and characterized using TEM. Images of the characterization can be observed in Figure 15. In

a), the sample of the non-treated samples, polymer vesicles can indeed be observed, but irregularly shaped contamination is also present. Figure 15b) is representative of the protease treated samples, where predominantly polymer vesicles could be observed. However, some limited amount of aggregate contamination could still be observed in other parts of the grid. Overall, the proteinase K treatment seemed to address the problems of aggregate contamination in the vesicles.

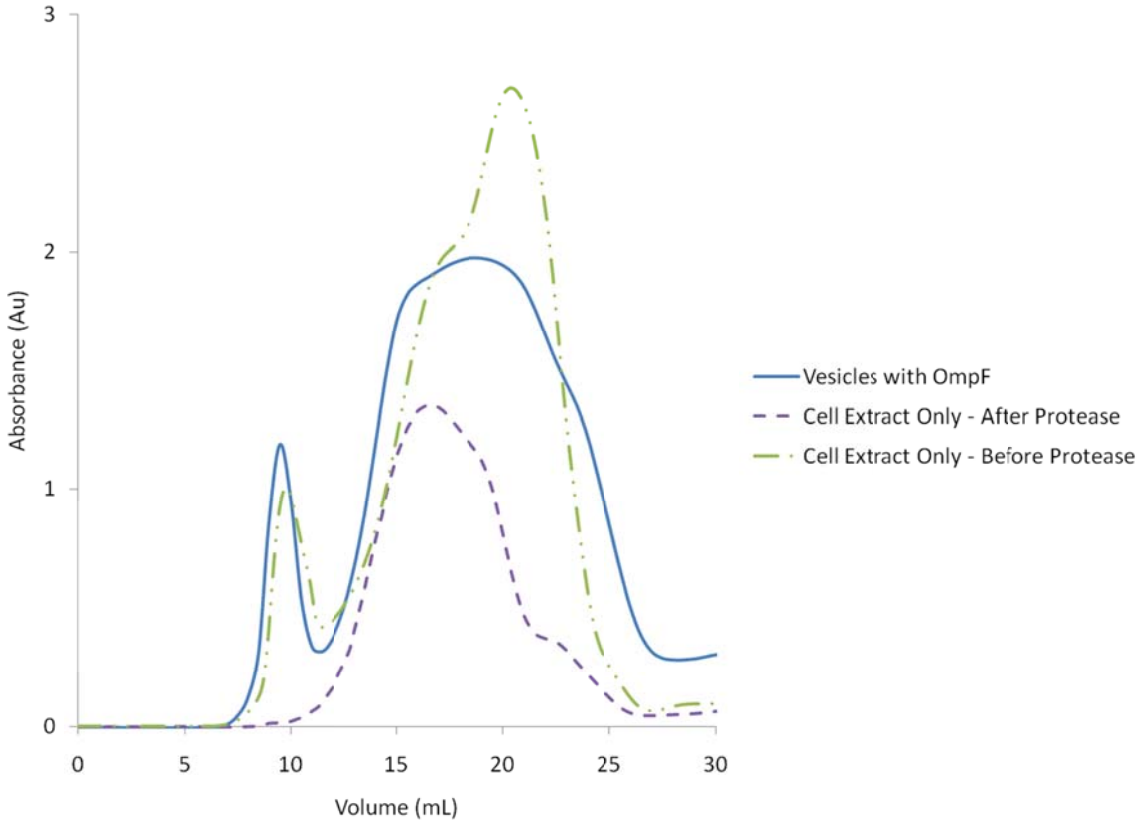
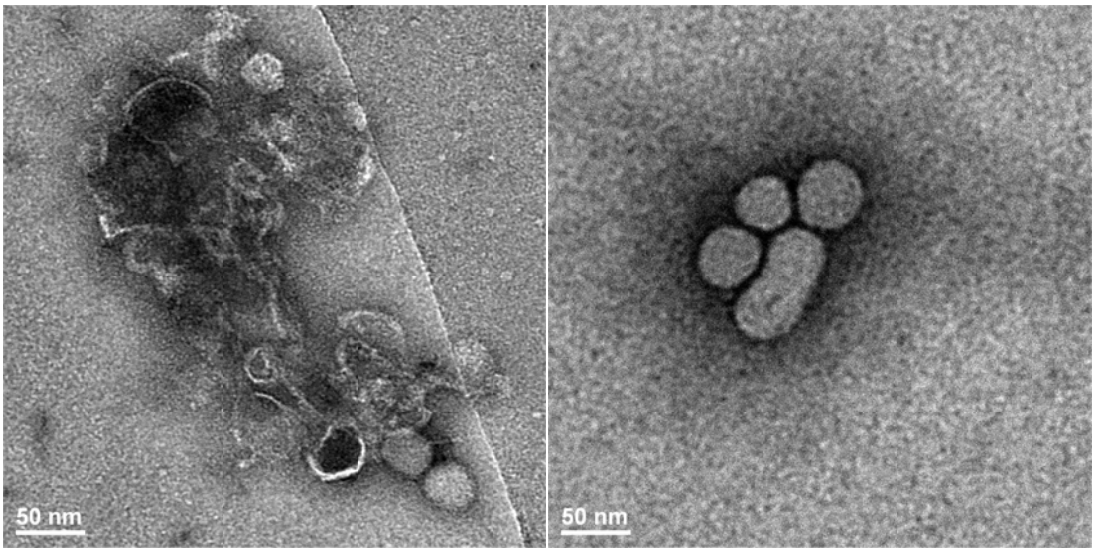


Figure 14 – Effect of Protease Treatment when Purifying Vesicles. The protease treatment breaks up the material in the cell extract above the exclusion limit. This allows for vesicles to be separated from unencapsulated protein.



a) b)

Figure 15 – TEM Images of Polymer Vesicles a) Void Volume Fraction without Protease Treatment b) Void Volume Fraction with Protease Treatment. The absence of the contaminating material in the protease treated sample suggests that the protease treatment is removing the aggregated material.

Encapsulating Perchlorate-Reducing Cell-Free Extracts:

Although this technique seemed to be successful in removing the contaminating protein aggregates, the activities of the perchlorate-reducing vesicles creating using this method were much lower than those in cell-free extracts and initially not easily distinguishable from the negative control. Raw data from initial activity assays are shown in Figure 16. A vesicle with no OmpF was the negative control in these experiments. These vesicles should not change in absorbance over time, as no methyl viologen should be able to diffuse into the core of the vesicles to allow the reaction to proceed. However, over the same time period required to observe a change in absorbance for the OmpF vesicles (indicating the catalysis of a perchlorate-reducing reaction), a noticeable decline in absorbance in the negative control (no OmpF) was observed, perhaps due to oxygen leaking into the cuvette. The vesicles with OmpF did seem to have a greater decline in absorbance over time than the negative control, but the difference was small enough that it is difficult to say if what was measured was just due to artifacts in the data (i.e. oxygen leakage), or if a real difference in signal was observed.

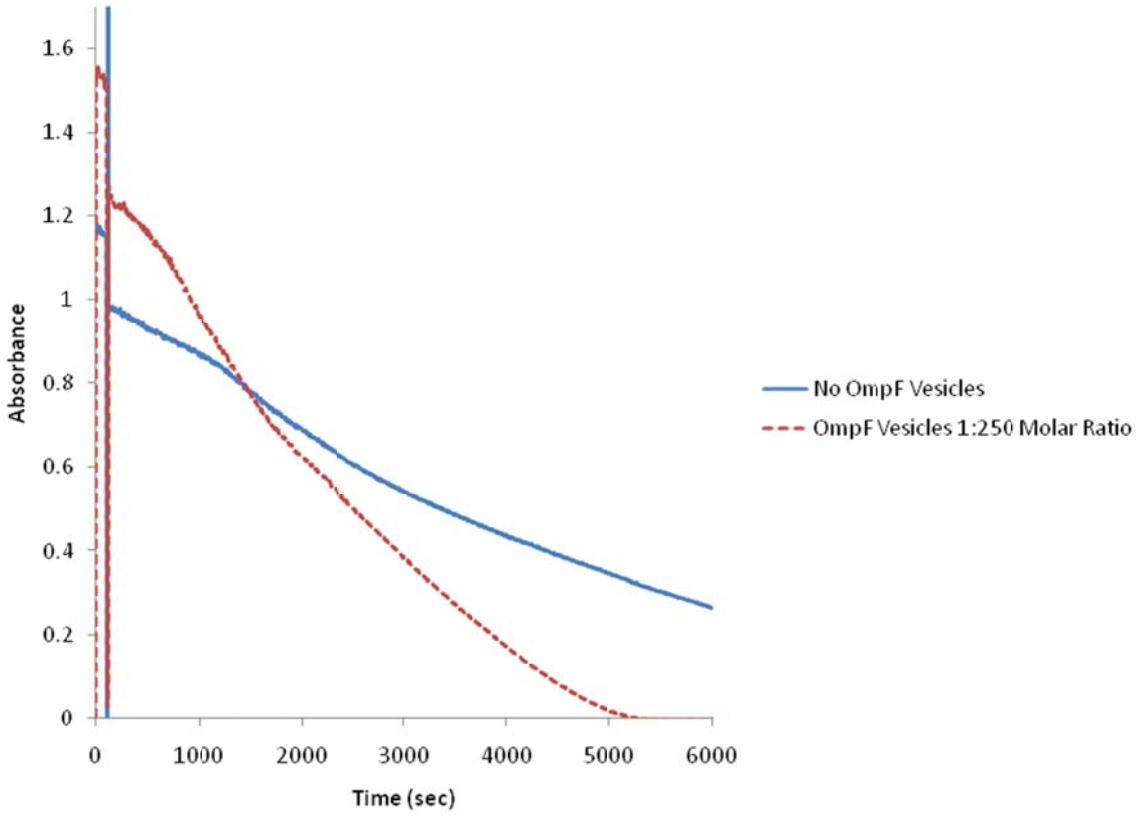


Figure 16 – Activity Assays of Vesicles Formed Using the Proteinase K Treatment Method.

To eliminate these artifacts, additional experiments were performed to obtain a high degree of anerobicity in the cuvettes. This is vital to getting good results with the methyl viologen assay, as oxygen will react with reduced methyl viologen much faster than with perchlorate [57]. If oxygen leaks into the cuvette, a stable baseline will be hard to obtain and measured activities will be artificially high. Several attempts to obtain anaerobic conditions were not successful, such as placing mineral oil on top of the assay mixture in a standard cuvette, and flowing nitrogen above the cuvette to purge the surroundings of oxygen. To achieve a good degree of anaerobicity, the assay mixture was pipetted into an anaerobic cuvette in an anaerobic glove box and then covered and capped with a septum. This sealed in an environment without oxygen and sealed out atmospheric oxygen. This resulted in initial stable baseline readings for the aerobic environment in Figure 17. This approach was sufficient when measuring activities of cell extracts, however the lower activities of the vesicles required a longer time frame. Over longer periods of time, oxygen leakage into the sealed cuvette could become a major problem in aerobic environments as can be seen in Figure 17. To get an even lower baseline, the spec was moved into a glove box and

measurements could be taken while the cuvette was kept in an anaerobic glove box. This resolved the issue and resulted in a stable baseline over two to three hours, as can be seen in Figure 17.

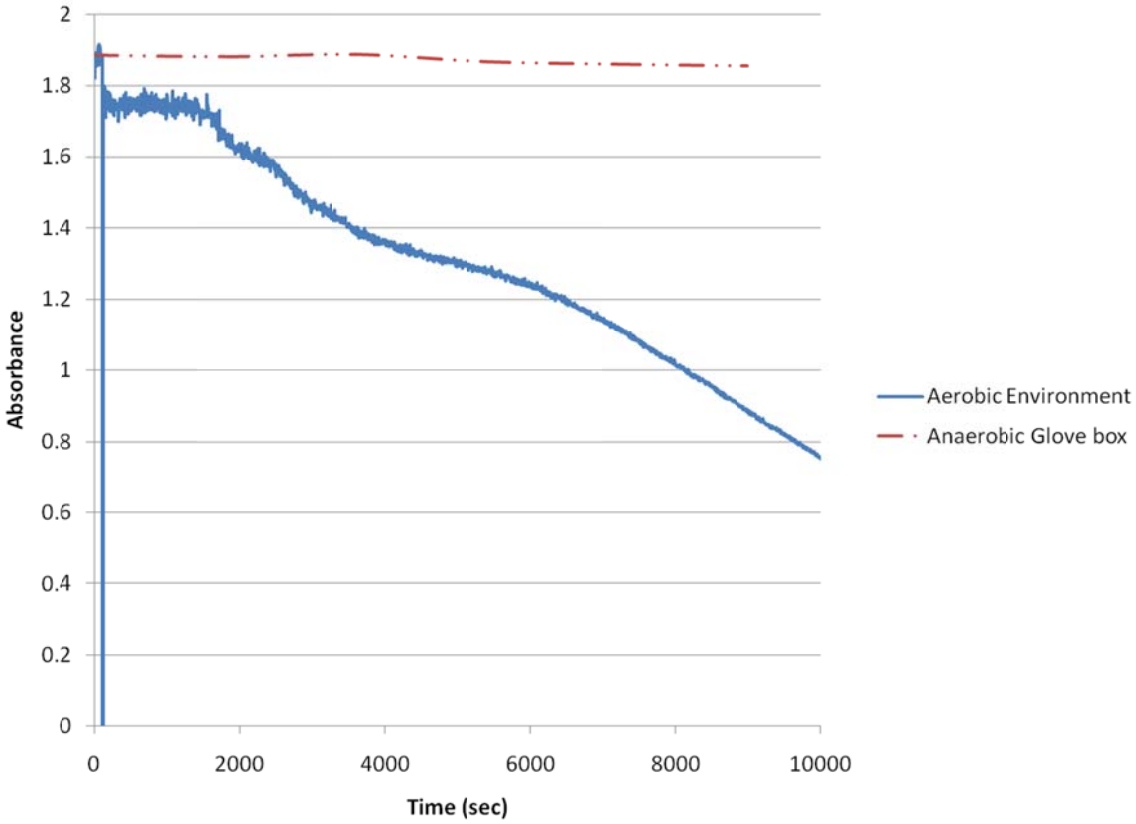
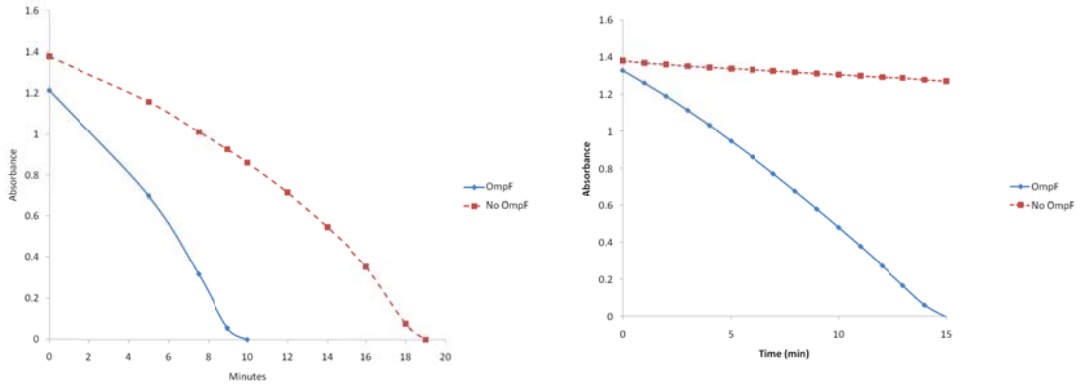


Figure 17 – Comparison of Aerobic and Anaerobic Methyl Viologen Assays. When the spec was placed inside an anaerobic glove box, the change in absorbance due to oxygen leakage was greatly reduced, leading to a more accuarate assay.

Activity assays for subsequent batches of vesicles were performed in an anaerobic glove box. This helped alleviate the ambiguity about the source of the measured activity, as the oxygen leakage was not an issue (Figure 17). Raw data for these experiments is shown in Figure 18 and summarized in Table 3. There is detectable activity in all of the samples and the OmpF vesicles consistently have more activity than the no OmpF vesicles. The only confounding factor is the occasional high activity of the no OmpF vesicles. As the cell-free extract is a very poorly defined source of protein, it is possible that some outer membrane porins could remain in some batches of the cell-free extract. These porins could then incorporate into the membrane, making it permeable to methyl viologen and thus able to

reduce perchlorate. Nevertheless, the data show that the vesicles have perchlorate-reducing activity.

The hydration mix had a perchlorate reductase concentration of about 1 μM and the activity the day of extrusion was usually between 6 U/mL to 13 U/mL (Table 3). Other encapsulation work has used an enzyme concentration of approximately 10 μM and a corresponding activity of 30-110 U/mL [14]. The encapsulated enzymes and the assays are different, but the previous work gives an idea of the bulk concentration needed to encapsulate protein. Despite the enzyme concentration being lower than previous work, I was able to encapsulate protein and detect activity in the vesicles. The variable activity in the no OmpF sample remains somewhat puzzling. Signal due to contaminating aggregates and oxygen leakage have been removed. However, as the cell-extract is a poorly defined system, outer membrane proteins could remain in the extract and incorporate into the membrane, causing the activity in the no OmpF vesicles. As an alternative negative control, the pH could be changed to close the OmpF pore (or by closing the pore in a different manner) and then assaying activity.



a) b)

Figure 18 – Encapsulation Experiments Performed in Anaerobic Glove Box a) 7/7/10 b) 7/10/10. These experiments were performed in an anaerobic glove box and thus the background decline due to oxygen leakage is greatly reduced.

Table 3 – Successful Encapsulation Experiments

Batch	Polymer (mg)	Molar Ratio OmpF:Polymer	Activity in Hydration Mix (U/L)	Specific Activity in Hydration Mix (U/mg)	Activity Encapsulated in Vesicles (U/L)
7/7/2010 no OmpF	12	NA	9896.1	0.71	24.3
7/7/2010 OmpF	12	250	13770.9	0.79	44.8
7/10/2010 no OmpF	12	NA	9884.4	0.79	2.8
7/10/2010 OmpF	12	250	9884.4	0.79	35.2

Digestion of Cell-free Extract With Proteinase K:

One control performed in previous encapsulation work is digestion with a protease to confirm that the vesicle is protecting the encapsulated enzymes [10]. The OmpF pore is resistant to proteases and is small enough to prevent the protease from entering the core of the vesicle, thus protecting the enzymes inside. Proteins outside the vesicle however, should be digested by the protease. Activity of polymer vesicles is measured before and after protease treatment and if activity is maintained, the proteins are considered protected by the vesicle [10]. I attempted similar experiments, but even without encapsulation in vesicles the perchlorate-reducing activity in the extracts was resistant to proteinase K. As shown in Figure 19, there was little to no difference in the protease-treated samples and non-treated samples. This may be because there is too much protein in the cell extract for the protease to digest, or because perchlorate reductase and chlorite dismutase proteins are somehow resistant to digestion. In any case, some other method or protease will have to be used to perform this type of control experiment.

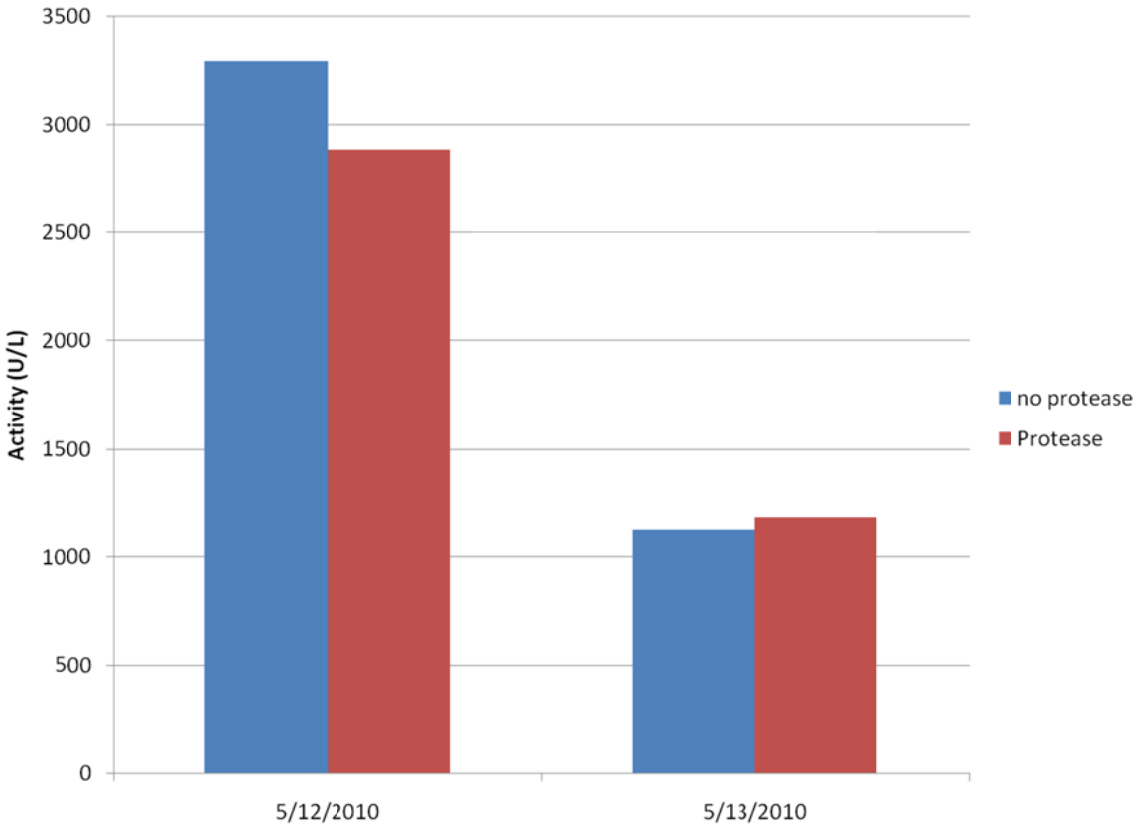
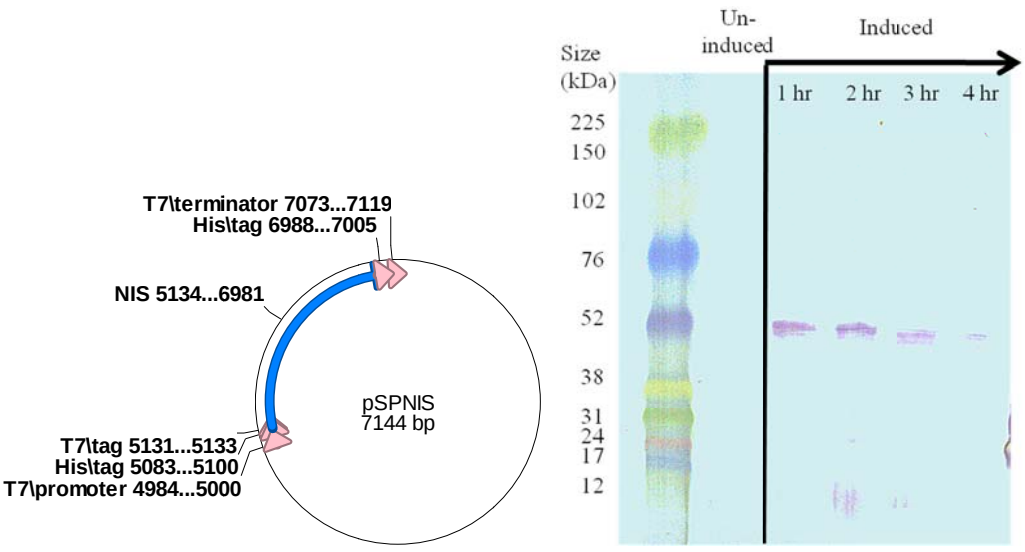


Figure 19 – Activities of Cell-Free Extract After Exposure to Proteinase K.

Cloning and Expressing the Sodium Iodide Symporter:

The sodium iodide symporter (NIS) is a transport protein that normally transports iodide, but it will also transport and concentrate perchlorate. As explained in the introduction, this active transporter could be used instead of the passive OmpF pore to concentrate perchlorate inside the vesicles. The first steps in cloning and expressing this gene have been completed. The NIS gene was PCR amplified, and inserted into the NdeI and XhoI sites of the pET 28b(+) vector. The insert was sequenced and shown to be free of errors (data not shown). A diagram of the plasmid is shown in Figure 20 a. Additionally, this plasmid was transformed into BL21 (DE3) as described [58] and a pilot induction was performed. The subsequent work was done by Emily Bozek under my direction. Using an anti-His antibody, a Western blot revealed the presence of a Histidine-tagged protein at around 50 kDa post induction (at 37°C), where the non-glycosolated form of NIS would be expected to run, as shown in Figure 20 b) [58]. This result was difficult to replicate and so we tried growing and inducing cells at 20°C. At present, we are able to express NIS at 20°C and visualize it using an anti-His antibody, and we are working on purifying the protein.



a) b)

Figure 20 – NIS Plasmid Construction and Expression a) pSPNIS Vector Diagram b) Western Blot of Pilot Induction.

CONCLUSIONS AND FUTURE WORK

The goal of my work was to create an engineered vesicle capable of reducing perchlorate, and this goal was met. I successfully prepared the components of the system: a membrane transport protein (OmpF) and perchlorate-reducing cell extracts. In preparation for using a specific perchlorate transporter, we have also achieved heterologous expression of the NIS protein. There were several issues with the procedure used to make and assay vesicles, such as aggregate contamination and oxygen leakage, but those issues have been addressed. At present, vesicle activity can be consistently detected using the procedures developed in my work, showing the creation of engineered vesicles capable of reducing perchlorate.

Now that these vesicles have been created, one may look towards the application of these vesicles. To be useable in a reactor, these vesicles will need to be immobilized onto a substrate. These vesicles are in the size range of a few hundred nanometers, a size that would be difficult to contain in a suspended-growth reactor. However, there is some precedent, as engineered vesicles have been immobilized on a glass substrate before [14]. Vesicles immobilized on spherical media could be packed into a flow through reactor for utilization as a pilot or full-scale reactor. One proposed reactor setup is illustrated in Figure 21. Vesicles could be immobilized onto beads at very high densities. These beads could then be packed into a flow-through reactor. As these vesicles would only contain the enzymes relevant to perchlorate degradation, the enzymes could be packed at much higher densities that would be possible in a conventional bioreactor. In addition, these reactors could utilize enzymes in conditions where they would not be expressed by bacteria and these reactors could safely incorporate genetically-engineered proteins.

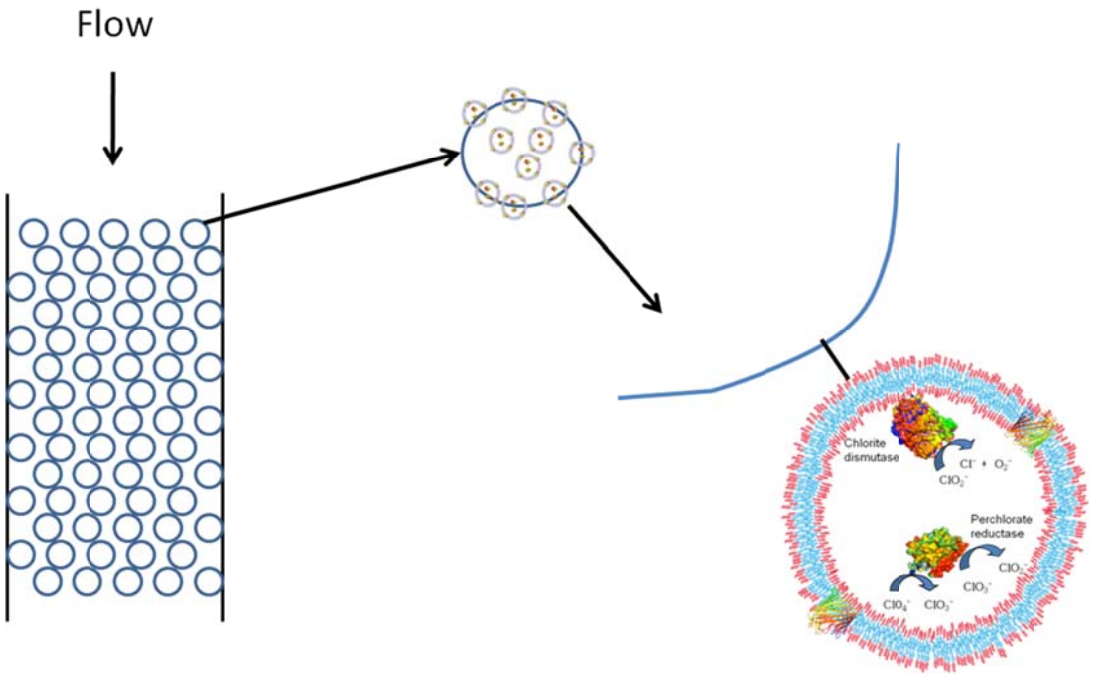


Figure 21 – Conceptual Model for a Fixed-bed Reactor with Immobilized Engineered Vesicles.

Figure 21 shows a means of applying my work to reactors. However, before reactors become a reality, these vesicles must be improved. A few possible methods to improve the vesicles are detailed here: encapsulation with protease, recombinant purification, encapsulating other types of catalysts, other sources of reducing power, and expanding to other contaminants.

Others have proposed applications for perchlorate-reducing enzymes derived from cell-free extract: use in sensors [59], or immobilized in alginate beads to create a bioreactor [60]. One possible advantage of vesicles is that the relevant enzymes could be encapsulated with a protease inhibitor to prevent proteolysis. Other cell-free extract systems such as hydrogels do not have this potential. One candidate might be alpha2-macroglobulin, a 720 kDa protein that acts as a broad spectrum protease inhibitor. This inhibitor is large enough to be contained by the OmpF pore, unlike a smaller protease inhibitor like PMSF. However, as the work with proteinase K showed some resistance of these enzymes to proteolysis, perhaps other processes cause the decrease in activity over time observed in the cell-free extract. If so, other additives could be encapsulated with the proteins to slow these processes as well.

To increase concentrations of the relevant enzymes, one could encapsulate purified proteins. Instead of using a crude source of the protein like cell-free extract, which has

extraneous material, one could tag these proteins and purify them using affinity chromatography. The yields using this method would be significantly increased as well. Chlorite dismutase has been expressed and purified recombinantly in the past, and work is ongoing to replicate this in our laboratory [37]. Purification of perchlorate reductase with similar methods has been attempted, but unsuccessfully. This is a very large protein with multiple subunits, and thus may be difficult to heterologously express [43]. If the perchlorate reductase cannot be expressed and purified heterologously, purification from its native host can be performed [49]. Combined with the heterologously purified chlorite dismutase, pure sources of these two enzymes could provide much higher enzyme concentrations and a better defined system than the cell-free extract utilized in this work.

Perchlorate reductase could also be coupled to a non-protein catalyst encapsulated in these vesicles. Chlorite dismutase is part of the superoxide dismutase family and research is being conducted to find catalysts that perform the same function as the enzymes in this family [61]. Chlorite dismutase has a limited number of turnovers before the enzyme ceases to function [37]. Thus, this system could couple protected perchlorate reductase and an artificial chlorite dismutase catalyst to create a novel enzyme/catalyst system.

Furthermore, this system could remove perchlorate using different sources of reducing power. Our study used methyl viologen as an electron donor due to its use in the literature for perchlorate kinetic assays, but other donors could be used. This could allow for the use of a cathode to act as a primary electron donor, reducing an electron shuttle that powers perchlorate reduction and obviating the need for large amounts of exogenous electron donor, as required in traditional biological perchlorate treatment. This has been attempted using a perchlorate bioreactor [62]. The addition of excess exogenous donor and the resulting regrowth potential in the distribution system is one issue that must be addressed in current perchlorate bioreactor designs. If a cathode is used as a donor, no residual exogenous donor remains, removing the risk of regrowth [62].

Using different enzymes, engineered vesicles could catalyze the removal of other contaminants. As a generalized system, one simply needs a source of enzyme, polymer, and membrane protein to make these vesicles. Technical hurdles must be overcome for sources of these materials to be commercially viable. However, engineered vesicle reactors have a number of advantages, such as the ability to utilize enzymes in conditions where they would not be expressed by bacteria and the ability to utilize genetically engineered proteins. As sources of polymer and recombinant enzymes become available, large-scale vesicle reactors could become a reality. My work has prepared the components of engineered vesicle reactors

and shown them to be functional. These components have been assembled in polymer vesicles and are active. These prototype vesicles can be used for the future development of perchlorate-reducing vesicle reactors.

REFERENCES

1. Vidali M (2001) Bioremediation. An overview. Pure and Applied Chemistry 73 (7):1163-1172.

2. Brown JC, Anderson RD, Min JH, Boulos L, Prasifka D, Juby GJG (2005) Fixed bed biological treatment of perchlorate-contaminated drinking water. Journal American Water Works Association 97 (9):70-81.

3. US Government Printing Office (2010) Code of Federal Regulations - Title 40 - Part 503 - Standards for the use or disposal of sewage sludge.

4. Rittmann BE, McCarty P (2001) Environmental Biotechnology: Principles and Applications. McGraw Hill, Columbus, OH.

5. Bosma T, Damborsky J, Stucki G, Janssen DB (2002) Biodegradation of 1,2,3-trichloropropane through directed evolution and heterologous expression of a haloalkane dehalogenase gene. Applied and Environmental Microbiology 68 (7):3582-3587.

6. Bosma T, Kruizinga E, de Bruin EJ, Poelarends GJ, Janssen DB (1999) Utilization of Trihalogenated Propanes by Agrobacterium radiobacter AD1 through Heterologous Expression of the Haloalkane Dehalogenase from *Rhodococcus* sp. Strain m15-3. Applied and Environmental Microbiology 65 (10):4575-4581.

7. Drobník J (1999) Genetically modified organisms (GMO) in bioremediation and legislation. International Biodeterioration & Biodegredation 44 (1):3-6.

8. Winterhalter M, Hilty C, Bezrukov SM, Nardin C, Meier W, Fournier D (2001) Controlling membrane permeability with bacterial porins: application to encapsulated enzymes. Talanta 55 (5):965-971.

9. Nardin C, Thoeni S, Widmer J, Winterhalter M, Meier W (2000) Nanoreactors based on (polymerized) ABA-triblock copolymer vesicles. Chemical Communications (15):1433-1434.

10. Axthelm F, Casse O, Koppenol WH, Nauser T, Meier W, Palivan CG (2008) Antioxidant Nanoreactor Based on Superoxide Dismutase Encapsulated in Superoxide-Permeable Vesicles. The Journal of Physical Chemistry B 112 (28):8211-8217.

11. Duncan R (2003) The dawning era of polymer therapeutics. Nature Reviews Drug Discovery 2 (5):347-360.

12. Banerjee R (2001) Liposomes: Applications in Medicine. Journal Biomaterials Applications 16 (1):3-21.

13. Kumar M, Grzelakowski M, Zilles J, Clark M, Meier W (2007) Highly permeable polymeric membranes based on the incorporation of the functional water channel protein Aquaporin Z. Proceedings of the National Academy of Sciences 104 (52):20719-20724.

14. Grzelakowski M, Onaca O, Rigler P, Kumar M, Meier W (2009) Immobilized Protein-Polymer Nanoreactors. Small 5 (22):2545-2548.

15. Nardin C, Hirt T, Leukel Jr, Meier W (1999) Polymerized ABA Triblock Copolymer Vesicles. Langmuir 16 (3):1035-1041.

16. Choi H-J, Montemagno CD (2005) Artificial Organelle: ATP Synthesis from Cellular Mimetic Polymersomes. Nano Letters 5 (12):2538-2542.

17. Stoenescu R, Graff A, Meier W (2004) Asymmetric ABC-triblock copolymer membranes induce a directed insertion of membrane proteins. Macromolecular Bioscience 4 (10):930-935.

18. Mecke A (2006) Biomimetic membranes designed from amphiphilic block copolymers. Soft matter 2 (9):751.

19. Government Accountablity Office (2005) Perchlorate: A System to Track Sampling and Cleanup Results Is Needed.

20. Environmental Protection Agency (2002) Perchlorate Environmental Contamination: Toxicological Review and Risk Characterization.

21. Greer M, Goodman G, Pleus R, Greer S (2002) Health effects assessment for environmental perchlorate contamination: the dose response for inhibition of thyroidal radioiodine uptake in humans. Environmental Health Perspectives 110 (9):927-937.

22. National Academy of Science (2005) Health Implications of Perchlorate Ingestion.

23. Environmental Protection Agency (2009) Drinking Water: Perchlorate Supplemental Request for Comments.

24. Environmental Protection Agency (2006) Unregulated Contaminant Monitoring Regulation Occurrence Data.

25. Coates JD, Achenbach LA (2004) Microbial perchlorate reduction: Rocket-fuelled metabolism. Nature Reviews Microbiology 2 (7):569-580.

26. Motzer WE (2001) Perchlorate: problems, detection, and solutions. Environmental forensics 2 (4):301.

27. Environmental Protection Agency (2008) Drinking Water: Preliminary Regulatory Determination on Perchlorate.

28. Environmental Protection Agency (2009) EPA Seeks Advice on Perchlorate in Drinking Water - Agency Issues Interim Health Advisory.

29. Environmental Protection Agency (2009) EPA Seeks Comments on its Reevaluation of the Chemical Perchlorate.

30. Tripp AR, Clifford DA (2006) Ion exchange for the remediation of perchlorate-contaminated drinking water. Journal American Water Works Association 98 (4):105-114.

31. Amy G (2004) Treatability Of Perchlorate-containing Water By RO, NF And UF Membranes. IWA Publishing, London.

32. Roquebert V, Booth S, Cushing RS, Crozes G, Hansen E (2000) Electrodialysis reversal (EDR) and ion exchange as polishing treatment for perchlorate treatment. Desalination 131 (1-3):285-291.

33. Na C (2003) Perchlorate removal via iron-preloaded GAC and borohydride regeneration. Journal American Water Works Association 95 (1):8-8.

34. Environmental Protection Agency (2005) Perchlorate Treatment Technology Update.

35. Carollo Engineers (2010) Development of an Innovative Oxidant-Laden Residuals Treatment Process.

36. Kengen SWM, Rikken GB, Hagen WR, van Ginkel CG, Stams AJM (1999) Purification and characterization of (per)chlorate reductase from the chlorate-respiring strain GR-1. Journal of Bacteriology 181 (21):6706-6711.

37. Streit BR, DuBois JL (2008) Chemical and steady-state kinetic analyses of a heterologously expressed heme dependent chlorite dismutase. Biochemistry 47 (19):5271-5280.

38. O'Connor SM, Coates JD (2002) Universal Immunoprobe for (Per)Chlorate-Reducing Bacteria. Applied and Environmental Microbiology 68 (6):3108-3113.

39. Rikken GB, Kroon AGM, van Ginkel CG (1996) Transformation of (per)chlorate into chloride by a newly isolated bacterium: reduction and dismutation. Applied Microbiology and Biotechnology 45 (3):420-426.

40. Chaudhuri SK, O'Connor SM, Gustavson RL, Achenbach LA, Coates JD (2002) Environmental Factors That Control Microbial Perchlorate Reduction. Applied and Environmental Microbiology 68 (9):4425-4430.

41. Bender KS, Shang C, Chakraborty R, Belchik SM, Coates JD, Achenbach LA (2005) Identification, characterization, and classification of genes encoding perchlorate reductase. Journal of Bacteriology 187 (15):5090-5096.

42. Xu J, Trimble JJ, Steinberg L, Logan BE (2004) Chlorate and nitrate reduction pathways are separately induced in the perchlorate-respiring bacterium *Dechlorosoma* sp. KJ and the chlorate-respiring bacterium *Pseudomonas* sp. PDA. Water Research 38 (3):673-680.

43. Sexton J (2007) Subcloning and partial purification of perchlorate reductase from *Dechlormonas aromatica* RCB. Thesis. Master of Science in Chemistry. University of Notre Dame.

44. Jap BK, Walian PJ (1996) Structure and functional mechanism of porins. Physiological Reviews 76 (4):1073-1088.

45. Tonacchera M, Pinchera A, Dimida A, Ferrarini E, Agretti P, Vitti P, Santini F, Crump K, Gibbs J (2004) Relative Potencies and Additivity of Perchlorate, Thiocyanate, Nitrate, and Iodide on the Inhibition of Radioactive Iodide Uptake by the Human Sodium Iodide Symporter. Thyroid 14 (12):1012-1019.

46. Dohan O, Portulano C, Basquin Cc, Reyna-Neyra A, Amzel LM, Carrasco N (2007) The Na+/I- symporter (NIS) mediates electroneutral active transport of the environmental pollutant perchlorate. Proceedings of the National Academy of Sciences 104 (51):20250-20255.

47. Nardin C, Hirt T, Leukel Jr, Meier W (2000) Polymerized ABA Triblock Copolymer Vesicles. Langmuir 16 (3):1035-1041.

48. Bruce RA (1999) Reduction of (per) chlorate by a novel organism isolated from paper mill waste. Environmental microbiology 1 (4):319.

49. Kengen SWM, Rikken GB, Hagen WR, van Ginkel CG, Stams AJM (1999) Purification and Characterization of (Per)Chlorate Reductase from the Chlorate-Respiring Strain GR-1. J Bacteriol 181 (21):6706-6711.

50. Prilipov A, Phale PS, Gelder P, Rosenbusch JP, Koebnik R (1998) Coupling site-directed mutagenesis with high-level expression: large scale production of mutant porins from *E. coli*. FEMS Microbiology Letters 163 (1):65-72.

51. Environmental Protection Agency (1993) Method 300.1 Determination of inorganic anions in drinking water by ion chromatography.

52. Environmental Protection Agency (2007) Perchlorate in water, solids and solid wastes using high performance liquid chromatography/electrospray ionization/ mass spectrometry (HPLC/ESI/MS OR HPLC/ESI/MS/MS).

53. Laemmli UK (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227 (5259):680-685.

54. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Molecular Systems Biology 2.

55. BW25113 from National BioResource Project (Japan) as ME9062.

56. McClatchey KD, Amin HM, Curry JL (2002) Clinical laboratory medicine: self-assessment and review, vol 2. Lippincott Williams & Wilkin, Philadelphia.

57. Thorneley RNF (1974) A convenient electrochemical preparation of reduced methyl viologen and a kinetic study of the reaction with oxygen using an anaerobic stopped-flow apparatus. Biochimica et Biophysica Acta 333 (3):487-496.

58. Levy O, Dai G, Riedel C, Ginter CS, Paul EM, Lebowitz AN, Carrasco N (1997) Characterization of the thyroid Na+/I- symporter with an anti-COOH terminus antibody. Proceedings of the National Academy of Sciences of the United States of America 94 (11):5568-5573.

59. Okeke BC, Ma G, Cheng Q, Losi ME, Frankenberger Jr WT (2007) Development of a perchlorate reductase-based biosensor for real time analysis of perchlorate in water. Journal of Microbiological Methods 68 (1):69-75.

60. Frankenberger Jr WT (2003) Perchlorate Removal in groundwater by perchlorate reductases from the perchlorate respiring bacterium, perc1ace. University of California, Riverside, CA.

61. Mitchell JB, Samuni A, Krishna MC, DeGraff WG, Ahn MS, Samuni U, Russo A (1990) Biologically active metal-independent superoxide dismutase mimics. Biochemistry 29 (11):2802-2807.

62. Thrash JC, Van Trump JI, Weber KA, Miller E, Achenbach LA, Coates JD (2007) Electrochemical Stimulation of Microbial Perchlorate Reduction. Environmental Science & Technology 41 (5):1740-1746.

63. Vishniac W, Santer M (1957) The thiobacilli. Bacteriological reviews 21 (3):195-213.

APPENDIX A: DETAILED PROTOCOLS

ENCAPSULATING CELL-FREE EXTRACT FROM 5000 ML OF *AZOSPIRA* CULTURE IN ABA3 VESICLES

Materials Required:

100 mL Rikken Media
5 L Rikken Media
Azospira oryzae glycerol stock
Stainless Steel Extruder
Akta Prime
Sephracyl 500-HR column
Anaerobic Cuvettes and Septa
Assay Mixture
Spectrophotometer
Zeta Sizer
Nutrient Agar Plate
50 mM Phosphate Buffer pH 6.0
Fisher Sonic Dismembrator
Ultracentrifuge and 10.9 mL ultra centrifuge tubes
Rotary Evaporator
ABA3 Polymer
20 mg/mL Proteinase K
Microplate and microplate reader
BCA protein assay kit (Pierce)

Step 1: Streak *Azospira oryzae* to a nutrient agar plate.

From a glycerol stock (labeled *Azospira oryzae)* in box labeled "SP Freezer stock" in the -80°C freezer, streak to a nutrient agar plate, and incubate at 30 deg C for 3 days.

Step 2: Transfer a colony to 100 mL of anaerobic Rikken Media.

Withdraw 0.5 mL of Rikken media from tube and transfer to a sterile microcentrifuge tube using a sterile needle and syringe. Pick a large colony from the plate using a sterile toothpick and transfer to the medium in the tube. Using a sterile needle, transfer the medium back to the larger tube and incubate at 30 deg C for 3-5 days until turbidity has developed in the media.

Step 3: Inoculate 5 L bottles.

Pour the starter culture into 5L bottles of Rikken Media. Cap with a rubber stopper. Incubate for 4-5 days at 30 deg C on a stir plate. When the OD_{600} is ≥ 0.6 (measure turbidity before pelleting), spin down the cells in the media in a centrifuge for 10 min at 6000 x g. Use pellets for cell-free extract preparation or freeze at -80 deg C for later use. For pelleting cells use the floor model centrifuge and tubes with purple caps.

Step 4: Resuspend pellets from 5 L culture in a total of 10 mL 50 mM PBS at pH 6.0 with 0.1 mg/L DNase in a 50 mL tube.

Step 5: Sonicate 3s on, 2 sec off for 3 min, with 5 min on ice in between. Set sonicator to ~ 30 % amplitude. Collect Sample (Lys 1).

Step 6: Centrifuge for 15 min at 5000 x g in 1.7 mL microcentrifuge tubes in a Fisher Legend Centrifuge at 4 deg C. Collect supernatant.

Step 7: Centrifuge supernatant from step 6 for 1hr at 40,000 rpm in Ti 70.1 rotor. Use Beckman ultracentrifuge bottles and be sure bottles are balanced to within 0.01 g.

Use supernatant in later steps of protocol (cell-free extract). Add protease inhibitor to the cell-free extract if desired. Collect Sample (Su 1). Be sure to avoid getting lipid material from pellet into supernatant when collecting supernatant.

Step 8: Test enzyme activity of Su1 using the protocol for enzyme assays of perchlorate with 10 uL of cell-free extract.

Step 9: Hydrate film made using 200 mL flask.

Form a film from 12 mg ABA 3 polymer using a rotary evaporator. Wash flask with soap and rinse with chloroform. Dissolve polymer in chloroform. Put flask onto rotary evaporator and remove chloroform first using a pressure of 150 mBar and then a pressure of 50 mBar until a film forms. Pull a full vacuum on the film for 4 hours. Add 1.5 mL cell-free extract as well as desired amount of OmpF (depending on molar ratio desired). Hydrate with stir bar for two days in fridge until polymer chunks are gone. Collect fraction (Hy 1) if desired.

Step 10: Extrude.

Spin down hydration mix for 1 min at 16,000 x g in microcentrifuge using a tabletop centrifuge. Use supernatant for extrusion.

Extrude 1 time through a 1 micron filter and then 10 times through a 0.4 or 0.6 micron filter using extruder in Clark Lab. Do not let pressure go above 50 psi. Collect fraction (Hy 2) and measure perchlorate-reducing activity.

Step 11: Treat with Protease.

Add 5% by volume 20 mg/mL solution of proteinase K to extruded vesicle mix. Sample should become slightly less turbid as protein aggregates are broken apart. Let sit at room temperature for 1 hour before size exclusion.

Step 12: Run vesicles on Sepharcyl 500 pressurized column.

Equilibrate Column using 50 mM PBS buffer for 1 hr. Inject vesicles onto column and record UV absorbance.

Size Exclusion Protocol:

Set UV-lamp to “on”; go to manual run, set pressure limit to 0.38 mPa; clean loading loop tubing (2 ml volume) with DI, then buffer (take off port and clean with syringe in sink); start run and click prompts to get run going.

Pause flow rate before loading the vesicle solution; set valve to “load!”; add sample; set valve to inject; resume flow rate (up to 0.5 ml/min); set collect fraction to 5.0 ml (for cleaning tubing in advance). Collect first peak, as this is the void volume-vesicle fraction.

Step 13: Measure activity of approximately 400 uL of vesicles.

Use PROTOCOL FOR ENZYME ASSAYS OF PERCHLORATE to measure activities.

Step 14: Quantify Size of vesicles using a 1:10 or 1:20 dilution of vesicles in buffer using DLS.

Dilute samples 1:10 or 1:20 in buffer (e.g. 50 uL vesicles in 500 uL buffer) in a disposable sizing cuvette. Using zeta sizer, make 3 size measurements. Set material to polystyrene latex.

Step 15: Measure protein concentration of cell extract batch, Hydration Mix (Hy2), and vesicles using BCA protein method, use a 1:10 to 1:20 dilution of the hydration mix and the cell extract batch.

Use microplate reader method for Pierce BCA method.

http://www.piercenet.com/products/browse.cfm?fldID=02020101

References:

Kengen SWM, Rikken GB, Hagen WR, van Ginkel CG, Stams AJM (1999) Purification and characterization of (per) chlorate reductase from the chlorate-respiring strain GR-1. Journal of Bacteriology 181 (21):6706-6711.

Rikken GB, Kroon AGM, van Ginkel CG (1996) Transformation of (per)chlorate into chloride by a newly isolated bacterium: reduction and dismutation. Applied Microbiology and Biotechnology 45 (3):420-426.

Buffers:

50 mM Phosphate Buffer, pH 6.0:

6g NaH2PO4 H20

1.6g Na2HPO4 7 H20 or 0.8 g Na2HPO4

Other buffers and materials are described in later protocols.

GROWING 5 L OF *AZOSPIRA ORYZAE* CULTURE [39]

Materials Required:

100 mL Rikken Media
5 L Rikken Media
Azospira oryzae glycerol stock

Step 1: Streak *Azospira oryzae* to a nutrient agar plate.

From a glycerol stock (labeled *Azospira oryzae)* in box labeled "SP Freezer stock" in the -80°C freezer, streak to a nutrient agar plate, and incubate at 30 deg C for 3 days.

Step 2: Transfer a colony to 100 mL of anaerobic Rikken Media.

Withdraw 0.5 mL of Rikken media from tube and transfer to a sterile microcentrifuge tube using a sterile needle and syringe. Pick a large colony from the plate using a sterile toothpick and transfer to the medium in the tube. Using a sterile needle, transfer the medium back to the larger tube and incubate at 30 deg C for 3-5 days until turbidity has developed in the media.

Step 3: Inoculate 5 L bottles.

Pour the starter culture into 5L bottles of Rikken Media. Cap with a rubber stopper. Incubate for 4-5 days at 30 deg C on a stir plate. Make sure stir bar is rotating. When the OD_{600} is $\geq$0.6 (measure turbidity before pelleting), spin down the cells in the media in a centrifuge for 10 min at 6000 x g and use for cell-free extract preparation or freeze for later use. For pelleting cells use floor model centrifuge and tubes with purple caps.

Rikken Media for Azospira [39]:

In 1 L Water (2 L) {5 L}:
1.55 g K_2HPO_4 (3.1) {7.75}
0.85 NaH_2PO_4 H_2O (1.7) {4.25}
1 g $NaClO_4$ or 1.15 g $NaClO_4$ H2O (2.3) {5.75}
2 g $NaCH_3COO$ (4g) {10 g}:
0.5 g $(NH_4)_2HPO_4$ (1) {2.5}
0.1 g $MgSO_4$ $7H_2O$ (0.2) {0.5}
0.02 g yeast extract (.04) {0.1}
0.2 ml trace elements (0.4) {1 mL}
0.17 mg Na_2SeO_4

Mix together ingredients in order shown.
Dispense in to smaller tubes if desired.
Degass smaller tubes (100 mL) under N2 gas using a canula (30 min for 100 mL cultures).
Flush headspace of smaller tubes (100 mL) with 80% N2 20% CO_2 gas using a canula (6 min for 100 mL cultures).
Add a stir bar into 5 L broth (5L broth is not degassed).
Autoclave 30L cycle.

Trace metal solution [63]

	1L	250 mL
Ethylenediamine tetraacetic acid	50.0 g,	12.5 g
ZnSO47H20	2.20 g,	0.55 g
CaCl2-2H20	5.54 g,	2 g
MnCl2-4H20	5.06 g,	1.265 g
FeSO4-7H2O	4.99 g	1.248 g
(NH4)Mo7024-4H2O	1.10 g,	0.275 g
CuSO4*5H20	1.57 g	0.3925 g
CoCl2.6H20	1.61 g	0.4025 g
H20	250 mL	1000 ml.

Add $ZnSO_4$ and EDTH Adjust to pH, 6.0 with KOH. Allow time for these two to dissolve, add other components slowly, maintaining pH 6.0 with KOH. Solution should have a green color after everything dissolves. Autoclave. After sitting on bench for a few weeks, solution will turn purple.

Nutrient Agar Plates:

Beef Extract	3.0 g
Peptone	5.0 g
Agar	15.0 g

1. Suspend 23 g of the powder in 1 L of purified water. Mix thoroughly.
2. Heat with frequent agitation to completely dissolve the powder.
3. Autoclave at 121°C for 15 minutes.

Procedure:
Autoclave the agar, cool to 45-50°C and pour into Petri dishes. Allow to solidify for at least 30 minutes.

References:

Kengen SWM, Rikken GB, Hagen WR, van Ginkel CG, Stams AJM (1999) Purification and characterization of (per) chlorate reductase from the chlorate-respiring strain GR-1. Journal of Bacteriology 181 (21):6706-6711.

Rikken GB, Kroon AGM, van Ginkel CG (1996) Transformation of (per)chlorate into chloride by a newly isolated bacterium: reduction and dismutation. Applied Microbiology and Biotechnology 45 (3):420-426.

Vishniac W, Santer M (1957) The thiobacilli. Bacteriological reviews 21 (3):195-213.

PROTOCOL FOR GROWING 2 L OF OMP8 CELLS AND OMPF PURIFICATION

Materials Required:

Glycerol Stock "New Omp8"
LB-Amp Plate
2 L LB Broth
20 mM Tris-CL pH 8.0
20 mM phosphate buffer pH 7.4
Octyl-PoE
20% SDS
Fisher Dismembrator
Wheaton 7 mL Homogenizer
Ultracentrifuge

Day 1: Streak Plate.

5 PM - Streak Omp8 (freezer stock labeled Omp8 in box labeled "SP Freezer Stocks") to an LB-Amp plate, grow for 18+ hours.

Day 2: Make Starter Cultures.

12 PM - take plate out, immediately pick colonies and inoculate three 5 mL Starter cultures in LB-Amp. DO NOT STORE PLATE IN FRIDGE BEFORE MAKING STARTER CULTURES. Plate is often bad after going into the fridge.

Day 3: Inoculate, Induce and pellet Omp8 Cells.

6 am – Inoculate each of the 1 L LB fernbach flasks with 1 starter culture. Grow Until OD_{600} 0.5 to 0.8 AU (Approx 3-7 hours) at 37 deg C at 200-250 rpm.

~12 pm – induce with 0.4 mM IPTG (0.0952 g IPTG per 1 L fernbach flask). Grow for another 6 hours at 37 deg C.

~6 pm – pellet cells for 15 min at 6000 x g using floor model centrifuge and tubes with purple caps.

OmpF Purification

Step 1: Lyse Cells.

Resuspend cell pellet in 10 mL buffer per g cell pellet (20 mM Tris-Cl pH 8.0).

Add 10 uL DNase I (1 U/uL) per 10 mL Buffer.

Disrupt cells using a sonicator: 3 sec on 2 sec off for 5 min at 30% amplitude. Leave sample on ice during and for 5 min between cycles. Sonicate three times.

Step 2: Add SDS and incubate.

Add 1 mL 20% SDS per 10 mL cell suspension. Make fresh SDS every day. Sample should turn clear after addition of SDS. If not, sonicate for additional time. Incubate for 1 hr at 60°C with gentle stirring.

Step 3: First Centrifuge cycle.

Centrifuge at 40,000 x g (~17,000 rpm) using Type 70.1 rotor at 4°C for 60 min.

Step 4: Preextraction.

Keep 100 uL of supernatant for SDS-PAGE. Wash pellet with 20 mM phosphate buffer pH 7.4 to remove residual SDS. Add 5 mL/g cell pellet 0.125% Octyl-POE in 20 mM phosphate buffer pH 7.4.

Homogenize pellet using Wheaton 7 mL homogenizer.

Shake for 1 hr at 37°C.

Centrifuge at 40,000 rpm (~145,000 x g) at 4°C for 45 min using Type 70.1 Ti rotor.

Step 5: Extraction.

Keep 100 uL of supernatant for SDS-PAGE. Add 2 mL/g cell pellet 3% Octyl-POE in 20 mM phosphate buffer pH 7.4.

Homogenize pellet using Wheaton 7 mL homogenizer.

Shake for 1 hr at 37°C.

Centrifuge at 40,000 rpm (~145,000 x g) at 4°C for 45 min using Type 70.1 Ti rotor. OmpF is in the supernatant.

Check samples on 12% SDS-PAGE.

Buffers:

20% SDS:

2 g SDS
Make up to 10 mL total volume.

Heat to approx 70°C and stir until SDS is dissolved.

20 mM Tris-Cl:

In 200 mL: .48 g Tris base
Adjust to pH 8.0 using HCL.

20 mM phosphate buffer:

Stocks to make 20 mM phosphate buffer:

0.2 M Na_2HPO_4 x H_2O (M=178.05 – 1.78 g/50mL)
0.2 M NaH_2PO_4 x H_2O (M=156.05 – 1.56 g/50mL)

For 500 mL 20 mM buffer: 40.5 mL 0.2 M NaH_2PO_4 + 9.5 0.2 M Na_2HPO_4 + 450 mL H_2O.

LB:

20 g Lennox LB Broth.

Dissolve in 1 L water.

Autoclave.

LB amp plate:

20 g Lennox LB Broth
15 g Agar

Dissolve in 1 L water.

Autoclave, allow to cool, add 50 mg Amp.

Pour into petri dishes and allow to cool.

PROTOCOL FOR ENZYME ASSAYS OF PERCHLORATE

Materials Required:

Assay Mixture
Anaerobic Cuvettes with 13 mm septa (SIGMA catalog number Z106496-100EA)
Spectrophotometer (to measure absorbance at 578 nm)
Dithionite Solution (0.2 M)
Perchlorate (1 M)

Purge 10 mL assay mixture with N_2 for 6 min beforehand to remove O_2. Use Finneran lab gassing station to do this.

Experimental Design:

Sample: hydration mix/cell extract/perchlorate reducing vesicles

Negative control 1: Add 30 µL DI water in lieu of perchlorate + cell free extract
Negative control 2: Add 30 µL 1M sodium perchlorate but no cell free extract

Step 1: Prepare Assay Mixtures and Capped Cuvette

Move degassed assay mixture, cuvettes, and septa inside anaerobic glove box. Pipette 2 mL assay mixture into cuvettes inside glove box. Cap with septa and screw cap and cuvette together tightly. Move cuvette from glove box, and add desired volume of sample to cuvette for cell extract activity measurments. For vesicles, add vesicle mix before capping with septa and use spectrophotometer in Strathmann glove box.

Place sample into spectrophotometer and blank at 578 nm.

Assay mixture volume: 2 mL

Components:
50 mM Tris-Cl^- Buffer
0.5 mM MV
20 µL enzyme mixture/cell fraction or approx 400 µL vesicle solution

Step 2: Prereduce Assay Mixture

Add:

Dithionite solution (0.2 M) until absorbance of 1.5 is reached (Range of 1.3 to 1.8 should be fine, usually requires around 5 uL of dithionite). Sample should turn royal blue as dithionite is added. If measuring activity of vesicles, let sample sit for 5 min to scavenge any remaining oxygen and to decrease blank measurement.

Step 3: Start reaction

Set up spectrophotometer to start logging blank for at least 100 seconds to get a baseline slope.

Add 20 µL chlorate or perchlorate (0.1 M) to samples and measure absorbance over 1-2 min if measuring activity of hydration mix/cell extract. Log for 1000-5000 seconds if measuring activity of vesicles.

Repeat with controls as desired.

Step 4: Calculate perchlorate-reducing activity

Use extinction coefficient of 13.1 mM^{-1} for MV and Equation 1.

Units of activity 1 µmol min^{-1} = 1 U.

References:

Grzelakowski M, Onaca O, Rigler P, Kumar M, Meier W (2009) Immobilized

Protein-Polymer Nanoreactors. Small 5 (22):2545-2548.

Preparation of Solutions:
Assay Mixture:
Make 1 M Tris-Cl

Tris-HCl is a solution frequently used in biochemistry made from Tris base and concentrated hydrochloric acid (HClaq). To make 1 mol/L Tris-Cl dissolve 121.1 g of tris base in 700 ml of double distilled water, bring to desired pH with concentrated HClaq (usually 7.5 or 8.0), add double distilled water to 1 L, filter with 0.5 μm filter, autoclave, and store at room temperature.

Make 50 mM Tris-Cl^- Buffer, MW = 121.13, = 12.5 mL 1 M Tris in 250 mL.
Adjust to pH 7.5.

Add Methyl Viologen (MV), MW = 257.16, (0.5 mM) = 0.032 g in 250 mL (Adjust for hydrate). Add enzymes per experimental design to cuvettes.

Dithionite Solution:

Dithionite Solution, MW = 174.10, (0.2 M) = 0.1745 g in 5 mL.

Perchlorate Solution:

Sodium Perchlorate, MW = 122.4 g/mol, (100 mM) = 0.124 g in 10 mL.

References:

Kengen SWM, Rikken GB, Hagen WR, van Ginkel CG, Stams AJM (1999) Purification and characterization of (per) chlorate reductase from the chlorate-respiring strain GR-1. Journal of Bacteriology 181 (21):6706-6711.

WESTERN BLOT PROTCOL

Materials Required:

Transfer buffer
Tris Tween Buffered Saline
Filter Paper
Membrane (Nitrocelluose or PVDF)
Antibodies
Gel electrophoresis and western blot setup
Shaker
Fiber pads

Step 1: Transfer Blotting

1. Prepare the transfer buffer. (Using buffer chilled to 4°C will improve heat dissipation.)

2. Cut the membrane and the filter paper to the dimensions of the gel or use precut membranes and filter paper. Always wear gloves when handling membranes to prevent contamination. Equilibrate the gel and soak the membrane, filter paper, and fiber pads in transfer buffer (15–20 min depending on gel thickness). It helps to soak the membrane in methanol first and then in water.

3. Prepare the gel sandwich.
Place the cassette, with the gray side down, on a clean surface.
Place one pre-wetted fiber pad on the gray side of the cassette.
Place a sheet of filter paper on the fiber pad.
Place the equilibrated gel on the filter paper.*
Place the pre-wetted membrane on the gel.*
Complete the sandwich by placing a piece of filter paper on the membrane.*
Add the last fiber pad.

* Removing any air bubbles which may have formed is very important for good results. Use a glass tube or roller to gently roll out air bubbles.

Current flows from the gray to the red (black to red) so that the protein comes out of the gel and onto the membrane.

4. Close the cassette firmly, being careful not to move the gel and filter paper sandwich. Lock the cassette closed with the white latch.

5. Place the cassette in module. Repeat for the other cassette.

6. Add the frozen blue cooling unit. Place in tank and fill to the "blotting" mark on the tank.

7. Add a standard stir bar to help maintain even buffer temperature and ion distribution in the tank. Set the speed as fast as possible to keep ion distribution even.

8. Put on the lid, plug the cables into the power supply, and run the blot.

A 30 V current run overnight (~16 hours) worked for us in the past for transfer.

9. Upon completion of the run, disassemble the blotting sandwich and remove the membrane for development. Clean the cell, fiber pads, and cassettes with laboratory detergent and rinse well with deionized water.

Step 2: Developing

1. Take out membrane mark the top (active) side of the membrane to make sure reaction is being conducted on the correct side of the membrane.

2. Soak the membrane in 30 ml blocking buffer (TTBS+ 0.9 g non-fat dry milk (1.5%)) for 45 minutes to 1 hour.

3. Incubate with 30 ml TTBS + anti His antibody –primary antibody (1:500 ratio, 60 uL) or anti NIS antibody –primary antibody (1:200 ratio, 150 uL) + 0.3 g non-fat dry milk (0.5%)) for 60 min. Antibody volume – 60 uL or 150 uL depending on antibody. Save TBS/antibody mix in fridge.

4. Wash membrane in TTBS with gentle shaking for 5 min X 3.

5. Incubate membrane in 30 ml TTBS + secondary antibody (1:1000 ratio, 30 uL) - 30 microL of secondary antibody + 0.3 g non-fat dry milk (0.5%)) for 60 min. Save TTBS/antibody mix in fridge.

6. Wash membrane in TTBS with gentle shaking for 5 min X 3.

7. Dry the membrane in air for a minute or two. Add developing solution, use sigma HRP solution.

Note:

1) Samples were run two ways, one as described here and another in which membranes were blocked overnight and the primary anti-His antibody was incubated for 5 hours before washing.
2) Blocking with a mix of BSA and milk and overnight seems to help (1.5% each). The same mix should be used for primary and secondary antibodies. Overnight blocking, longer primary antibody incubation time (2-3 hours) and only 45 min for secondary antibody incubation are other conditions that seem to work. Instead of 3 x 5min washes in between the primary and secondary antibody incubation, 6 x 5min seemed to work better (also the same for after the secondary antibody incubation, before the substrate reaction).

Buffers:

Towbin transfer Buffer (w SDS)
25 mM Tris 3.03 g Tris
192 mM glycine 14.4 g glycine
20% methanol
1g SDS
Bring to 1 L.

pH should be around 8.3.

TTBS (Tris Tween Buffered Saline)

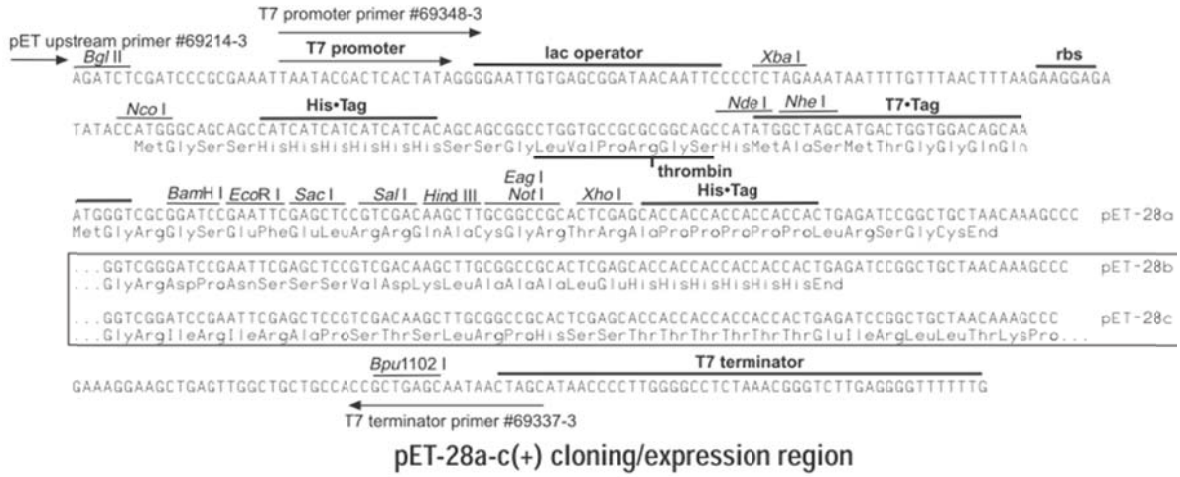
20mM Tris, pH 7.5 20 ml 1 M Tris (or 3.15 g Tris)
0.1 M NaCl 100 ml 1 M NaCl (or 5.85 g NaCl)
0.1% Tween 20 1ml Tween 20
Upto 1L with water, adjust pH to 7.5.

APPENDIX B: PRIMER DESIGN

PCR design for amplifying OmpF:

The program ApE, a plasmid editor, was used when analyzing the sequences for this design. NCBI was used to obtain the sequence of the OmpF gene.

Experimental plan to directionally insert OmpF gene into Nde I and Xho I sites of pET 28 B (+) vector.



pET-28a-c(+) cloning/expression region

(Source: pET 28 map by Novagen)

OmpF Base sequence: (OmpF Sequence is 1.09kb region from base 985117 to 986205, GenBank: U00096.2 Escherichia coli str. K-12 substr. MG1655)

5’ATGATGAAGCGCAATATTCTGGCAGTGATCGTCCCTGCTCTGTTAGTAGCAGGTACTGCA
AACGCTGCAGAAATCTATAACAAAGATGGCAACAAAGTAGATCTGTACGGTAAAGCTGTTGG
TCTGCATTATTTTTCCAAGGGTAACGGTGAAAACAGTTACGGTGGCAATGGCGACATGACCT
ATGCCCGTCTTGGTTTTAAAGGGGAAACTCAAATCAATTCCGATCTGACCGGTTATGGTCAG
TGGGAATATAACTTCCAGGGTAACAACTCTGAAGGCGCTGACGCTCAAACTGGTAACAAAAC
GCGTCTGGCATTCGCGGGTCTTAAATACGCTGACGTTGGTTCTTTCGATTACGGCCGTAACT
ACGGTGTGGTTTATGATGCACTGGGTTACACCGATATGCTGCCAGAATTTGGTGGTGATACT
GCATACAGCGATGACTTCTTCGTTGGTCGTGTTGGCGGCGTTGCTACCTATCGTAACTCCAA
CTTCTTTGGTCTGGTTGATGGCCTGAACTTCGCTGTTCAGTACCTGGGTAAAAACGAGCGTG
ACACTGCACGCCGTTCTAACGGCGACGGTGTTGGCGGTTCTATCAGCTACGAATACGAAGGC
TTTGGTATCGTTGGTGCTTATGGTGCAGCTGACCGTACCAACCTGCAAGAAGCTCAACCTCT
TGGCAACGGTAAAAAAGCTGAACAGTGGGCTACTGGTCTGAAGTACGACGCGAACAACATCT
ACCTGGCAGCGAACTACGGTGAAACCCGTAACGCTACGCCGATCACTAATAAATTTACAAAC
ACCAGCGGCTTCGCCAACAAAACGCAAGACGTTCTGTTAGTTGCGCAATACCAGTTCGATTT
CGGTCTGCGTCCGTCCATCGCTTACACCAAATCTAAAGCGAAAGACGTAGAAGGTATCGGTG
ATGTTGATCTGGTGAACTACTTTGAAGTGGGCGCAACCTACTACTTCAACAAAAACATGTCC
ACCTATGTTGACTACATCATCAACCAGATCGATTCTGACAACAAACTGGGCGTAGGTTCAGA
CGACACCGTTGCTGTGGGTATCGTTTACCAGTTCTAA 3’

Reverse complement of downstream end of sequence:

5’TTAGAACTGGTAAACGATACCCACAGCAACGGTGTCGTCTGAACCTACGCCCAGTTTGTT
GTCAGAATCGATCTGGTTGATGATGTAGTCAACATAGGTGGACATGTTTTTGTTGAAGTAGT
AGGTTGCGCCCACTTCAAAGTAGTTCACCAGATCAACATCAC 3’

Upstream Primer Complementary Sequence (just chose beginning of OmpF sequence, see highlighted region in OmpF sequence):
ATGATGAAGCGCAATATTCTG

Add Nde I Restriction Site (CA^TATG); upstream bases to allow for restriction (TGA); and remove extra ATG:

CAGCA^TATGATGAAGCGCAATATTCTG

Number of base pairs of total primer: 27
Tm = 58˚C
GC = 41%

Number of base pairs of complementary sequence of primer: 21

ATGAAGCGCAATATTCTG

Tm = 49˚C
GC = 39%

Upstream primer will be:

CAGCATATGATGAAGCGCAATATTCTG

Downstream primer anti sense sequence (used primer selection tool from ApE): 5’ GAACTGGTAAACGATACC 3’(See reverse complement sequence after stop codon above)

Add XhoI Restriction sequence for downstream (CTCGAG), 3 base pairs to allow for restriction (TAC)

Final Primer Complete Sequence:
TATC^TCGAGGAACTGGTAAACGATACC

Number of base pairs: 27
Tm = 59˚C
GC = 44%

Final primer complementary sequence only:

GAACTGGTAAACGATACC

Tm = 48˚C
GC = 44%

TATCTCGAGGAACTGGTAAACGATACC

Comparison of Melting Temperatures:

	Upstream Primer	Downstream Primer
Complementary Sequence	49	48
Full Primer	58	59