Capture and Cultivation of Microorganisms Using Magnetic, Lipid-Bound Antibodies

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Dedication

This thesis is dedicated to Billy, who nudged me along.

Abstract

In order to uncover and fully understand the genetic potential of the microbial "uncultivable majority," novel methods for isolation are needed. Here we report on efforts to develop a method for physical separation and isolation of viable microorganisms. This method uses differences in the molecular composition of membrane lipids between bacteria and archaea to segregate the desired organisms while keeping them alive and viable for reproduction. Magnetic antibodies bound to the molecule squalene, which is found in the cell membranes of certain archaea, but not bacteria, may enable separation of archaea from bacteria in mixed samples. We tested our technique using the squalene-bearing archaeon, *Halobacterium NRC-1*, and an analogous bacterium, *Salinibacter ruber*. Cells were subject to partial fixation and digestion to allow for antibodies to access the cell membrane. Viability of cells was demonstrated by growth of treated cells in batch culture. Specificity of the antibody probe to archaeal cells was demonstrated using immunofluorescence microscopy. Evaluation of separation efficiency using qPCR indicated that samples were not enriched in the target strain and that archaeal cells were preferentially lost throughout the procedure. FISH-probed cell counts suggested that cell clumping during centrifugation negatively impacted separation efficiency. Future efforts will aim to increase efficiency by minimizing the existence of cellular aggregates throughout the procedure.

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1 Introduction

In classical microbiology, organisms are studied by isolating a target species from its environment to enable study of a pure culture. Through rigorous experimentation with an isolated strain, we can gain insight about its physiology and genetic potential. Unfortunately, less than 2% of the estimated number of microbial species in the environment have been isolated and described, a number that has changed little in recent years, despite technological advances (Amann et al, 1995; Rappé & Giovannoni, 2003). Many of these "uncultivable" organisms evade isolation simply because it is difficult to replicate important aspects of their natural environment in the lab (Stewart, 2012). Additionally, species that grow slowly or prefer scant nutrients may be outcompeted by those that can metabolize a wide variety of nutrients or grow quickly on the same substrate (Taylor & Williams, 1975; Hsu et al, 1977).

Recently, "-omics" techniques have been adapted and expanded to work around the isolation problem. These approaches characterize the genetic potentialof a species or a consortium of microbes without the need for lab growth. Metagenomics, for example, involves screening the DNA sequences of an environmental sample to help determine which species are present and what metabolic processes they have the potential to use (Streit & Schmitz, 2004). Single cell sequencing allows analysis of one community member, but this approach is susceptible to contamination and the risk of amplification bias is high (Lasken, 2012). Although these culture-independent techniques are useful, the advantages of culture-dependent methods should not be ignored. Increasing both sets

of available tools will provide maximum benefits by allowing us to chip away at the "uncultivable majority."

Isolation of a single species is not always preferable, such as when cells will grow only in co-culture, or in a host (Moore et al, 2002; Goodman et al, 2007), but there exist various benefits to growth in pure culture. Microbial bioprospecting holds potential for drug and antibiotic discovery (Liu et al, 2010; Mahajan, 2012), as well as "green" energy production (Ratha et al, 2012). In the geosciences, researchers hope to gain a better understanding of microbe-mineral interactions across mineralogies and lithologies, to know which organisms are key players in mineral precipitation and alteration over time. Clade-specific lipids preserved in sediments may be extracted and analyzed to determine which organisms were present. However, characterization of the organisms is required in order to understand the paleoenvironment and determine the specificity of certain lipids as clade-specific "biomarkers", such that the information provided by this method is limited by the paucity of isolated strains strains.

A few current cultivation methods include natural environment simulation in a diffusion chamber (Kaeberlein et al, 2002), extinction dilution to decrease cell counts to a manageable few (Rappé et al, 2002), and single-cell, long term cultivation (Sizova et al, 2011). Our proposed enrichment method involves physical separation by attachment of magnetic particles to a taxon-specific component of the cell membrane.

We have developed a procedure targeting a known physiological variant in a selected test species with lipid-based antibodies. Antibodies are proteins produced by higher animals used for the detection of foreign molecules that will be tagged for

destruction by the immune system (Janeway et al, 2001). The hypervariable region of an antibody fits like a key in a lock to its target molecule or *antigen*, and its specificity is defined by how "loose" the fit is, or by how well it will bind with the target [Figure 1]. When specificity is high, the antibody should bind to only one antigen; when specificity is low, the antibody may bind its antigen and a range of other, similar molecules. We have adopted this concept to target the lipid, squalene, which is found in the cell membranes of some archaea. A cell wall typically surrounds the membrane, the components of which vary between taxa (Woese & Fox, 1997). According to the fluid mosaic model of cell membranes, lipids exist in a mono- or bilayer where only the head group may be accessible at the surface (Nicolson, 2014). Thus, it seems necessary to treat cells so that squalene is exposed and accessible by antibodies. This must be done without affecting reproductive viability of cells.

Squalene antibodies were first obtained by introducing squalene-containing liposomes into mice (Matyas et al, 2000). Injection of squalene alone did not trigger an immune response; in fact, a mixture including squalene and the highly immunogenic compound Lipid A was needed (Matyas et al, 2000.) This led to production of monoclonal and polyclonal squalene antibodies—the latter also binds to squalane, which is squalene's hydrogenated form (Matyas, 2000). These advances enabled development of a squalene-specific assay, used to detect and characterize squalene antibody binding activity (Matyas, 2002). An assay does not typically test antibody binding to non-specific molecules. For example, bacteria use squalene as a precursor to hopenoids, but are not known to contain squalene in their membranes. The exact antibody binding site (epitope)

on the squalene molecule is unknown, so initial testing must determine whether the antibody will bind non-specifically to similar molecules [Figure 2].

Once specific antibodies are bound to membrane lipids, we can add *secondary* antibodies using paramagnetic beads coated with secondary antibodies that are specific to the primary antibody [Figure 3]. Application of a magnetic field to the sample enables capture of the bead-bound cells while other cells are washed out. This should, in principle, result in a sample enriched in the target organism. RNA-bound magnetic probes have enabled physical separation of taxa with dead cells in a similar manner, (Pernthaler et al, 2008). Surface proteins of flagella have also been bound to magnetic antibodies with some success, (Chakaborty et al, 2011), but ours is the first method to target lipids in live cells.

Various unique archaeal membrane lipids hold high potential for lipid-based immuno-magnetic capture (van de Vossenberg, 1998). Recent drilling of Lake Vostok's ancient polar waters and an ongoing search for the deep biosphere's limits are bringing more microbe-bearing samples to the surface, increasing potential for discovery of new species, distinct metabolisms, and novel enzymes (Bulat et al, 2014; Treude et al, 2014). Addition of this culture-dependent method to our toolbox of cultivation approaches will help us study more species, as they are uncovered.

2 Methods

2.1 Cultivation

A halophilic archaeon and analogous bacterium were selected based on similar metabolic needs and salinity tolerances that would allow them to grow in the same medium. The archaeon, *Halobacterium NRC-1*, (Carolina, 154777), and bacterium, *Salinibacter ruber*, (Deutsche Sammlung von Mikroorganismen und Zellkulturen, DSM-13855), are morphologically distinct in growth phase—the former grows in wide, straight rods while the latter forms long, slender, slightly curved rods. This morphological distinction disappears as they age or become subject to less-than-favorable conditions.

Growth experiments were performed to determine a favorable medium suitable for both strains. The following broths were tested, with varying organic compositions: Antón et al.'s "Medium B" (2002), Halohandbook's "Modified Growth Medium" (2009), and DSMZ's "936 *Salinibacter ruber* medium." These media were mixed in sterile bottles, using Nanopure-filtered water. Solutions were buffered to pH=7.2 with NaOH and HCl, and stored at 4°C.

Both strains require oxygen for respiration, and must be aerated for proper growth, so a large surface area to volume ratio and breathable cap were ideal. Cultures were initially grown in rectangular, flat-lying filter-cap bottles (CellTreat, 229500) so that media was only a few millimeters deep. Later, cultures were grown in autoclaved Pyrex bottles with filter caps. Strains were grown in isolation at 42°C to 107/ml before experimentation, either in stagnant media or on a 100 rpm shaker.

2.2 Probe Specificity Testing with Immunofluorescence

The squalene antibody probe, SQE #14, was produced by Dr. Alan Epstein's lab at the University of Southern California using the hybridoma clone PTA6538-SQE14 (ATCC, United States). These were grown in hybridoma growth medium with 3% characterized fetal calf serum (GIBCO 06-5012EL; Life Technologies, United States) to a concentration of 10^6 cells/ml, and then aerated for 3-5 additional days of growth. Cell suspensions were spun in 500 ml conical tubes at 2,400 rpm and 4°C for 15 minutes before the supernatant was passed through a 0.2 μm filter, into a sterile bottle. Aliquots were stored, frozen, until use.

Cell Preparation

Prior to magnetic separation, the probe's specificity had to be determined. If the probe was specific to squalene, it should bind only to archaeal cells containing squalene and not to the ultrastructure of bacteria that contain different lipid components (Matyas et al, 2000). Both strains were subject to the following treatment in isolation. All experimental media were amended with 25% w/v NaCl to maintain osmolarity.

Initially, 800 μl of growth phase cells in media were incompletely fixed with 200 μl paraformaldehyde, mixing gently at room temperature for 15 minutes. Cells were then chilled on ice for 40 minutes before rinsing 4 times with 1 ml phosphate buffered saline (PBS). For each rinse, cells were vortexed briefly and centrifuged for 1 minute at 11,000 rcf, then the supernatant was removed and new PBS added. After the fourth rinse, cells

were suspended in 100 µl of 2.5 µg/ml lysozyme/GTE solution. Each well of an 8-well glass slide was treated with 25 µl of Poly-L-Lysine (Ted Pella). After 10 minutes, the wells were aspirated using a QuikSip bottletop aspirator (BrandTech Scientific, Inc) and allowed to dry completely. Wells were re-hydrated with 50 µl 2% bovine serum albumin (BSA) in PBS blocker and incubated in a humid chamber for 15 minutes. The blocker was aspirated before application of primary antibodies.

In later tests, 10 µl cells were dropped onto wells of an 8-well slide. These were allowed to dry partially, but not completely. The slide was immersed in a 2% acetic acid solution for 5 minutes, then wells were aspirated, but not dried completely. Ten µl of Proteinase K solution (Millipore; 20 mg/ml in 10 mM TrisCl, pH 7.5) was added to each well. The slide was incubated in a humid chamber at 37°C for one hour. Wells were then rinsed and aspirated 3 times with one drop of Milli-Q water. A drop of 0.01 M HCl was added to each well, and then incubated at room temperature for 20 minutes. Wells were washed twice with Milli-Q water before application of primary antibodies.

Antibody binding

Twenty microliters (µl) of monoclonal Immunoglobulin M (IgM) SQE #14 primary antibody in 2% BSA/PBS was added to each well containing cells. Various concentrations of primary were tested—1:10, 1:40, 1:80, and 1:100. Control wells were treated with 2% BSA/PBS. Slides were incubated in a humid chamber at room temperature for one to two hours. Following incubation, wells were washed 10 times by adding and aspirating one drop PBS, without completely drying. In a dark room, FITC-labeled goat anti-mouse secondary antibody (IgM) was added to each well at a

concentration of 1:1000 or 1:2000 in 2% BSA/PBS. Slides were incubated in a humid, dark chamber for 1 hour. Wells were washed in the dark 10 times with PBS.

Analysis

For cells fixed with PFA, these slides were counter-stained with 3 µl/well of 1 µg/ml FM4-64. Vectashield was added to the slide before placing a coverslip and analyzing with fluorescent microscopy. In experiments with Proteinase K or with no digestion, 10 µl PBS was added to each well and a cover slip was placed for immediate microscopic analysis.

Slides were analyzed using an Olympus BX61 microscope with epifluorescence capabilities (DAPI, FITC, and Texas Red filter sets). Bright spots indicating antibody binding were compared with phase-contrast microscopy in the same field of view (FOV) for identification of tagged objects. Fluorescence of the squalene-bearing archaeal cells, and not the bacteria, was interpreted as a positive result.

2.3 Immuno-Magnetic Capture

After determination of the probe's specificity, the following immuno-magnetic separation method was developed to isolate targeted cells for enrichment. Media for this experiment, with the exception of acetic acid, were amended with 25% w/v NaCl to maintain osmolarity. Cells were not allowed to dry completely at any stage. Resuspension of cells from pellets was executed using aspiration and release with a pipet tip rather than vortex mixing.

For each strain, 15 ml of cells in culture (approximately 10^7 cells/ml) were centrifuged 5 minutes at 7000 rcf. All but 0.5 ml of supernatant was removed before cells were resuspended and combined in a single 1.5 ml tube. These were spun down again for 5 minutes at 7000 rcf, resuspended with 0.5 ml 2% acetic acid in Modified Growth Medium base, and then centrifuged 5 minutes at 13,000 rcf before supernatant was removed.

Cells were suspended and incubated in 200 µl Proteinase K solution (Millipore; 20 mg/ml in 10 mM TrisCl, pH 7.5) for 1 hour at 37°C in a humid chamber. One ml of NaCl/Milli-Q water was added to the sample to aid pelleting, and then cells were centrifuged 5 minutes at 9700 rcf. The supernatant was removed and cells were suspended and incubated in 200 µl 0.01 M HCl for 20 minutes at room temperature. After 4 minutes of centrifugation at 12,000 rcf, cells were rinsed and spun 3 times (12,000 rcf) with 0.5 ml Milli-Q water.

The water was aspirated and 400 µl primary antibody, SQE #14, diluted 1:40 in 2% BSA/PBS (blocker) was added to cells and incubated 2 hours at room temperature. These were centrifuged 4 minutes at 9700 rcf, then rinsed and suspended 5 times with 0.5 ml PBS. After the last rinse, cells were suspended in 1 ml blocker. This suspension was added to 0.5 ml PBS in a 3-ml syringe with a coarse filter (<20 µm retention) in a filter holder. Cells were forced through this filter to remove large clumps of cells. The filter was discarded and the filtrate retained. From this, a 200 µl sample was removed for the "Before" fraction, to quantify separation efficiency.

Dynabead magnetic antibodies were washed according to Invitrogen's protocol, using 25 µl beads to 1 ml 2% BSA/PBS blocker in a 1.5 ml tube. The cells were added to this tube, incubated in a 10 rpm rotisserie at 4°C for 20 minutes, and then placed in a Magna-Sep Magnetic Particle Separator (Invitrogen), along with two empty tubes. Due to the small magnetized area, not all Dynabeads could be captured by the magnet, so after 1 minute, the supernatant was transferred from tube 1 to tube 2, allowing more beads access to the magnet. One-half ml blocker was added to the first tube, re-suspending any loose cells. Following a one-minute incubation, the supernatant was transferred again, stepwise, through the sequence of tubes. After 3 magnetization steps, the "Wash" fraction fluid was collected in a 15 ml tube. The tubes were all removed from the particle separator, Dynabeads in each of the 3 tubes were suspended with 350 µl of blocker, and this "Magnetized" material was combined into one collection tube.

The two antibody-treated samples were centrifuged 5 minutes at 9700 rcf, and supernatant in excess of 1 ml was removed. Cells were re-suspended and split out for various analyses: "Before", "Wash", and "Magnetized." Additionally, supernatant from each series of acetic acid, water, and PBS rinses were sampled. 100 µl of each was fixed with 22 µl of 37% paraformaldehyde overnight at 4°C; 100 µl of the Magnetized fraction was used to inoculate 10 ml halophile media containing 1% organics, and the remainder was frozen for DNA extraction.

2.4 DNA Extraction & Quantitative Polymerase Chain Reaction (qPCR) Analysis

In order to test extraction efficiency prior to implementation, various DNA extraction, clean-up, and concentration kits were tested using acetic acid-fixed cells of both strains:

1. DNeasy Blood & Tissue kit (Qiagen, Germany), using protocol for cultured cells
2. PowerSoil DNA Isolation kit (MoBio Laboratories, Inc., United States). We also attempted amplification and the following kits with this product:
    a. DNA Clean & Concentrator kit (Zymo Research, United States)
    b. One-Step PCR Inhibitor Removal kit (Zymo Research, United States)
3. DNeasy Blood & Tissue kit (Qiagen, Germany), using protocol for mouse or rat tail digestion

Following kit testing, samples of the 3 experimental fractions were thawed and DNA was extracted using Qiagen's DNeasy Blood & Tissue kit. Each sample was centrifuged 5 minutes at 9700 rcf, excess buffer removed, and then the kit's protocol for extracting DNA from rat tail tissue was followed, with a 6-minute incubation at 56°C after addition of Buffer ATL. Samples were vortexed briefly after addition of lysis buffers and again halfway through incubation. DNA yields were tested using a NanoDrop 2000c UV-Vis spectrophotometer (Thermo Scientific, United States) before samples were shipped, frozen, to Dr. Karen Lloyd's lab at University of Tennessee-Knoxville for quantitative PCR analysis (qPCR).

qPCR analysis followed previously described methods (Kubo et al, 2012). Archaeal 16S rRNA was quantified using ARCH806F and ARCH915R primers; bacterial genes were quantified using BAC340F and BAC515R. R-squared values for qPCR standard curves were between 0.981 and 0.999. No low melting point primer-dimers were detected. Results were normalized to reflect dilution of samples during separation.

2.5 Cell Counts from Fluorescence In-Situ Hybridization (FISH)

Following qPCR analysis of the first separated batch, a second set of separations was completed. These were analyzed immediately in the Bailey lab using fluorescence in-situ hybridization (FISH) and later in the Lloyd lab with qPCR. We hoped to determine with FISH whether the separation was working before submitting samples for qPCR analysis.

FISH procedures were based on the Halohandbook (Dyall-Smith, 2009) with the following specifications:

From separated fractions, 100 µl of sample had been mixed with 22 µl 37% formaldehyde and incubated overnight at 4°C. For each filter, 10 ml PBS was added to a filtration tower over a 22 mm, 0.2 µm filter. Ten µl of the fixed sample was added and filtered down using a pump. Another 10 ml PBS was added and filtered down. Filters were allowed to air dry, then were stored short-term at 4°C (less than 24 hours) or long-term at -20°C.

For each FISH slide, 2 ml of 30% stringency hybridization buffer was prepared using the following: 360 µl 5M NaCl, 40 µl 1M Tris-HCl, (pH 8.0), 600 µl formamide, and 1 ml Milli-Q water. EUB338-II+III (combined GCA GCC ACC CGT AGG TGT and GCT GCC ACC CGT AGG TGT) and Arch915 probes (GTG CTC CCC CGC CAA TTC CT) were used, with FITC and TXred labels, respectively (Daims et al., 1999; Stahl and Amann, 1991). Probes were excluded from negative controls.

Wash buffers contained the following, based on 30% stringency: 1020 µl 5M NaCl, 1 ml 1M Tris-HCl (pH 8.0), 50 µl SDS, and Milli-Q water up to 50 ml. These were incubated for 20 minutes at 46°C. Filters were retrieved using forceps and allowed to

dry, face-up. Filters were stained for one minute, face-down, with 10 µl of 1 µl/ml DAPI. These were dipped in Milli-Q water, then ethanol, and placed on a clean slide to air dry with cells facing up. After all filters were placed and dried, small drops of Vectashield (Vector Laboratories, Inc.) were added to the slide between filters. A coverslip was added and sealed at the edge with clear nail polish to hold in place during microscopy. Slides were stored in the dark at 4°C before analysis.

Using an Olympus BX61 microscope and CellSens software, for each filter piece, 10 random fields of view (FOV) were chosen, away from the outer edge of the filter, to avoid regions that had been handled with forceps. Cells were counted manually, across the appropriate focal depths containing cells, within the microscope's ocular counting grid. For each counted FOV, upper and lower focal planes were set based on the topography of the filter paper and presence of cells. Exposure times were set to hold constant for all images: TXred at 100 ms and FITC at 200 ms. A z-stack image was compiled from each filter set, first of archaeal cells in TXred and second, of bacterial cells visible in FITC. Counts were compiled to determine the relative percentages of each cell type before and after separation.

2.6 Batch Culture

Sterile halophile broth was inoculated with *H. NRC-1* cells derived from the separation experiment's magnetic fraction. Inoculated growth media was later examined for evidence of cell growth using an Olympus BX61 microscope.

2.7 Statistical Analysis

qPCR and FISH results were analyzed using Tukey's Honest Significant Difference (HSD) test to determine the significance of sample composition changes following magnetic separation (Tukey, 1949). The "One-way ANOVA (Analysis Of Variance) with post-hoc Tukey HSD (Honest Significant Differences) Test Calculator for comparing multiple treatments" was used (Vasavada, 2014). Samples from qPCR Batch 1 were compared to each other, and samples 1-3 of Batch 2 were also compared. Batch 2, sample 4 was omitted, as it did not contain the same starting composition as the others in the same batch. Additionally, Before and Wash counts analyzed by FISH were compared.

3 Results

3.1 Cultivation

We first tried to identify a common growth medium so that cells would survive when combined for experimentation. Additionally, this mimics a natural environment where cells exist in the same medium. *Halobacterium NRC-1* cells approached exponential growth using the Halohandbook's Modified Growth Medium with a 10-fold increase in organic concentration (Dyall-Smith, 2009). *H. NRC-1* thrived with 1% yeast, 1% peptone, or 0.05% yeast and 0.05% peptone. The latter was used, with MOPS as the buffer.

Salinibacter ruber cells did not appear to grow significantly in the type strain media strain description growth medium described by Antón et al. (2002). The Halohandbook's Modified Growth Medium (Dyall-Smith, 2009)-used by Makhdoumi-Kakhki et al (2012) for isolation-was also tested, with negative results. DSMZ's 936. *Salinibacter ruber* medium yielded some growth, but was not robust. Finally, this strain was grown successfully in Modified Growth Medium (Dyall-Smith, 2009), amended with MOPS buffer, and 0.05% yeast and 0.05% peptone as organic sources.

Aeration was needed to ensure exponential cell division. Test tubes did not support growth. Positive growth results were obtained in shallow, stagnant media and in deeper, agitated media. Filter-top caps were used to increase oxygenation and avoid contamination. Prior to experimentation, *H. NRC-1* inoculates were grown for 1 day and *S. ruber* for 3 days, on a 100 rpm shaker at 42°C.

3.2 Probe Specificity and Binding

We suspected that the archaeal membrane lipid, squalene, would not be sufficiently accessible on the exteriors of cells for antibodies to bind without pre-treatment to expose the lipid membrane. Some antibody-based assays demonstrate positive results with digestion of cells, (Brun et al, 2004), so we sought a similar method to expose the antigen. Fixation with paraformaldehyde and digestion with lysozyme did not produce any observable antibody binding in either strain.

Acetic acid fixation is suitable for halophiles and Proteinase K digestion is widely used for permeabilizing cells prior to FISH (Dyall-Smith, 2009; Pinkel et al, 1986). This combination did appear to permeabilize the cells and allow antibody binding, as discussed in the following section. No antibody binding was observed without fixation and digestion.

H. NRC-1 cells became spherical during acetic acid fixation [Figure 4]. We used acetic acid plus Modified Growth Medium base to maintain osmolarity and cell structure, but this did not appear crucial--cells remained alive and motile without addition of salts during fixation. *S. ruber* cells did not undergo any visible morphological alteration during fixation.

The development of our antibody assay required optimization of reagent concentrations and incubation conditions specific to the experiment. No antibody binding was observed in either strain after 1 hour of incubation, in primary concentrations of 1:10, 1:40, 1:80 and 1:100. Secondary antibodies were incubated for 1 hour at a concentration of 1:2000.

Binding to archaeal cells was observed after 2 hours of incubation with primary concentrations of 1:10, 1:40, and 1:80. Secondary antibodies were incubated at 1:1000 for 1 hour. At a dilution of 1:10, cells were clustered and fluorescence was high. At a dilution of 1:40, cells were clear and visibly tagged [Figure 5a]. At a dilution of 1:80, the dimly fluorescing cells were more difficult to observe. No fluorescence was observed in the control well. Bacterial cells did not appear to be tagged, as no fluorescence was viewed in most fields of view. The presence of cells was verified using phase contrast microscopy [Figure 5b]. Some fluorescing spots were identified across bacterial wells [Figure 6]. Material that did not bind with antibodies was also observed in the archaeal wells. No correlation was made between these variably shaped objects and antibody binding.

3.3 Magnetic Separation—qPCR and FISH Analyses

DNA was extracted from each separated fraction for qPCR analysis of separation efficiency. Various DNA extraction kits, clean-up kits, and concentration kits were tested using fixed cells, but no DNA peaks were observed with the Nanodrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Delaware).

Finally, the DNeasy Blood and Tissue kit's procedure for extracting DNA from rat tail tissue yielded a range of 16.5 to 52.6 ng/µl nucleic acid concentration across all samples. This DNA was successfully amplified using general bacterial and archaeal primers. Results were normalized to account for dilution during separation.

Comparison of the three sample fractions-Before, Magnetized, and Wash-from both batches of qPCR analyses indicated that the samples became enriched in bacterial

cells throughout the procedure [Figure 7]. Total archaeal cell counts decreased in both cases, but to a lesser extent in the second batch. Analyzed samples from different rinse media contained greater quantities of archaea than bacteria [Figure 8].

We hoped to determine qualitative separation efficiency before sending a second batch of samples for qPCR by counting FISH-probed cells from the separated samples. Dynabeads in the Magnetized fraction obstructed visualization of cells, making direct counts of captured cells impossible. Instead, we counted only the Before and Wash fractions to determine whether or not archaeal depletion had occurred throughout the experiment, which would suggest that the missing cell fraction had been captured by the magnet.

Subsamples from the second batch of Mag-Sep experimental cells were analyzed with cell counts before the remaining samples were sent to collaborators at the University of Tennessee for qPCR analysis. Some replicates of counts from FISH on filters indicated that the Wash fractions of some samples were depleted in archaea after separation, but other replicates did not show this [Figure 9]. A significant portion of cells was lost between primary antibody binding and magnetic separation, seen by the decreased total cell counts. According to these counts, less than half the starting quantity of cells was present in the Wash fraction.

In images from FISH counts, archaea in the Wash fraction [Figure 10] are generally associated with clusters of cells. In the Before fraction, [Figure 11], they are found both in clusters and as singular cells.

3.4 Viability

Success of our separation method hinges on the ability of these treated cells to continue to divide. Media inoculated with SQE#14-tagged cells grew up in dense culture. This was noted 8 days after inoculation, when a color change of the media was macroscopically evident. Cell growth and motility were verified microscopically. The morphology of these cells was consistent with growth phase *Halobacterium NRC-1*. No *Salinibacter* cells were identified in the medium.

4 Discussion

4.1 Media Standardization

At a glance, comparison of media for the two strains suggested that they could easily be grown in the same medium. In reality, the two thrive in the same mix of ions, but this bacterial strain prefers media with lower organic content than does the archaeal strain. Bacterial growth is stunted when organic concentration is too high.

The recommended Tris buffer did not work as well as MOPS. We believe MOPS was better suited to the pH range of the media.

4.2 Fixation and Digestion

Incomplete fixation of cells was performed to increase structural stability during subsequent enzymatic partial digestion. We expected that this digestion would be necessary in order to access membrane lipids. We infer that this hypothesis is correct, given that no binding was observed without fixation and digestion, while binding was observed after implementing partial digestion.

Fixation with paraformaldehyde and digestion with lysozyme did not result in cells that produced positive binding results. There was likely minimal digestion of the archaeal membrane with this enzyme because lysozyme targets peptidoglycan, which is useful for bacteria who construct their cell walls from peptidoglycan, but less so for archaea that use protein or pseudopeptidoglycan. When using this protocol to target bacterial molecules, however, lysozyme may be the better option. We also moved away from using PFA as fixative—it may be too harsh on cells. The utility of dilute acetic acid

as fixative in other halophile-specific procedures led us to test and adopt this medium in our procedure (Dyall-Smith, 2009).

Low concentrations of acetic acid generally cause *Halobacterium* to assume a coccoidal morphology (Kushner et al, 1964). We used acetic acid in Modified Growth Medium base to maintain osmolarity and cell structure, but this did not appear crucial—cells remained alive and motile without addition of salts during fixation. It is unclear whether or not this morphological change affects permeability of the cell membrane. Bacterial cells did not undergo any obvious morphological changes during this step.

Proteinase K-often used for complete cell lysis-proved useful in partial digestion of these cell membranes, enabling access to the target molecule. Longer digestion times or higher enzyme concentrations may increase membrane lipid availability and thereby enhance binding, but at the upper limits, cells will lyse. Further work should be done to test these limits and determine whether greater enzyme exposure would increase antibody binding.

4.3 Antibody Binding

Archaeal cells incubated with primary squalene antibodies for 2 hours and fluorescent secondary antibodies for 1 hour resulted in FITC-tagged objects in all fields of view, at all concentrations. Wells containing bacteria had few-to-no tagged objects. From these results, archaeal cell fluorescence was interpreted to mean that the antibody probe bound specifically to archaeal squalene, but not to bacterial cell membrane components.

Many un-tagged archaeal cells were also observed, indicating that these cells were not permeable to antibodies or that the amount of primary used was not sufficient to tag

all cells. At 1:10 concentration, overall fluorescence was high, causing all substances on the slide to fluoresce brightly. At 1:80 concentration, tagged cells were evident, but not sufficiently bright against the background, indicating that antibody concentration was too low. It does not seem necessary to bind with all cells, and there is greater danger of non-specific binding at too-high concentrations, so we determined we would not increase primary concentration above 1:40 for separation experiments. No quantification was done to determine whether a greater proportion of cells were tagged when primary antibody concentration was increased.

Although bright spots matched up with objects on the slide, the identity of some objects was unclear. Morphologies of archaeal cells had been altered by acetic acid fixation [Figure 4], and the high salt concentration of media used led to salt precipitation on the slide surface [Figures 5 & 6]. These two factors impaired positive identification of all objects in the wells. However, after applying outline detection to the FITC-tagged objects in Figure 5, it seemed that tagged objects correlated with spherical and triangular objects, consistent with late-phase *Halobacterium* morphologies.

In *Salinibacter* immunofluorescence images, [Figure 6], rare, small dots appeared to indicate tagged objects. These did not match up with clearly bacillus-shaped cells, typical of *S. ruber*. Additionally, large clusters of *S. ruber* cells were not tagged, where non-specific binding should be obvious if it did exist. Instead, these fluorescent dots seemed to mark spheres, which we interpreted to be *Halobacterium* cells that came loose from other wells on the slide earlier in the procedure. The acicular mineral precipitates

were also evident in the bacterial wells, and were more clearly recognized as non-cellular when compared with the rounded shape of *Salinibacter* bacilli.

4.4 Magnetic Separation—qPCR

Various DNA extraction kits were tested, but spectrophotometric peaks indicative of DNA presence were not observed, initally. This was likely due to the high ionic concentrations of these halophilic cells, which may affect DNA extraction (Dair et al, 2001). After successfully retrieving nucleic acids from untreated cells with the DNeasy kit, but failing with treated cells, we suspected that the increased cellular stability from fixation was inhibiting extraction. The additional digestive buffer used in the DNeasy rat tail digestion protocol seemed to effectively lyse the fixed cell membrane, allowing DNA extraction. It is unclear with these analyses whether this extraction protocol lysed both bacterial and archaeal cells with the same efficiency. It is important to note that if extraction efficiencies differ between clades, results will be skewed.

In the first batch of magnetic separations, qPCR results for archaeal and bacterial "Before" fractions suggested that each of these contained similar cell concentrations [Figure 7]. "Wash" fractions were slightly depleted in bacteria in all cases, but contained approximately an order of magnitude fewer archaea. "Magnetized" fractions contained significantly fewer archaea. This indicates a loss of archaea throughout the experiment, while bacterial presence remained fairly constant before and after separation.

In order to determine at which step archaea were being lost, the rinse supernatant was sampled for DNA during the second batch of separations. Archaea appeared to be preferentially lost during centrifuge steps. qPCR results [Figure 8] appear to support this

hypothesis, where roughly 10x fewer bacterial cells were lost in each rinse test. Archaea did not seem to spin down as readily as bacteria, but centrifugation also caused cells to clump, so high spin forces and times were generally avoided. This suggests that more optimization should be done to avoid cell loss throughout the separation protocol. Alternatively, a method that does not involve centrifugation may be more successful.

Results from the second batch of qPCR analyses appear to indicate that the proportions of each clade were similar in the Wash fraction, and that bacteria, again, became enriched in the Magnetized fraction. Overall, there were fewer cells lost during the procedure, but the samples did not appear enriched in the target strain.

4.5 Magnetic Separation—FISH

Successful magnetic separation should be indicated directly by archaeal enrichment in the Magnetized sample or indirectly by archaeal depletion in the wash sample. The former could not be quantified with FISH due to visual obstruction caused by the Dynabeads, so cell counts were completed with the latter.

Archaeal cell counts of samples A and C [Figure 12] were proportionally depleted relative to bacteria, suggesting that they may have been bound and removed by the magnetic separation procedure. However, the absolute count numbers [Figure 9] indicate that total cells decreased significantly. This may or may not support the conclusion that antibody binding and magnetic separation preferentially separated archaea. Sample D had a higher proportion of archaea in the wash fraction, but archaeal counts remained stable while bacteria were lost. Sample B was fairly constant between the two stages, due to the

similar proportions of both cell types lost during the procedure. These varying results make it difficult to draw conclusions when the Magnetized fraction counts are missing.

It is possible that clusters containing archaea such as those observed in the Wash fraction [Figure 9] comprise the only archaeal cells remaining, and that singular cells were either removed by immuno-magnetic separation or lost during rinses. In that case, only clustered cells would remain to be counted. Also, potentially, clusters containing a threshold amount of Dynabead-bound archaea could be magnetized and removed as an aggregate. When these magnetized clusters contain mostly bacteria, separation will appear unsuccessful.

Throughout the separation protocol, cells were rinsed by centrifugation and aspiration of media. With each of these steps, extracellular polymeric substances (EPS) caused pelleted cells to stick to each other, resulting in cell clusters. Increasing spin times or velocities should decrease cell loss during rinses, but this also seemed to promote clumping.

We explored various means to mitigate clumping, with some success, but were unable to eliminate the clustered cells entirely. By growing cells on a shaker and filtering out clumps >20μm, we believe separation efficiency was enhanced, but these modifications were made before quantitative data were obtained. Enzymatic treatment of cells to dissociate clusters should be done before addition of antibodies to avoid interference with antibody binding.

More alterations to the separation technique should be explored. Addition of a detergent such as Tween may keep cells from sticking to one another, but it may need to

be included in all media while wash steps are performed using centrifugation. Another potential option would be to forgo centrifugation altogether, avoiding pelleting. This could be possible if the procedure were performed on a filter, but cells would need to be re-suspended in the end before magnetizing the sample.

According to our Tukey HSD results [Figure 13], the proportion of archaea to bacteria did not change significantly between Before and Wash samples, in any of the three separations analyzed. Similarly, comparison of Before and Wash samples in both Batch 1 and Batch 2 indicated that there was not significant change [Figure 14]. However, these results also indicate that a significant change was in fact present between Before and Magnetized samples of both batches [Figure 14]. According to this analysis, the bacterial enrichment during separation is statistically significant. Additionally, we see agreement between FISH and qPCR results with these statistical analyses.

4.6 Viability

The 8-day incubation of treated cells indicates a longer-than-normal lag phase before cells reached exponential growth. Typically, media inoculated with only 10µl of *H. NRC-1* cells would reach a similar density in 3-4 days. We interpreted this to mean that cells were affected, but remained viable after incomplete fixation, partial digestion, and binding of antibodies to membrane lipids.

5 Conclusions

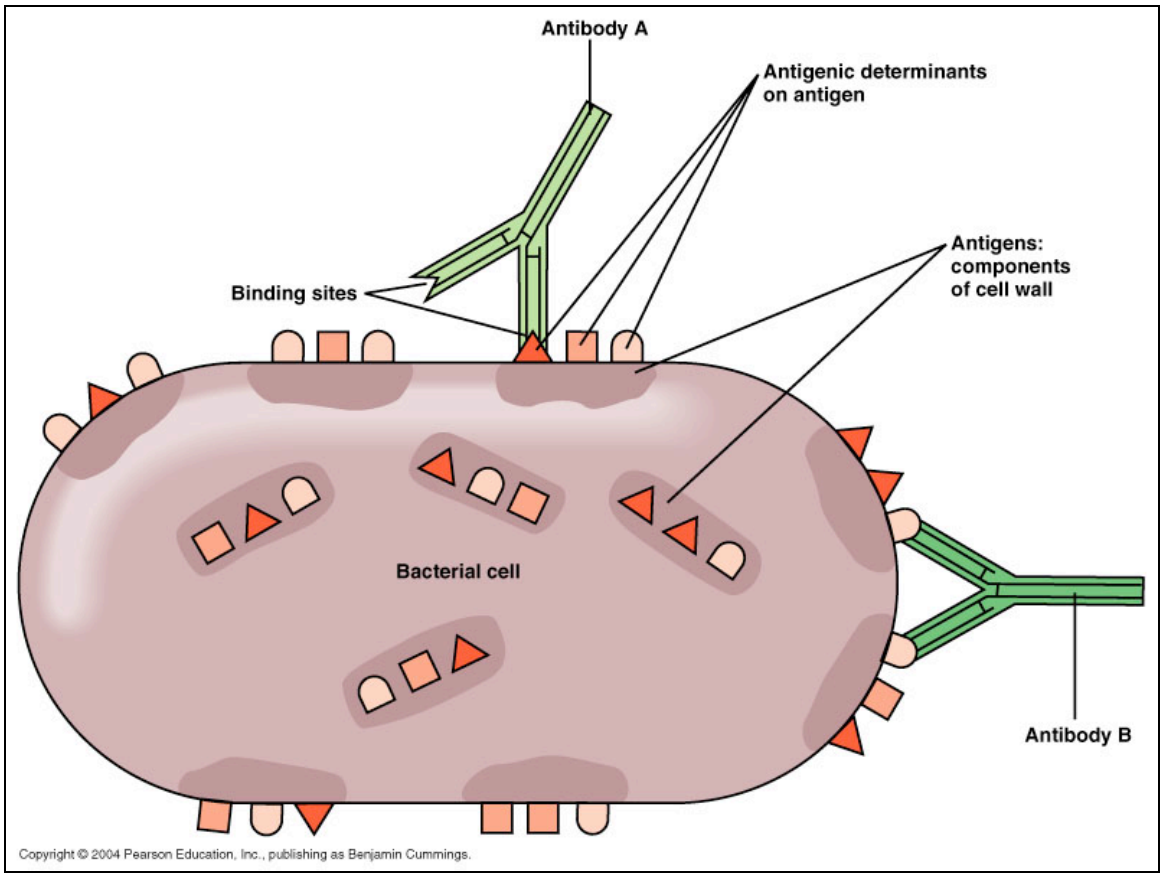
One potential application of this method is to isolate the Crenarchaeota whose sediment-preserved membrane lipids are extracted and correlated with the TEX_{86} paleothermometer proxy (Powers et al, 2004). These uncultivated archaea record surface temperatures in fresh and salt waters by slightly altering their membrane lipid structure with respect to the temperature of their surroundings (Damsté et al, 2002). Their lipid remains can be extracted from sediments to determine seasonal temperature variations, but only the surface water signal seems to be recorded (Wuchter et al, 2006). Additionally, the signal seems distorted in subpolar regions (Ho et al, 2014). Since the organisms are found throughout the water column, we would like to have a better understanding of how and why membrane composition appears to be vary (Woltering et al, 2012). Isolation and controlled temperature experiments using isolated species would provide insight about the control mechanisms for their membrane expression.

This procedure holds promising potential, but optimization is still needed. Initial immunofluorescence experiments demonstrated that anti-squalene antibodies could be bound to archaeal membrane without binding non-specifically to bacterial membrane. After fixation and partial digestion to enable antibody binding, cells remained viable for reproduction. However, cell counts and qPCR results indicated that the separation was not efficient, such that the Magnetized fraction was not enriched in archaeal cells. Statistical analysis indicated that there was a significant shift in proportions of the two taxa after magnetic separation, but it did not enrich for the target species. We observed agreement between both FISH and qPCR results with this analysis.

The demonstration of antibody specificity and viability of treated cells, both observed in this study, are two key components needed to validate this approach. Separation efficiency is the remaining barrier. Clumping of cells must be minimized in order to increase separation efficiency. Future work should explore extracellular polymeric substance removal or performance of the procedure in suspension, avoiding centrifugation.

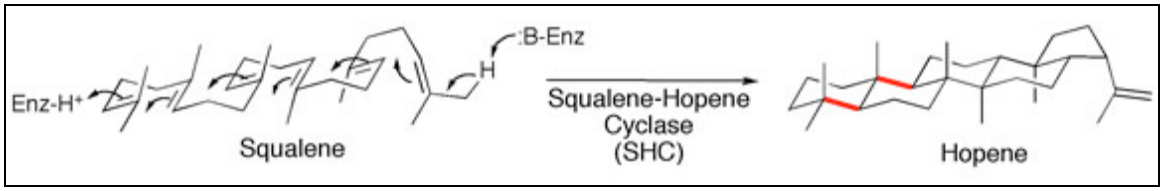
Figures

Figure 1



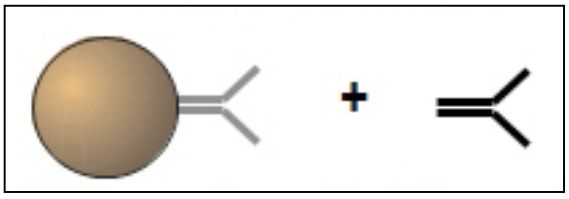
Cell with various antigens on its cell wall. Antibodies bind specifically to the antigens for which they are produced (Madigan, 2006).

Figure 2



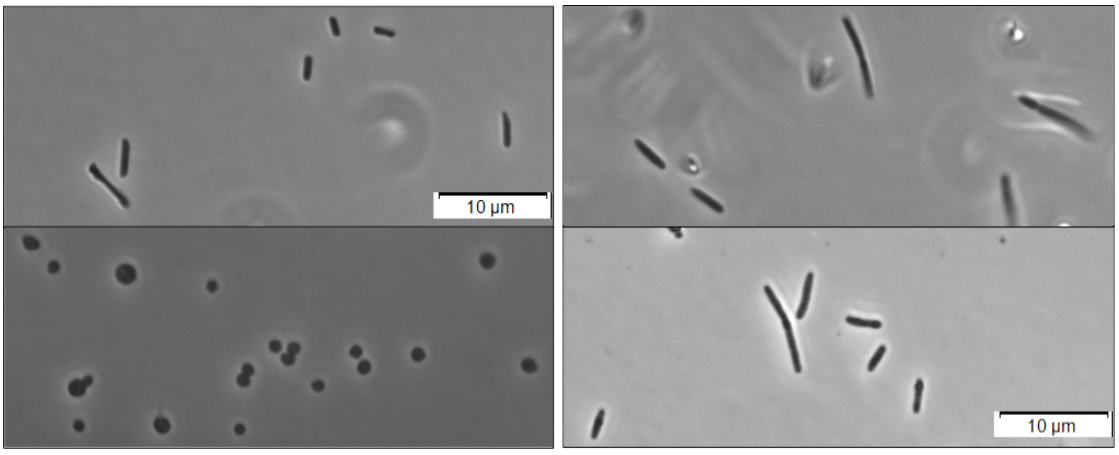
Formation of hopene from squalene. In this study, the two similar molecules were tested with the squalene-specific antibody, SQE #14 (UNC Chemistry Michel Gagné).

Figure 3



A paramagnetic bead coated with secondary antibody will bind to the squalene-specific primary antibodies (Life Technologies).

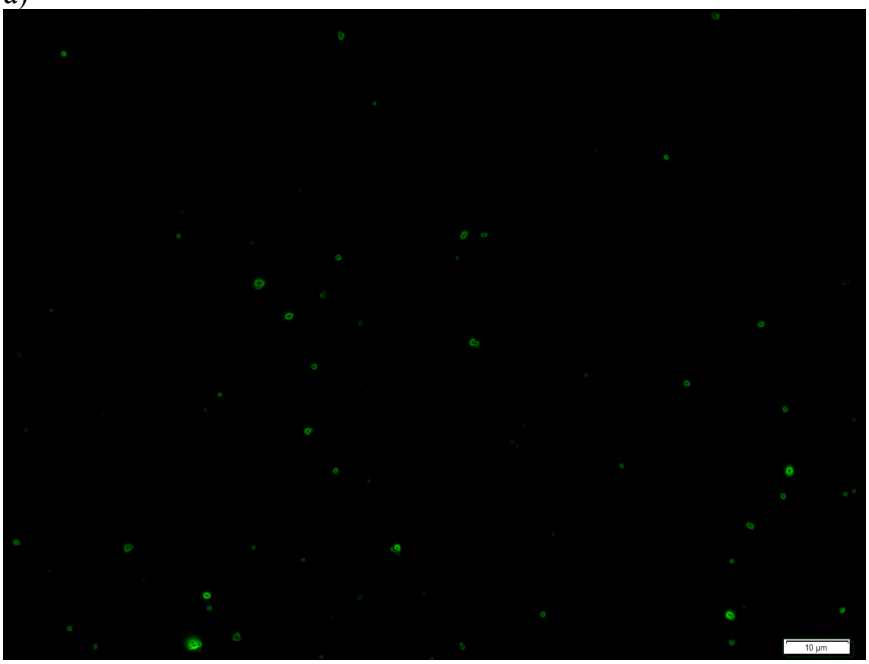
Figure 4



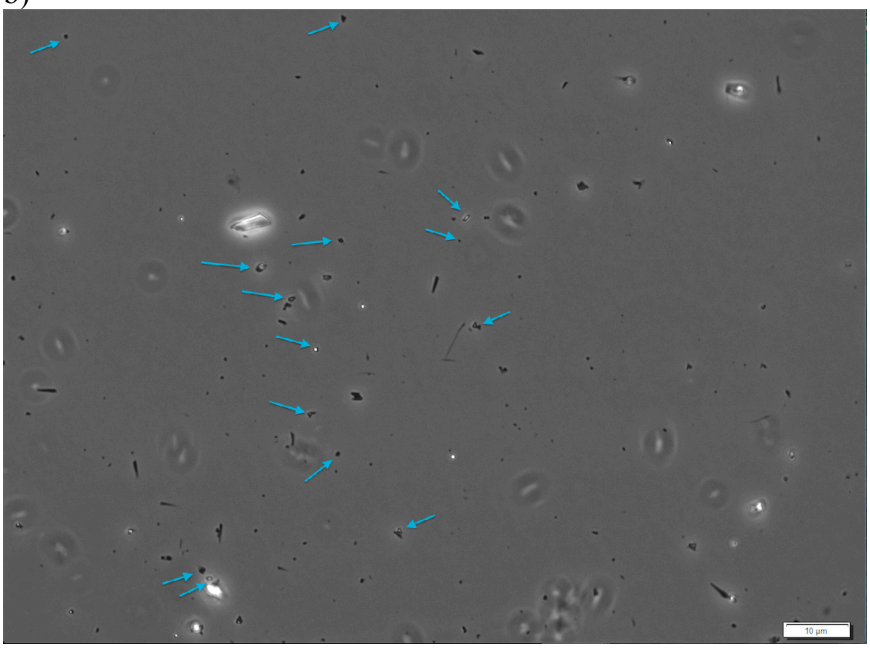
Effects of acetic acid treatment on the two test species, using phase-contrast microscopy: *Halobacterium* cells before (upper left) and after acetic acid fixation (lower left), and unaltered *Salinibacter* cells before (upper right) and after fixation (lower right).

Figure 5

a)



b)

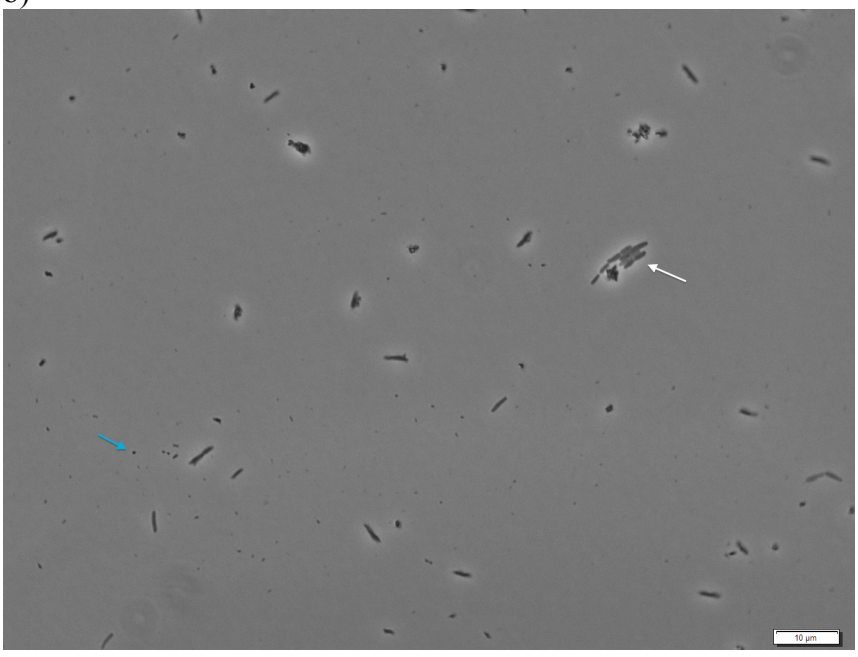


Immunofluorescence test of antibody specificity: Epifluorescent (a) and phase contrast (b) images of *Halobacterium* cells tagged with SQE#14 primary antibody (1:40) and secondary FITC fluorophore (1:1000). Arrows point to antibody-bound objects.

Figure 6

a)

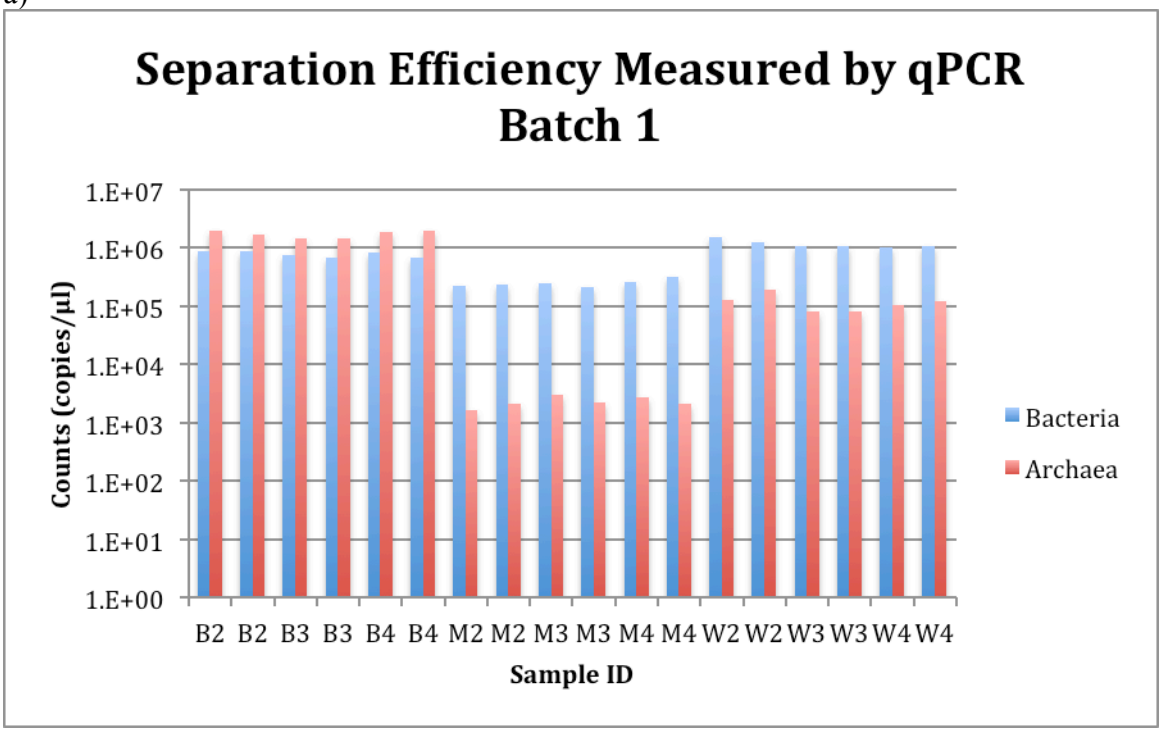
b)



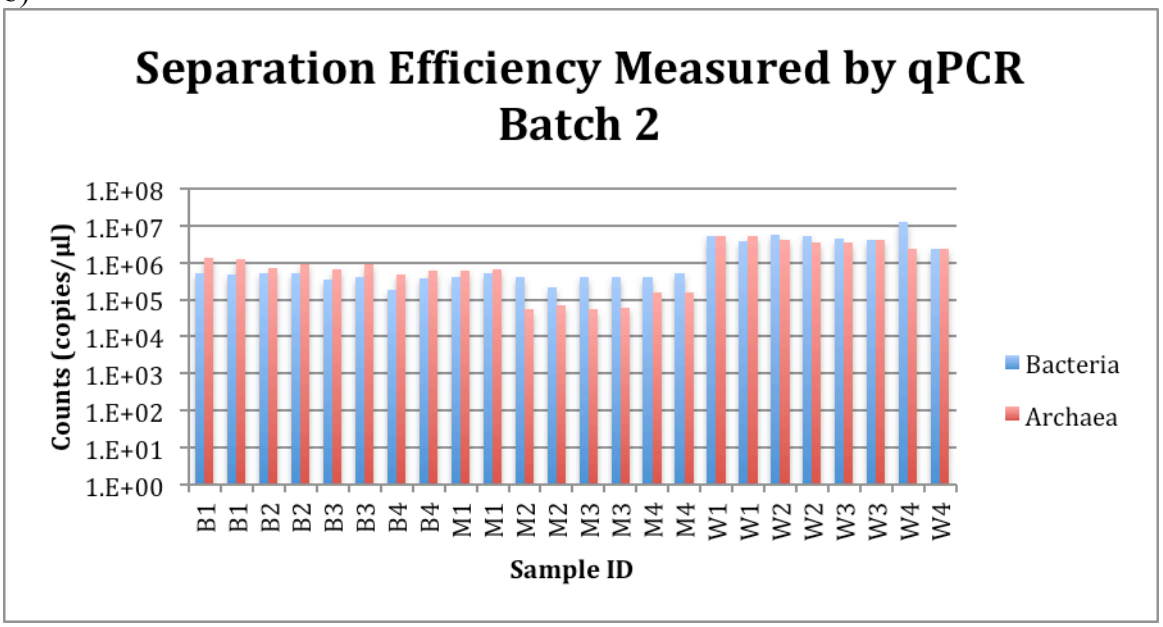
Immunofluorescence test of antibody specificity: Epifluorescent (a) and phase contrast (b) images of *Salinibacter* cells treated with SQE#14 primary antibody and secondary FITC fluorophore. Blue arrow points to brightest spot on epifluorescence image. White arrow points to a cluster of cells. No fluorescence is visible in the region of the cell cluster. Fluorescent images were taken with same exposure; background subtraction, outline detection, and sharpen filters were applied with same values.

Figure 7

a)

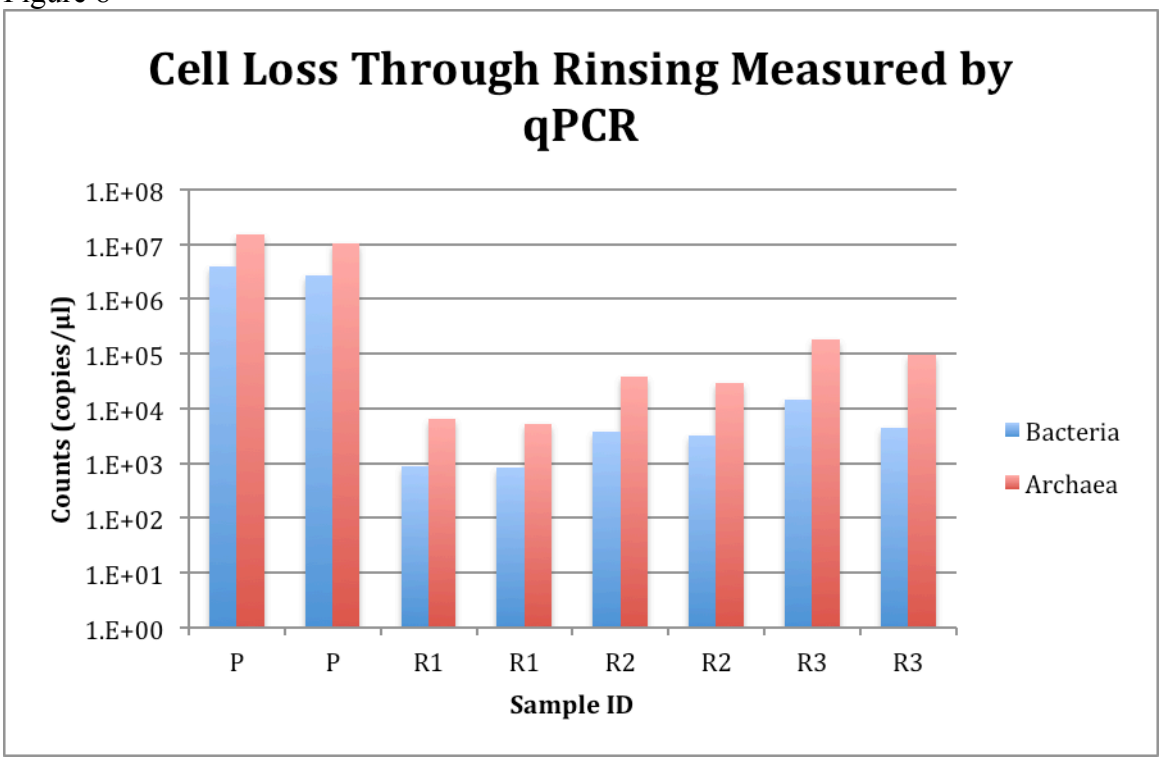


b)



qPCR results from two separation batches, before and after optimization: Batch 1 results (a) comprise triplicate separations. Batch 2 results (b) include triplicate separations 1-3 in addition to one singular separation. qPCR was performed in duplicate for each sample fraction following magnetic separation.

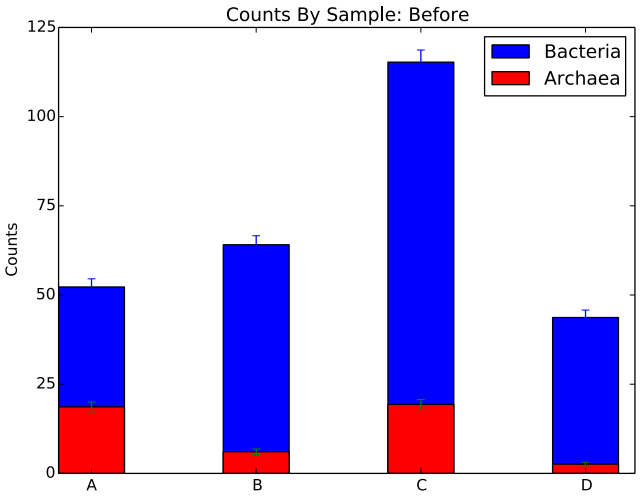
Figure 8



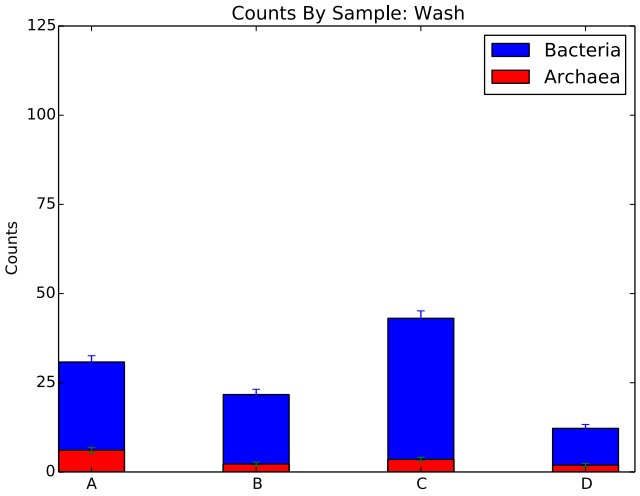
Quantification of cell loss during separation procedure by clade: Duplicate qPCR results of cell quantities before treatment and cluster filtering (P-samples), followed by samples of each rinse supernatant. R1 = acetic acid, R2 = ddH_2O, R3 = PBS.

Figure 9

a)

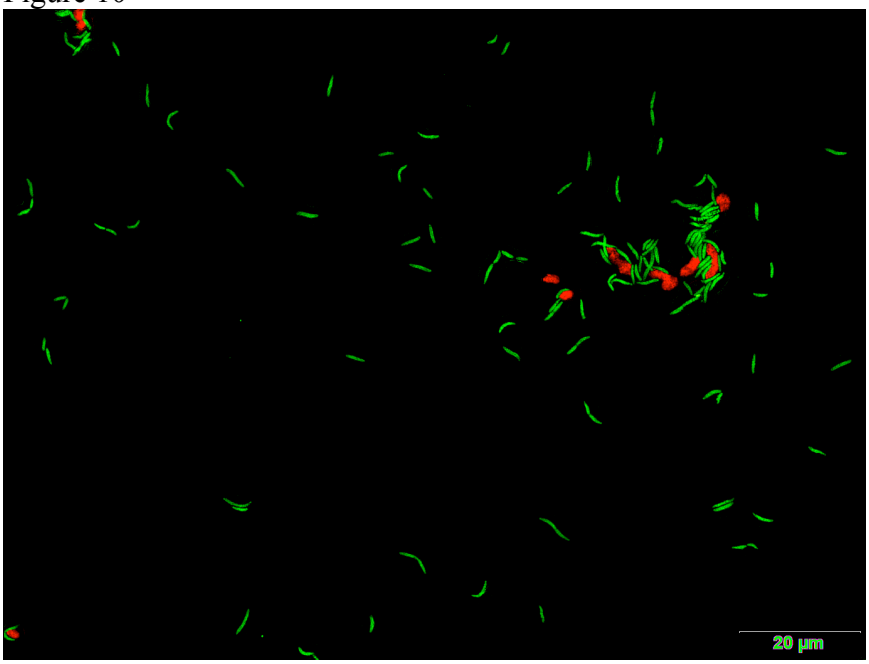


b)



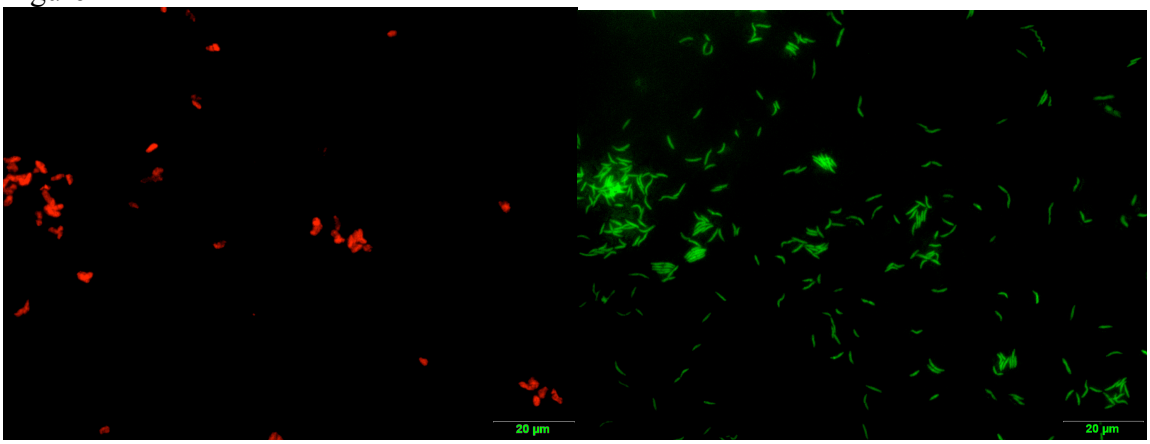
Summary of manual FISH cell counts from Before (a) and Wash (b) fractions for 4 samples of Batch 2 separations: A, B & C were completed side-by-side, in triplicate, starting from the same cultures. Archaeal depletion was expected to denote successful separation.

Figure 10



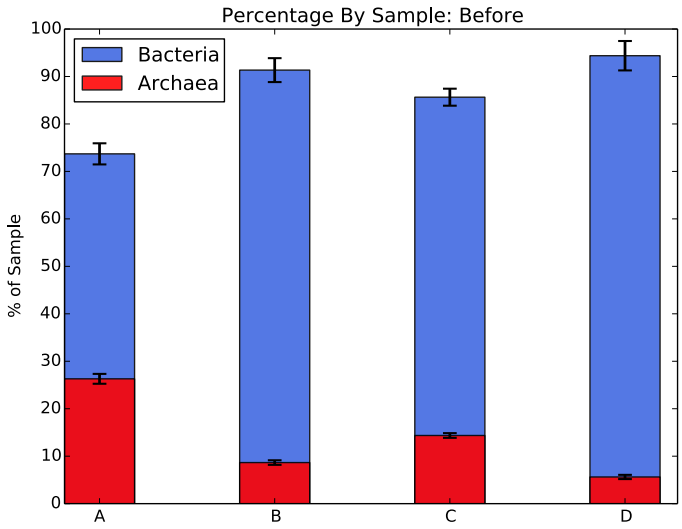
FISH image of a Wash fraction sample, with FITC (bacteria) and TXred (archaea) images overlaid: Bacterial cells (green bacilli) are found both free-floating and clustered, while archaeal cells (red, spheroidal) are mostly associated with cell clusters.

Figure 11

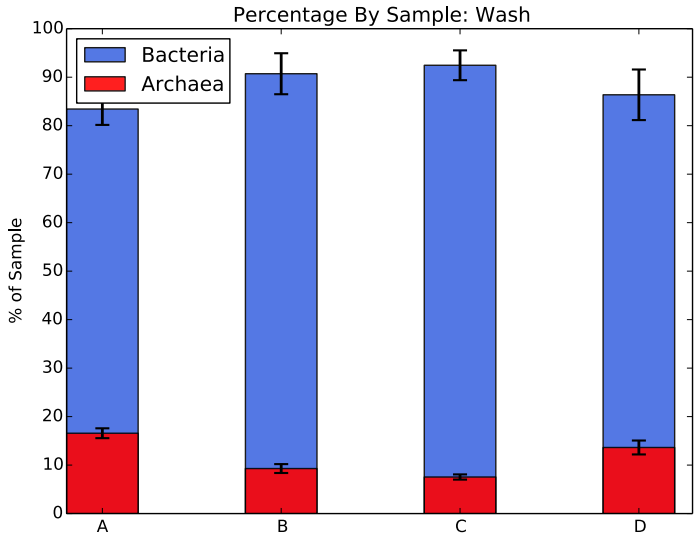


Fish images of a Before fraction sample: Archaeal cells (left) may be associated with cell clusters or exist as singular cells. The two images of archaea (left) and bacteria (right) from the same microscopic field of view (FOV) are overlaid to view cell associations in each fraction, as in Figure 10.

Figure 12
a)



b)



Summary of proportional FISH cell counts, calculated from numerical counts depicted in Figure 9: Compares percent compositions of archaeal vs bacterial cells from FISH counts in the Before (a) and Wash (b) samples.

Figure 13

a) **Slide 1**

	Before	Wash
Before	0	1.414 ns
Wash		0

+ p < .10 (q-critical[2, 18] = 2.4523362705)
* p < .05 (q-critical[2, 18] = 2.9711524428)
** p < .01 (q-critical[2, 18] = 4.070729555)

b) **Slide 2**

	Before	Wash
Before	0	2.081 ns
Wash		0

+ p < .10 (q-critical[2, 18] = 2.4523362705)
* p < .05 (q-critical[2, 18] = 2.9711524428)
** p < .01 (q-critical[2, 18] = 4.070729555)

c) **Slide 3**

	Before	Wash
Before	0	1.822 ns
Wash		0

+ p < .10 (q-critical[2, 18] = 2.4523362705)
* p < .05 (q-critical[2, 18] = 2.9711524428)
** p < .01 (q-critical[2, 18] = 4.070729555)

Tukey Honest Significant Difference Table of q-statistics comparing Before and Wash counts on each FISH slide.

Figure 14

a) **Batch 1**

	Before	Magnet	Wash	
Before	0	16.343 **	1.498 ns	
Magnet		0		14.845 **
Wash			0	

+ p < .10 (q-critical[3, 15] = 3.13894287274)
* p < .05 (q-critical[3, 15] = 3.67194020829)
** p < .01 (q-critical[3, 15] = 4.83414977432)

b) **Batch 2**

	Before	Magnet	Wash	
Before	0	4.971 **	0.810 ns	
Magnet		0		4.161 *
Wash			0	

+ p < .10 (q-critical[3, 15] = 3.13894287274)
* p < .05 (q-critical[3, 15] = 3.67194020829)
** p < .01 (q-critical[3, 15] = 4.83414977432)

Tukey Honest Significant Difference Table of q-statistics comparing Before, Magnetized, and Wash counts from each qPCR batch. These results include all samples of Batch 1 and samples 1-3 of Batch 2.

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