

*Growing
Halobacterium
sp. NRC-1*

Michael Enos
APS Honors Biology Research Lab VI
Twelfth Grade
Dr. Joseph Francis
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In collaboration with
Dr. J. Francis and Jordan Thiesen

Growing *Halobacterium sp.* NRC-1

Objectives

- Work with a research team
- Learn to grow colonies of *Halobacterium sp.* NRC-1
- Gain experience working in a research laboratory and using techniques from microbiology
- Begin experiments on how various substances, both natural and artificial, and environmental conditions affect *Halobacterium sp.* NRC-1

Introduction

Halobacterium sp. NRC-1 is a salt-loving extremophile from Kingdom Archaea. Archaeans have genetic and biochemical similarities to both prokaryotes and eukaryotes. Many Archaeans are extremophiles, meaning that they are able to survive extreme conditions that would quickly kill other microbes. These conditions include extreme heat, extreme cold, or, in the case of *Halobacterium sp.* NRC-1, extreme salinity. *Halobacterium sp.* NRC-1 can survive in salt concentrations of 3–5M, conditions that would quickly kill any other microbes. *Halobacterium sp.* NRC-1 can also tolerate high levels of UV light and other forms of solar radiation, high temperatures, and low concentrations of oxygen and nutrients.

Halobacterium sp. NRC-1 is able to survive hypersaline conditions because its cells take in KCl to keep the concentration of KCl inside the cell equivalent to the concentration of NaCl outside the cell. This prevents the osmotic stress that kills other microbes subjected to such hypersaline solutions. Because other microbes are, for the most part, unable to grow in the hypersaline solution *Halobacterium sp.* NRC-1 is grown in, keeping a solution of *Halobacterium sp.* NRC-1 sterile is relatively easy. This semester, research focused on how to grow *Halobacterium sp.* NRC-1 using both the spread method and the streak method and how to obtain large individual colonies of *Halobacterium sp.* NRC-1.

Materials

- *Halobacterium sp.* NRC-1 solution from Carolina Biological Supply Company (Burlington, North Carolina 27215; www.carolina.com)
- Plates of *Halobacterium sp.* NRC-1 from last semester's Medical Microbiology lab
- Spreaders
- Deionized water
- Graduated glass beakers
- Graduated cylinders
- Petri dishes
- Refrigerator
- Test tube holder
- Test tubes
- Trisodium citrate•2H₂O
- Bacto™ Tryptone (pancreatic digest of casein). 10kg. Ref# 211701; Lot #4121065 from Beaton, Dickinson, and Company (Sparks, MD 21152 USA; Le Pont de Claix, France).
- Oxoid peptone LP0037. Bacteriological Peptone IVD. Lot 535714. Expires 04/2012. 500 grams; from Oxoid LTD, Basingstoke, Hampshire, England.
- Agar (Criterion Dehydrated Culture Media Agar Bacteriological Grade; 500gw; Catalog #C5001; Lot #06310. Expires August 2011. Hardy Diagnostics)
- Autoclave (Harley Sterile Max)
- Stirring pad
- Stirbars
- Plastic pipettes
- Micropipettes
- Sycamore leaves
- Tobacco
- Paper towels
- Lined paper
- Plain scratch paper
- Digital camera attached to stand and connected to computer
- Computer for taking and storing photographs
- Dissecting microscope
- Incubator set at 42°C (we used two different ones over the semester)
- Spectrophotometer (Spectronic 200+ from ThermoSpectronic)
- Cuvettes for spectrophotometer
- 0.1N NaOH
- 1N HCl
- Inoculating loops
- Bunsen burner

Methods

Making NRC-1 liquid growing media: To make 1L of *Halobacterium sp.* NRC-1 liquid growing media, the following ingredients were mixed in less than one liter of deionized water:

- 250g NaCl
- 20g MgSO₄
- 3g Trisodium citrate
- 2g KCl
- 10g Bactopeptone (originally) or 10g Oxoid peptone (on 11/7/07 and subsequent dates)

The solution was then autoclaved for a liquids cycle in the autoclave (15–20 minutes) and allowed to cool to 65°C. 0.1mL of a trace metals solution was then added. The solution was then filled up to one liter, and the pH was adjusted to 7.2 using 0.1N NaOH and 1N HCl.

Making trace metals solution: To make 200mL of a trace metals solution, the following ingredients were added to 200mL of deionized water:

- 1.32g ZnSO₄•7H₂O
- 0.34g MnSO₄•H₂O
- 0.78g Fe(NH₄)₂SO₄•6H₂O
- 0.14g CuSO₄•5H₂O

Making salt agar plates: 10g of agar was added to 500mL of growing media while the solution was stirred. The new solution was then autoclaved for a liquids cycle on the autoclave (15–20 minutes). The solution was then poured into Petri dishes, where it was allowed to cool and solidify.

Making a 25% NaCl solution: To make 100mL of a 25% NaCl solution, 25g of NaCl were added to 100mL of deionized water.

Preparing plates (spread method): 100µL of a solution of *Halobacterium* was placed onto a salt agar plate and spread thoroughly with a spreader so that the solution was spread across the entire plate. Before placing the plates in the incubator, the *Halobacterium sp.* NRC-1 solution was allowed to soak into the agar for five minutes to an hour. The plates were then turned over and placed in the incubator.

Preparing plates (streak method): An inoculating loop was dipped in a solution of *Halobacterium* and was moved up and down across one side of the plate. The loop was then sterilized using a Bunsen burner. Starting at the extreme edge of the first pattern formed, the loop was moved up and down across another side of the plate perpendicular to the first side of the plate. The loop was then sterilized again using the Bunsen burner. This process was repeated for each of the two remaining sides of the plate. The purpose of the streak method is to dilute the

solution originally placed on the plate so that isolated colonies can be formed, even from an initially strong solution of bacteria.

Determining the optical density of a sample: The spectrophotometer was first autozeroed at a wavelength of 620nm using 2mL of a salt solution in a cuvette. At least 2mL of a solution of *Halobacterium* was placed in another cuvette, and the optical density of that solution was read and recorded. This optical density provided a good idea of the concentration of bacteria in the solution: the higher the optical density, the higher the concentration of bacteria in the solution. It also allowed us to make a solution one week that had approximately the same concentration of *Halobacterium* as one used the week before because it allowed one to compensate for the growth of the *Halobacteria* in the solution over the intervening week.

Observations

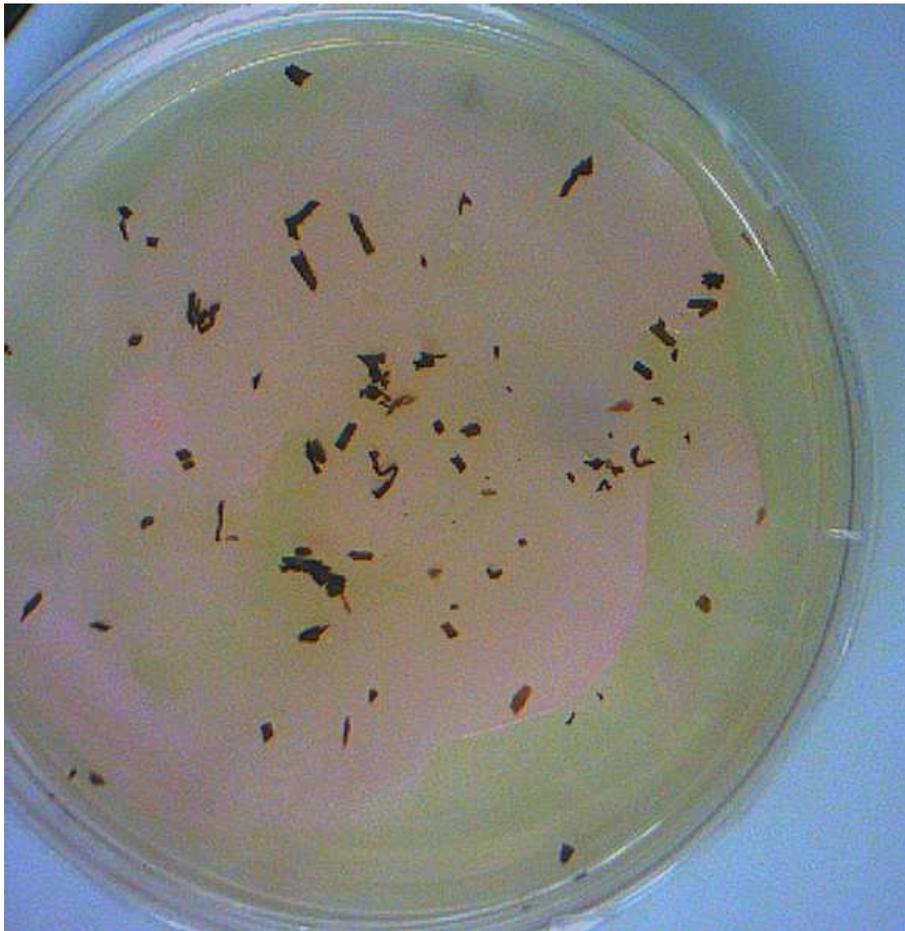
9/19/07: I transferred one colony of *Halobacterium sp.* NRC-1 from a plate from last semester's medical microbiology lab into a sterile test tube filled with *Halobacterium sp.* NRC-1 liquid growing media. I transferred another colony from the same plate into another sterile test tube filled with *Halobacterium sp.* NRC-1 liquid growing media. I then filled a third sterile test tube with *Halobacterium sp.* NRC-1 liquid growing media as a control. Sterile technique was observed throughout this experiment. No pictures were taken on this day.

9/26/07: I attempted to make plates by mixing 10g of agar with 500mL of *Halobacterium sp.* NRC-1 liquid growing media. However, the autoclave malfunctioned, the solution was not autoclaved properly, and the experiment had to be ended at that point. No pictures were taken on this day.

10/10/07: I made 100mL of a 25% NaCl solution. I calibrated the incubator to 42°C. I measured the optical density of one tube of *Halobacterium sp.* NRC-1 as 0.102. I made four plates of bacteria using the spread method. I added nothing to the first and second plates but added twelve to eighteen small pieces of dried sycamore leaves to the third and fourth plates. I let the agar soak into the third and fourth plates for about five minutes before adding the small pieces of dried sycamore leaves to them. I let all four plates sit for a total of one hour before turning them over. I was not able to put the plates in the incubator because the incubator had heated to a temperature of 58°C. No pictures were taken on this day.

10/17/07: I observed the plates that Jordan and I had made the previous week. On his first plate, Jordan put small pieces of tobacco on the plate five minutes after making it using the spread technique. There was a nice, non-uniform lawn of *Halobacterium* growth. The bacteria definitely seemed to be avoiding the tobacco pieces and the area immediately around them.

On his second plate, Jordan put small pieces of tobacco on the plate two hours after making it using the spread technique. There was a thinner but more uniform growth of bacteria on this plate than on the first plate. The bacteria did not seem to be avoiding the tobacco as consistently on this plate as they were on Jordan's first plate. In some cases, the bacteria did appear to be avoiding the tobacco pieces and the area immediately around them. In other cases, however, the bacteria appeared to even be growing right underneath the tobacco. A picture of this plate is shown below.



On his third plate, Jordan put small pieces of tobacco on the plate two hours after making it using the spread technique. On this plate, the tobacco looked longer, thicker, and strandier than on the other two plates. The bacteria had grown thickly and uniformly. While the bacteria

sometimes did appear to be avoiding the tobacco pieces and the areas immediately around them, in other cases the bacteria seemed to be growing right under the tobacco.

My first plate was a control plate; that is, no substances were added to the plate. The plate was made using the spread technique. The lawn of bacteria looked thinner than the lawns on Jordan's plates but was fairly uniform, with a few patches with a higher concentration of bacteria and a few patches with very few or no bacteria.

My second plate was also a control plate made using the spread technique. It was less homogenous than my first plate was. There were more and larger areas with no bacteria and no areas with a concentration of bacteria.

On my third plate, I added small pieces of dried sycamore leaves to the plate five minutes after making the plate using the spread technique. The bacteria growth was somewhat thin and not very homogenous. The bacteria did not seem to be avoiding the sycamore leaves, except near the edge of the plate where there were fewer bacteria to begin with. A picture of this plate is shown below.



I added small pieces of dried sycamore leaves to the fourth plate five minutes after making it using the spread technique. The bacteria growth was much more scattered than on any of the previous plates. Where the bacteria were growing, they were fairly thick. The bacteria did not seem to be avoiding the sycamore leaves or the areas around them. No red (mutated) bacteria were observed on any of the five plates.

The optical density of 2mL of salt water was 0.000. The optical density of a 1/10 solution of bacteria from the previous week was 0.243. To make a solution of bacteria roughly equal in concentration to the solution used on the previous five plates, I diluted 30.0 μ L of bacteria solution from tube B in 70.0 μ L of a 25% salt solution. I made two plates from this solution using the spread technique. I added small pieces of scratch paper to both plates about five minutes after adding the *Halobacterium* solution. I tried flipping the plates after twenty minutes. The agar ran on plate 1 but not on plate 2, so I flipped them both back over and let them sit out. Dr. Francis flipped them over and put them in the incubator later. When these plates were observed later, it was found that the *Halobacteria* were not growing under or near the small pieces of scratch paper. All *Halobacteria* from these plates were pink. Pictures from that day can be found in "My Pictures\Fall 2007\Archaeobacteria\10-17-07".

10/24/07: The optical density of a 25% salt solution was 0.001. The optical density of a full strength *Halobacterium* solution was 0.463. I made four plates using the spread method. I added small pieces of tobacco, lined paper, paper towel, and sycamore leaves to plates one, two, three, and four respectively after I had let the solution soak into the plate for about five minutes. When I observed these plates on 10/31/07, the bacteria were not growing under the substance or near it on any of the plates. Thus, the fact that the *Halobacteria* did not grow near the tobacco does not seem to have had anything to do with the chemical properties of the tobacco itself. Perhaps tobacco or similar porous substance affects the *Halobacteria* in some other way. It is plausible that a porous substance such as tobacco soaks up all the *Halobacteria* initially around it, leaving none to grow under or around the substance. All *Halobacteria* observed on these plates were pink. No pictures were taken on this day.

10/31/07: The optical density of a half-strength dilution of *Halobacterium* was 1.000. The optical density of a quarter-strength solution of *Halobacterium* was 0.732. This quarter-strength solution served as the first of seven serial dilutions, which I used to make seven plates using the spread method. No pictures were taken on this day. I observed these plates on 11/7/07 and

11/28/07. Plates one through three featured a fairly uniform lawn of *Halobacteria* with a few barren patches. Plate four featured a relatively uniform lawn of *Halobacteria* with a few pinpoint colonies in some of the barren patches. Plate five featured a lawn comprised of extremely dense pinpoint colonies. The barren patches in the lawn had a lower density of pinpoint colonies. Plate six featured dense pinpoint colonies throughout. All *Halobacteria* observed on these plates were pink. No pictures were taken on this day.

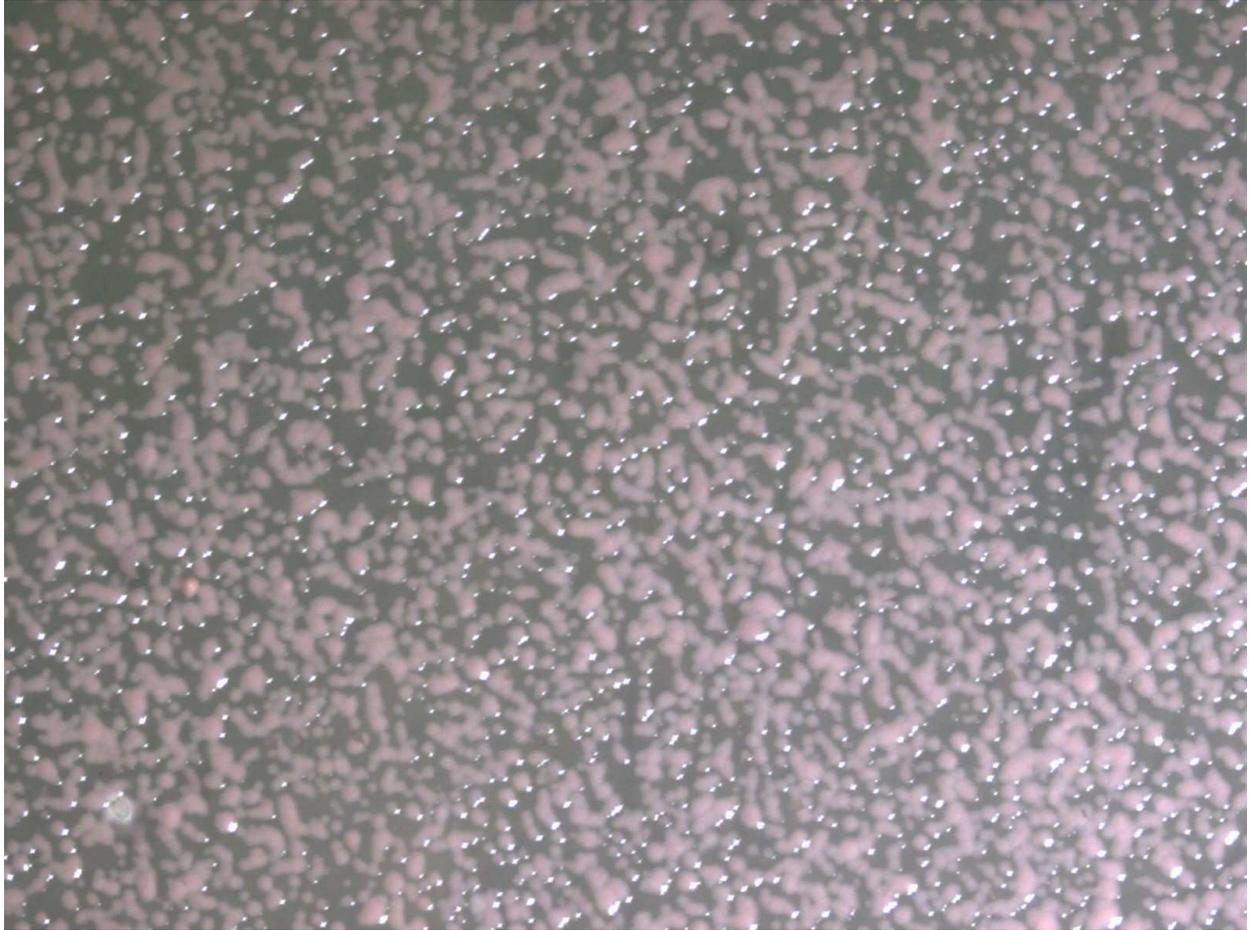
11/7/07: We made plates after adding 9.6 grams of agar to 480mL of *Halobacterium sp.* NRC-1 liquid growing media and autoclaving it. We then made one liter of NRC-1 liquid growing media but did not pH it, fill it up to one liter, or autoclave it. Three plates were made using the streak method. These plates were observed on 11/14/07 and 11/28/07. All three plates featured thick lines of *Halobacteria* at the beginning of the streak method and rather large isolated colonies at the end. All *Halobacteria* observed on these plates were pink. No pictures were taken on this day.

11/14/07: We pH'd the *Halobacterium sp.* NRC-1 liquid growing media to 7.2 using 0.6N NaOH and 1N HCl. I transferred a red (mutant) colony of *Halobacterium sp.* NRC-1 to a tube full of *Halobacterium sp.* NRC-1 liquid growing media using sterile technique. We made four plates. Plate one was made using the spread method and 100mL of the solution of mutant *Halobacterium*. Plate two was made using the spread method and 100mL of a 1:250 dilution of last week's sixth serial dilution. Plates three and four were made using the streak method and a highly concentrated solution of *Halobacteria* (undiluted from tube B). These plates were observed on 11/28/07. On plate one, only a few extremely small, barely visible colonies could be observed. These colonies appeared pink, though it was difficult to tell. Plate two was densely populated with pinpoint-sized colonies. Plates three and four featured lawn growth wherever the inoculating loop touched the plate. Plates two, three, and four contained only pink *Halobacteria*. When plate one was examined on 12/5/07, it was found to have a few small drops of a reddish solution, presumably containing red *Halobacteria* and possibly some pink *Halobacteria*. No pictures were taken on this day.

11/28/07: We made four plates. Plate one was made using 100μL of the solution of mutant *Halobacteria* and the spread method. Plate two was made from 100μL of a $\frac{1}{8}$ dilution of the fifth serial dilution from 10/31/07 using the spread method. Plate three was made from 100μL of a $\frac{1}{80}$ dilution of the fifth serial dilution from 10/31/07 using the spread method. Plate

four was made from 100 μ L of a $\frac{1}{800}$ dilution of the fifth serial dilution from 10/31/07 using the spread method. Plate five was made from 100 μ L of a $\frac{1}{8000}$ dilution of the fifth serial dilution from 10/31/07 using the spread method. These plates were observed on 12/5/07. On the plate of mutant bacteria, no individual colonies could be observed. There seemed to be streaks of a reddish solution, presumably containing some red *Halobacteria* and possibly some pink *Halobacteria* (it was impossible to tell because no colonies could be observed). No pictures were taken on this day. On the $\frac{1}{8}$ dilution plate, the bacteria formed a pink lawn with occasional isolated colonies. Some of the colonies were larger than others. All the *Halobacteria* on this plate were pink. The $\frac{1}{80}$ dilution plate was densely populated with pink pinpoint colonies, with a few streaks. On the $\frac{1}{800}$ dilution plate, the *Halobacteria* formed dense pink pinpoint colonies clustered in one region of the plate. On the $\frac{1}{8000}$ dilution plate, no colonies were visible with the naked eye or under the microscope. Salt crystals had formed on half the plate.

12/5/07: I examined nine plates of *Halobacteria* under the dissecting scope. The first plate I examined was plate two from 11/14/07. The *Halobacteria* formed densely packed pinpoint colonies. All colonies were pink as far as could be determined under the dissecting scope. The colonies sometimes ran together, but generally each colony was distinct. The area of one typical colony was approximately 0.1152mm². The area of another typical colony was 0.1137mm². Pictures of this plate can be found in “My Pictures\Fall 2007\Archaeobacteria\12-5-07”. They are pictures 1-1 and 1-2. Picture 1-2 is shown on the following page.



The second plate I observed was the plate of mutant (red) *Halobacteria* from 11/14/07. No individual colonies could be observed. There seemed instead to be a few drops of a reddish solution of (presumably red) *Halobacteria*. Because no colonies were observed, it was impossible to tell whether any pink *Halobacteria* were present. Since no colonies were observed, no colony sizes could be measured. A picture of this plate can be found in “My Pictures\Fall 2007\Archaeabacteria\12-5-07”. It is picture 2-1.

The third plate I observed was the plate of mutant (red) *Halobacteria* from 11/28/07. No individual colonies could be observed. There seemed to be streaks of a reddish solution of (presumably red) *Halobacteria*. Because no colonies could be observed, it was impossible to tell whether there were any pink *Halobacteria*. Moreover, no individual colony sizes could be measured. Pictures of this plate can be found in “My Pictures\Fall 2007\Archaeabacteria\12-5-07”. They are pictures 3-1 and 3-2. Picture 3-2 is shown on the following page.



The fourth plate I observed was the streak plate from 11/7/07. Almost the entire plate was covered in salt crystals. The *Halobacteria* on the salt crystals were red, while the *Halobacteria* that were not on the salt crystals were pink. It is not certain whether the red *Halobacteria* on the salt crystals were a result of a mutation (perhaps brought about by the stress of the hypersaline environment) or a result of a physical change (due, for example, to dehydration). The size of one typical pink colony was 2.45mm². The size of another typical pink colony was 3.9243mm². The size of a typical red colony could not be determined because no individual red colonies were observed. All the red *Halobacteria* observed formed a semi-homogeneous streak. Pictures of this plate can be found in “My Pictures\Fall 2007\Archaeobacteria\12-5-07”. They are pictures 4-1, 4-2, 4-3, 4-4, 4-5, and 4-6. Picture 4-2 is shown on the following page.



The fifth plate I observed was the $\frac{1}{8}$ dilution from 11/28/07. The *Halobacteria* formed a pink lawn with occasional isolated colonies. Some of these colonies were significantly larger than the others. The size of one of the larger colonies was 0.1772mm^2 . The size of one of the smaller colonies was 0.0092mm^2 . All *Halobacteria* observed were pink. Pictures of this plate can be found in “My Pictures\Fall 2007\Archaeobacteria\12-5-07”. They are pictures 5-1 and 5-2.

The sixth plate I observed was the $\frac{1}{80}$ dilution from 11/28/07. The *Halobacteria* on this plate were densely packed pinpoint colonies with a few streaks where the colonies ran together. The size of a large colony was 0.0557mm^2 . The size of a moderate-sized colony was 0.0285mm^2 . The size of a small colony was 0.0036mm^2 . The size of a particularly large colony found near one of the streaks was 0.4584mm^2 . Pictures of this plate can be found in “My Pictures\Fall 2007\Archaeobacteria\12-5-07”. They are pictures 6-1 and 6-2.

The seventh plate I observed was the $\frac{1}{800}$ dilution from 11/28/07. The *Halobacteria* were dense pink pinpoint colonies clustered in one region of the plate. The size of a large colony was

0.8929mm². The size of a small colony from another region of the plate was 0.0925mm². The size of another large colony from the same region of the plate as the small colony was 0.8689mm². No red *Halobacteria* were observed. Pictures of this plate can be found in “My Pictures\Fall 2007\Archaeobacteria\12-5-07”. They are pictures 7-1 and 7-2.

The eighth plate I observed was the $\frac{1}{8000}$ dilution from 11/28/07. No colonies were visible with the naked eye or under the microscope. Salt crystals had formed on about half the plate. No pictures were taken of this plate.

The ninth plate I observed was another streak plate from 11/7/07. The *Halobacteria* on this plate formed thick streaks with large individual colonies at the end. All *Halobacteria* observed were pink. The size of an average colony was 5.6621mm². Other colony sizes observed were 5.8957mm², 5.2891mm², and 6.8759mm². In another region, colony sizes of 4.9750mm² and 1.4013mm² were observed. Pictures of this plate can be found in “My Pictures\Fall 2007\Archaeobacteria\12-5-07”. They are pictures 9-1, 9-2, 9-3, and 9-4. Picture 9-2 is shown below.



Conclusion:

During this semester, a procedure for growing *Halobacteria sp. NRC-1* was developed. Many plates of *Halobacteria* were successfully made using differing concentrations of *Halobacteria* solution. Both the streak method and the spread method were used. The spread method yielded more bacteria, but tended to result in a homogenous lawn of bacteria at high concentrations. This lawn may not be the best platform for future experiments. For example, it would be difficult to determine the effects of a potential mutagen on *Halobacteria* if the mutagen was added to a lawn of *Halobacteria* because few if any distinct colonies of *Halobacteria* would be present and any kind of quantitative analysis would be extremely difficult. The streak method, on the other hand, results in large, individual colonies. However, while its colonies are the largest, there are also very few distinct individual colonies. Thus, the streak method too may not provide a good “platform” for future experiments because there would be too few distinct colonies on which to perform quantitative analysis. Perhaps the best platform for future experiments is using the spread method with a solution of *Halobacteria* of a relatively low concentration. When that procedure was used this semester, it resulted in densely packed pinpoint colonies of *Halobacteria*. These colonies could, however, be counted. Perhaps the best dilution used this semester was the $\frac{1}{800}$ dilution from 11/28/07. When the spread method was used with this dilution, a densely packed cluster of *Halobacteria* was formed on one part of the plate. This dilution, combined with the spread method, resulted in a few hundred distinct pinpoint colonies. That is enough colonies to perform quantitative analysis on, but not so many that it becomes overwhelming (as it would be, for example, if quantitative analysis had been performed on the $\frac{1}{80}$ dilution, where thousands of pinpoint colonies were spread across the entire plate).

During the entire semester, red *Halobacteria* were only observed under two conditions. The first condition was when a colony of red *Halobacteria* from last semester was grown in NRC-1 liquid growing media and then plated onto two different plates over two weeks. In that case, however, no red colonies were observed because no colonies were observed. Rather, a reddish solution (presumably of red and possibly pink *Halobacteria*) was observed on both plates. The second time was when salt crystals formed on a plate. The *Halobacteria* on top of the salt crystals had turned red. However, it was uncertain whether the change was due to a mutation or had a physical cause (such as dehydration). No individual red colonies were observed the

entire semester, and none of the pink colonies could definitively be said to have mutated to form red colonies.

The size of the largest colony on a plate was inversely proportional to the concentration of the solution. This decrease followed the shape of roughly a $1/x$ graph as long as the concentration was strong enough for any colonies to form. While the cause of this reduction in colony size is unknown, there are a few possibilities. The *Halobacteria* may release quorum sensors that allow them to “know” how many other *Halobacteria* are in their general region. Thus, the presence of many *Halobacteria* in a highly concentrated solution may reduce the maximum colony size.

Another possibility is that there is a substance in the media in the plates themselves that limits the maximum colony size. This semester, we started making growing media with tryptone. By the end of the semester, we acquired and switched to peptone, which was recommended to us by a leading researcher of *Halobacteria*. However, all the plates were made from the growing media made using the tryptone. The use of tryptone instead of the higher-quality peptone for the plates may be another reason that the colonies were not large. It is possible that the plates, made with the poorer-quality tryptone, were not providing enough food for the bacteria. It is also possible that other, unknown inhibitors could be present in the media.

Next semester’s research could focus on increasing the colony sizes in plates made using the lawn method. One way to search for an inhibitor released by the bacteria is to spin down a tube of a solution of *Halobacteria*, remove the supernatant, and add the supernatant to a normal plate of pink *Halobacteria* and see whether the supernatant inhibits the growth of these bacteria. Another option for research next semester would be to add different chemicals to plates of pink *Halobacteria* in an attempt to cause the *Halobacteria* to mutate. This semester has laid the foundation for future research that will reveal more about this little-known bacterium that clearly displays God’s handiwork in its remarkable ability to survive in harsh environments.