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Acknowledgements and Dedication

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THE RELATIONSHIP BETWEEN HALOTOLERANCE AND IONIZING RADIATION RESISTANCE OF ANTARCTIC BACTERIA
Thesis Abstract- Idaho State University (2019)

An interconnection between microorganisms capable of growing under hypersaline conditions and their effectiveness in surviving high doses of ionizing radiation has been previously demonstrated. It has been proposed that radiation resistance is the result repair mechanisms that deal with damage resulting from desiccation. We isolated 955 organisms from samples collected from hypersaline ponds in Antarctica. The ability of these organisms to grow in broth containing 0-25% NaCl was assessed. The ionizing radiation tolerance of a select number of organisms was determined by the use of an electron beam linear accelerator. Those organisms with a wide range of growth in salt were able to withstand moderate or high levels of radiation. Therefore, using salinity to screen for organisms that grow well in a wide range of salt concentrations may allow for finding organisms that are also tolerant to radiation, and may perhaps have unique mechanisms for their tolerances.

There were vast differences in tolerance to both salt and radiation among isolates identified as the same organism. This shows that genes and proteins are evolving to provide tolerance at a faster rate than the 16S ribosomal DNA is changing, and has major implications for analyses where 16S sequencing is used to identify community structure, since divergent evolution may have rendered some of the individuals completely different than the commonly accepted type species.

Key Words: salt, radiation, desiccation
Chapter 1

Introduction

Extreme Environments

Human life can only exist under a very limited range of conditions. If these conditions are altered and our bodies cannot adapt, we die. Oxygen is crucial to our survival; deprived of this, within minutes, our bodies begin shutting down. Our survival under conditions including extreme temperatures, pH, and pressure is very limited as well. There are many environments in which we could not exist. However, this does not mean that these environments are devoid of life. Perhaps because of our own anthropocentrism, we see environments which would not be hospitable to us as extreme.

Environments where nothing else would be able to survive, termed “extreme environments,” some microbes not only find hospitable, but thrive in. Researcher and author T. D. Brock described extreme environments as being those “in which species diversity is low and some taxonomic groups are missing” [2]. Extreme environments can be defined by various conditions, such as extreme temperature, pH, pressure, dryness, radiation, as well as salinity. Any organism that can survive in an extreme environment is termed an “extremophile.” Members of the Bacterial and Archaeal Domains, and even some Eukaryotes, live under conditions much different than our own.

Many environments have multiple extreme components. For example, Mono Lake, located in the Sierra Nevada Mountains of California, is not only hypersaline, containing approximately 84 to 94 g of salt per liter of water, but at a pH of 9.8, is extremely alkaline as well [3]. Not only are many different types of Bacteria and
Archaea present, but more complex organisms such as brine shrimp and alkali flies also flourish [3].

Another example of an extreme environment is that of Rio Tinto in Spain. Rio Tinto, also known as the “River of Fire,” is both highly acidic, with a pH of approximately 2, as well as very rich in heavy metals (per liter of water, it contains 3-20 g of iron alone). There is an enormous amount of biological diversity within the river, as there are several types of organisms representing each of the three Domains [4].

Yellowstone National Park, known for its extreme environments, contains thousands of geothermal pools with a variety of conditions, including very high temperatures, varied pH, and heavy metal content. Organisms from all three Domains of life are found in these pools [5, 6].

Of all the extreme environments on earth, perhaps the most well-known is Antarctica, as it is the coldest, highest, driest, and windiest place on earth [7]. The populations of microbes found within the ponds and ice of Antarctica are both extensive and diverse, branching across all three Domains of life [8-11]. Even though these ponds and lakes are very close in proximity, they are radically different from one another in terms of pH, salinity, and ion content and concentration (Fig. 1). This allows us to eliminate the variables they share, such as elevation and temperature, and study only those specifically attributed to each pond, making Antarctica an ideal place to study the diversity of extremophiles.
Organisms that can live under multiple extreme conditions are termed polyextremophiles. Just as polyextremophiles can be found in all different types of extreme environments, they can also be found in all Domains of life, including Eukarya. As an example, the tardigrade, or water bear, a well-known eukaryotic polyextremophile, is known to live under both very high and very low temperatures, as well as high pressure, radiation, desiccation and salinity [12].

Aside from our egocentric view that environments inhospitable to us are extreme, the fact is that these abnormal conditions may lead to cellular damage. This damage can include denaturation of proteins, organelle damage, and double-strand DNA breaks.

FIG. 1. Various salt ponds located on Bratina Island, Antarctica [1]
High salinity, high radiation, and desiccation are three stressors that can cause extensive damage to cells.

**High Salinity**

There is no clear definition as to what constitutes a hypersaline environment. Varying definitions include that it is: (i) any environment that contains a certain concentration of salt [13], (ii) an environment in which the high salinity makes it inhospitable to most organisms [14], (iii) an environment in which the salinity is much greater than that of seawater [15], or (iv) that high salinity alone does not constitute an extreme environment, but, that it must also be accompanied by other extreme factors such as elevated temperatures and/or high levels of radiation [16]. Even though the definition of a hypersaline environment is unclear, what is clear is that the environment must contain enough salt that most organisms are unable to survive.

Salinity is not found in just one type of environment; it can be found in both aquatic and non-aquatic environments, such as soil. The idea that in order for soil to be considered saline, it must contain greater than 0.2% soluble salts, appears to be widely agreed upon [17, 18]. While NaCl is the most abundant salt found in the environment, saline environments are not limited to one particular type of salt [18, 19]. The Dead Sea, for example, is a highly saline environment, containing multiple cations, including Mg$^{2+}$, Na$^+$, Ca$^{2+}$, and K$^+$, and the anions Cl$^-$, and Br$^-$ [19, 20].

Hypersaline environments can arise due to various conditions, such as the evaporation of water from seawater or the dissolution of salts in freshwater. The evaporation of seawater is a very common cause of high salinity environments. As the water evaporates, the concentration of salt rises. If water is added back into the system,
the concentration will decrease. There is a constant fluctuation in the salt concentration as water is gained and lost. Another cause of hypersaline environments is the dissolution of salts in freshwater. This occurs when either loss of water or addition of salts shifts the equilibrium of salt and water. When salt is in equilibrium with water, the salt molecules split apart and form bonds with the water molecules. At this point, the salt will go into solution. However, there is a saturation point at which no more salt will dissolve in the water. When this saturation point is reached, the salt molecules will release from the water molecules and rejoin, once again forming a solid state. Dissolution is commonly seen in environments in which there is little rainfall, high evaporation, and no connecting water sources, such as other lakes, rivers, or oceans. Deserts fit this description well, as they have little rainfall and very dry winds. Freshwater systems are dominated by different ions than those found in seawater. In freshwater systems, calcium, magnesium, and sulfate are mainly seen, in contrast to the sodium and chloride which are typically found in oceans [19, 20].

While most organisms cannot tolerate high salt concentrations, there are some that not only tolerate it but actually require it. Halotolerants, as the name implies, are organisms that can tolerate high salt concentrations but grow best under less saline conditions. Halophiles, however, are organisms that prefer or even require high salt concentrations for their survival [21, 22]. Organisms that strictly live in extremely saline environments generally do so because high solute concentrations are required for the stability and activity of their enzymes and cellular integrity. Halophiles are not confined to one genus, or even one Domain; they can be found across all three Domains of life [20, 23].
Halophiles are generally placed into one of two groups: moderate halophiles or extreme halophiles. Moderate halophiles (such as the Archaeon *Haloferax volcanii*) generally live in environments containing salt concentrations between 0.5 and 2.5 M NaCl (3-15% NaCl), but can survive in concentrations as high as 3.5 M NaCl [22, 24]. Extreme halophiles (*Halobacterium salinarum*, another Archaeon, is one example) generally cannot live in environments containing salt concentrations lower than 2 M NaCl, and prefer concentrations of 2.5 M to 5.2 M NaCl (15-30% NaCl) [15, 22, 25, 26]. Halotolerant organisms, on the other hand, are able to grow in relatively high salt concentrations (approximately 8% NaCl or more) as well as in the absence of any salt [24].

**Radiation**

Radiation, and ultraviolet (UV) radiation in particular, is another stress that organisms can encounter in the natural environment. Some of the radiation we encounter in the environment is man-made and comes from sources such as medical equipment and radioactive uranium (which is used to create electricity), as well as from consumer products such as tobacco, fertilizer, and welding rods. Natural radiation can be either terrestrial or extraterrestrial [27]. Terrestrial radiation, or radiation that originates on Earth, comes from radioactive elements that are found in igneous and sedimentary rocks, as well as from other natural sources such as radon gasses. Extraterrestrial radiation, or radiation from space, occurs when radiation from beyond the Earth’s atmosphere reaches and breaks through the atmosphere. As the cosmic rays pass through the Earth’s atmosphere, they collide with molecules (mainly oxygen and nitrogen) to create radioactive particles which can then be distributed into the air and to the Earth’s surface
Unlike desiccation and salt-tolerance, radiation-tolerance is inherently different in that there is no known advantage to being radiation-resistant in the natural world, as high levels of radiation are not naturally encountered. Yet some organisms, including *Deinococcus sp.*, *Halobacterium sp.*, tardigrades, some algae, and some yeast, are known to tolerate high levels of radiation. So, this raises the question: Where does this ability for repairing damage caused by ionizing radiation originate? Many believe that since radiation-resistance cannot be an adaptation to encountering natural radiation in the environment, it must be an incidental use of the cell’s DNA repair capabilities. More specifically, radiation-resistance exists because those repair capabilities that are used to provide the cell with desiccation-resistance also confer radiation-resistance [13, 29-31].

**Desiccation**

Desiccation results when there is significant loss of moisture from an environment, and in turn, the organisms inhabiting that environment. While most organisms require water in order to live, some organisms are able to survive for extended periods of time in its absence. This phenomenon is known as anhydrobiosis, or “life without water.” Desiccation-tolerant organisms are found in all three Domains of life [32]. Organisms such as tardigrades, as well as some nematodes, yeast, algae and Bacteria, can tolerate anhydrobiosis at any stage in their life, while others can only tolerate it at certain stages. Endospores and seeds of plants tolerate anhydrobiosis very well, yet the vegetative Bacteria and plants they are derived from cannot go without water for extended periods [33-36].
Even though desiccation is thought of most when it comes to hot environments, it is not limited by temperature. Desiccation can and does occur in cold environments as well. For example, as mentioned earlier, in spite of being covered by ice and surrounded by water, Antarctica is one of the driest places on earth. Winds, solar radiation, and salinity can account for environments becoming desiccated.

**Types of Damage Caused by These Extreme Environments**

Desiccation and ionizing radiation are found to cause not only single-strand breaks and DNA crosslinks, but also more deleterious DNA double-strand breaks (DSBs) [37, 38]. These breaks can also occur under high saline environments, through the formation of reactive oxygen species (ROS). It is well known that ROS accumulation induced by salt stress is one cause of oxidative damage to a cell [39, 40].

Upon exposure to IR, free radicals can form in the cell via radiolysis of water. One radical in particular, the hydroxyl radical, is very dangerous to the cell as it is highly reactive and often interacts with DNA, resulting in DNA DSBs. Protein oxidation is also prevalent, which results in the disruption of protein function caused by carbonyl groups [16, 40, 41].

**Mechanisms of Resistance**

Just as there are many different types of cellular damages, there are also many different mechanisms for repairing this damage. High intracellular Mn\(^{2+}\)/Fe\(^{2+}\) concentrations have been linked to increased resistance to IR [30, 40, 42, 43]. It is postulated that the high intracellular Mn\(^{2+}\) leads to the scavenging of a specific group of ROS that targets proteins. If the proteins are not damaged, the cell can then work to
repair damage to the DNA [40, 43].

It is imperative that the cells either prevent ROS accumulation or remove ROS [39, 40, 44]. One way of preventing ROS accumulation is by the scavenging of ROS by intracellular halides. This process occurs in a two-step chemical reaction in which the halide reacts with the hydroxyl radical, and the radical is transferred to the halide from the hydroxyl. Scavenging of the radical provides protection against both nucleotide modification and carbonylation of protein residues [41]. Radiation damage in *Halobacterium* cultures was quantified by GC/MS and compared to *in vitro* damage of plasmid DNA, suspended in a potassium phosphate solution, supplemented with either KBr or KCl. It was determined that KBr provided greater protection than KCl. Bromide and chloride radicals are much less damaging to the cell than hydroxyl radicals because bromide and chloride are less reactive than oxygen. While salt did provide protection against IR damage, increasing the concentration of salt above 2 M did not increase protection [41].

In addition to the role of salt in protection against IR, many genes have also been linked to increased radiation-tolerance. One pathway known to play a vital role in homologous recombination DNA repair in many Bacteria is the RecBCD pathway. The RecBCD pathway is clearly not the only pathway, however, as it has been discovered that the model radiation-resistant organism, *Deinococcus radiodurans*, does not possess this pathway, nor does it possess any orthologous genes of that pathway [31]. Another pathway, the RecFOR pathway, has been found in many Bacteria, including *Deinococcus radiodurans* [31]. Xu, *et al.* (2008) created a null recO *Deinococcus radiodurans* mutant. The recO gene is believed to be an essential gene in the RecFOR pathway. This
mutant not only had a decreased growth rate, but was also very sensitive to radiation, confirming that DNA repair in *Deinococcus radiodurans* occurs mainly via the RecFOR pathway [45].

A number of mechanisms to protect against DNA damage have been demonstrated for haloarchaea. Enzymatic scavengers such as superoxide dismutase and catalase, as well as proteins such as thioredoxin (involved in redox homeostasis), have been shown to play a role in both scavenging and minimizing the number of superoxide radicals formed [44]. Bacterioruberin, a large carotenoid pigment, has also been shown to protect against both IR (*in vitro*), by reducing the amount of DNA damage and thymidine degradation by the scavenging of free radicals [16, 41, 46, 47], and high salt, by reducing osmotic stress [48]. This phenomenon has also been demonstrated *in vivo*, as colorless *H. salinarum* mutants lacking bacterioruberin have decreased survival to radiation [46].

Both osmotic and ionic regulation are vitally important for the survival of halophiles. The reason for this is that their membranes are permeable to water, and it is not energetically favorable for the organism to have to pump water into the cell to account for the water lost by the osmotic process [23]. Therefore, in order for halophiles to survive in the high salt environment in which they live, there must be a way to balance the osmotic pressure. One way of doing this is by using compatible solutes to establish an intracellular concentration in their cytoplasm that is equal to that of the surrounding environment. This is achieved by sequestering potassium and chloride, and at the same time, eliminating sodium, which balances out the osmotic pressure as the solute concentration inside the cell is the same in concentration (but not composition) as the
solute concentration outside the cell [23, 25, 41, 46, 47]. In order for this to work, however, all of the intercellular enzymatic machinery must adapt not only to the high solute concentration, but also to the counter-balancing solute that was used, in order for the proteins to remain folded and, therefore, active. Since these organisms require such extreme adaptation, they generally cannot survive in environments in which there is a low concentration of salt [23].

Halotolerant organisms, on the other hand, do not require such extreme methods of adaptation, and instead will either take up from their environment, or synthesize their own organic osmotic solutes [23]. Instead of increasing the salt in their cytoplasm, as the strict halophiles do, they will exclude the salt as much as possible, while they accumulate these osmotic solutes [23, 49]. There is a wide array of organic molecules that are used as osmotic solutes, such as sugars and derivatives, amino acids and derivatives, betaines, ectoines, poluols and derivatives, and occasionally, peptides [49, 50]. One of the big advantages of using osmotic solutes instead of salt to balance out the cytoplasm is that these molecules do not require extensive changes to the intercellular machinery and, therefore, cellular function is not inhibited [49]. In fact, it has been shown that the accumulation of these organic solutes not only balances out the osmotic pressure, but also increases protein stability [49].

Linking Desiccation and Ionizing Radiation

The link between desiccation and ionizing radiation has been well-studied [13, 29, 33, 41, 51-60] and many organisms embody this correlation. *Deinococcus radiodurans* is perhaps the most well-known organism for being able to survive high levels of radiation.
A lesser-known fact is that *Deinococcus radiodurans* is also highly desiccation-tolerant [13, 51]. Many studies have been conducted examining the relationship between desiccation-tolerance and ionizing radiation-resistance. In one study, 41 ionizing radiation-sensitive strains of *Deinococcus radiodurans* were desiccated and their survival measured both quantitatively and qualitatively. Through gel electrophoresis, it was demonstrated that not only did desiccation induce DNA DSBs but that this resulting damage was comparable to the damage that occurred when the cell was exposed to high doses of ionizing radiation (5200 Gy). It was also shown that the DSBs accumulate as a function of time. Even five days post-rehydration, these radiation-sensitive cells were unable to repair the DSBs caused by the desiccation. In fact, some of the desiccation-sensitive strains were 100 to 250-fold less viable than their desiccation-resistant parents, when rehydrated. This finding leads to the suggestion that *D. radiodurans*’ radiation-resistance is a consequence of its desiccation-resistance [29].

This correlation was also examined in a recent study, in which microarray analysis was used to compare relative transcript levels of *Deinococcus radiodurans* R1 cells after being subjected to either desiccation or ionizing radiation. Thirty-two out of the 72 loci up-regulated after desiccation were also up-regulated in response to ionizing radiation stress, demonstrating a commonality in response [52].

As mentioned earlier, *D. radiodurans* is both highly desiccation-tolerant as well as radiation-tolerant; this too is the case with *Rubrobacter* sp.. Though *Rubrobacter* is not as well-known as *Deinococcus*, its tolerance to ionizing radiation rivals that of *Deinococcus*, as both organisms survive doses of 25,000 Gy or greater [13, 53-56, 58]. Since the natural habitats of these Bacteria are not ones in which there are high levels of
radiation, it would be unnecessary for them to have repair mechanisms for coping with
damage due to IR. Therefore, since these organisms obviously possess mechanisms
conferring resistance to IR, yet clearly have no reason to have this, it seems highly likely
that these mechanisms play some necessary role in repairing damage caused by
desiccation.

The relationship between desiccation and radiation-resistance was also
demonstrated in a separate experiment in which the radiation-tolerances of 10
Chroococcidiopsis strains, known to be desiccation-tolerant, were evaluated. All 10
strains survived doses of 2500 Gy or more. In fact, four of the ten strains actually
survived doses of 15,000 Gy, which rank them as being highly radiation-resistant.
Because of the relationship between desiccation and radiation, it is logical to believe that
the reason this organism is so radiation-resistant is because of its ability to survive
prolonged desiccation [33, 57].

Another experiment demonstrating the link between desiccation-tolerance and
radiation-tolerance was done by irradiating soil samples from a dry, arid environment, to
determine what organisms survived each dose of radiation. When comparing the arid to
non-arid environmental samples, there was a great deal of diversity among the organisms
as well as the levels of radiation they were able to survive. In fact, some organisms
isolated from the arid environment (various different Deinococcus, Geodermatophilus,
and Hymenobacter species) were able to survive 30,000 Gy [13]. These findings further
support the correlation between desiccation and radiation tolerance.

Desiccation and radiation-tolerance were further linked in another experiment in
which colonies of Moraxella and Acinetobacter species, which had survived periods of
prolonged desiccation, were exposed to radiation. Little reduction in survival was seen when the colonies were exposed to doses of 8000 Gy. In fact, all but three of the isolates survived a 10,000 Gy dose, classifying the colonies as highly radiation-tolerant. This evidence further supports the idea that radiation-tolerance and desiccation-tolerance are linked [59].

**Linking Salt-tolerance and Desiccation**

In addition to being linked to radiation, desiccation has also been linked to salt, as halophiles are also known to be desiccation-resistant [61, 62]. These stressors are likely linked because a cell can become desiccated due to high salt concentrations in the environment. A highly saline environment surrounding a cell will cause water to diffuse out of the cell, thus, leaving the cell desiccated. Therefore, halophilic organisms must be able to deal with becoming desiccated since it is highly likely to occur in highly saline environments.

**Linking Salt-Tolerance and Ionizing Radiation-Tolerance**

Having provided a link between salt tolerance and desiccation, and a link between desiccation and ionizing radiation tolerance, it would seem that there is a possible link between salt tolerance and ionizing radiation tolerance. In fact, one study used *H. salinarum* to examine the effects of salt on the structure of chromosomal DNA, as well as radiation-induced damage to the DNA. It was shown that even in the presence of high KCl concentration, DNA remained in its native B-form structure. It was further demonstrated, using a CD spectrum to measure the amount of damage caused by $\gamma$
radiation, that single-stranded breaks were reduced significantly (1/50 of the breaks seen with no KCl were seen with the addition of KCl) when KCl was added to the medium before being irradiated, suggesting that KCl provides protection against the formation of single-strand breaks caused by radiation, presumably by scavenging hydroxyl radicals [46]. In another experiment, bacterioruberin was shown to provide protection against radiation-induced damage, also by the scavenging of hydroxyl radicals [16, 41, 46, 47].

A pattern in the NaCl-tolerance of various Clostridium botulinum strains and their radiation-resistance was investigated by Kiss et al. [63]. The strains that were more radiation-resistant were also more resistant to NaCl, whereas the more radiation sensitive strains were less resistant to NaCl.

As mentioned earlier, one of the methods that radiation-resistant organisms employ is using Mn$^{2+}$ in the scavenging of ROS that attack proteins. If the proteins can remain undamaged and in their native confirmation, the cell can then work on repairing the other types of damage that have occurred, namely, that to the DNA [40, 42, 43]. Also mentioned earlier, halotolerant organisms will use compatible solutes to counteract the osmotic pressure of a high salinity environment, and these compatible solutes have been shown to increase protein stability [23, 49, 50]. Thus, both halotolerant organisms and radiation-resistant organisms will utilize different solutes to protect their proteins under stressful conditions.

**Linking Tolerance to High Salt, Desiccation and Ionizing Radiation**

While many studies have been conducted to establish links between salt tolerance and desiccation, desiccation and ionizing radiation, and salt and ionizing radiation, very
few have been done to demonstrate a link between all three. Since *Deinococcus radiodurans* is both desiccation-tolerant and radiation-resistant, it must have certain mechanisms that confer its extreme resistances. The *irrE* gene encodes a global regulator that has been linked to its resistance to radiation [51]. To determine if IrrE also provided tolerance to other stressors, including oxidative damage, thermal shock and osmotic shock, a plasmid containing the *irrE* gene was inserted into *E. coli*, and the transformant cells were tested against various stressors, including high salinity. IrrE did, in fact, provide protection for the cells against the stressors they tested, including salt shock. Further analyses revealed that IrrE is a regulator of a wide range of proteins and enzymes including stress-responsive proteins, kinases, detoxification proteins, and growth factors. It was also found that, following irradiation of the *E. coli* transformants, the transcription of *recA* and *pprA*, which encode recombinase and a radiation inducible protein, was stimulated by the IrrE protein. This study provided a link between not only desiccation and radiation-tolerances, but salt-tolerance, as well [51].

**Purpose**

Enzymes and proteins obtained from extremophilic organisms have been marketed and used in numerous industries, such as molecular biology, agriculture, food technology, and pharmaceuticals. For example, Taq DNA polymerase, the enzyme used in the polymerase chain reaction (PCR) is a vital molecular biology tool. It was isolated from the thermophile *Thermus aquaticus*, which was discovered in a geothermal pool in Yellowstone National Park. A glycoprotein produced by the psychrophile *Pseudoalteromonas antarctica* has been patented as an effective treatment for scars and
re-epithelization of wounds [64]. Extremophilic organisms provide endless biotechnological possibilities, and as such, are the focus of our laboratory.

The universal goal of this thesis was to study survival in an extreme environment, mainly that of high salinity. This is important because we believe that future experiments linking salt-tolerance and radiation-resistance could possibly provide support for the use of salinity screening as a way to identify radiation-resistant organisms. Upon further study, these organisms could lead to the discovery of novel mechanisms and/or genes. These mechanisms could pave the way for great advances not only in the field of molecular biology, but also in the healthcare industry if these genes could be found in humans. Identifying these genes and finding homologous genes in humans could allow us to enhance our own radiation-resistance by creating drugs to target and up-regulate these specific genes. Being able to test tolerance to ionizing radiation is generally not feasible for many laboratories as it is both a costly and time-consuming process, and there are few facilities available. Therefore, if a correlation was established, the use of salinity screening could prove to be an invaluable method for identifying radiation-resistant organisms.

We first investigated the degree of salt-tolerance that organisms from a saline Antarctic pond displayed. The abilities of these organisms to grow in salt were then compared to other published studies. Once we had identified organisms with differing salt-tolerances, radiation was then used as a second variable to determine if the organism was a polyextremophile. We knew two very important things about the relationship between salt and radiation. First, there is a reason for salt-tolerance, yet not for radiation-
resistance, except that it could be conferred by another stressor. Second, double-strand DNA breaks are known to be caused by both high salt and ionizing radiation. We hoped that by investigating these polyextremophiles we would to begin to lay the foundation for establishing a correlation between salt-tolerance and radiation-resistance.

In the second part of the study, we identified new organisms with unique salt-tolerances. These organisms have the potential of being either new species, or more likely, new subspecies. Future experiments may be done with these isolates to establish them as novel organisms, which could lead to numerous publications. Furthermore, the unique salt-tolerances of these organisms could also mean unique mechanisms of resistance. These organisms would be ideal for future experiments in which repair mechanisms are the focus.
Chapter 2

Materials and Methods

Collection and Isolation of Antarctic Samples

Since Antarctica is the coldest, highest, driest, and windiest place on earth [7], it was believed that samples collected here would be likely to contain new salt-tolerant organisms, that could also have high levels of radiation-tolerance. Ponds in Antarctica are very diverse in terms of their mineral content, and many contain some type of salt, usually sodium chloride. Samples were collected from a hypersaline pond (Son of Salt Pond) on the McMurdo Ice Shelf, near Bratina Island, in Antarctica, January 1993 by Dr. Peter Sheridan (Fig. 2) [65]. After collection, the samples were inoculated into liquid enrichment cultures. The enrichment cultures were then plated onto solid media and further purified based on colony color and morphology, resulting in 76 different mixed cultures, which were stored at 4°C and used as the starting point for the project.
Colony Characteristics of Isolates

In order to separate the mixed cultures into isolates, cultures were streaked onto plates. After the colonies had grown, distinct colonies were streaked onto new plates. Using colony color and morphology as distinguishing characteristics, the process continued until there was only one type of colony per plate. At this point, the colony color and morphology was recorded and a colony was removed from the plate and inoculated into broth. These cultures were considered to be pure, and each of the 955 isolates was given a unique identification number.

Determination of Identity of Isolates

During this investigation, another graduate student determined the identities of 660 of the isolates using 16S rRNA sequencing [66]. The identities were then used to
compare the characteristics and results obtained in this study to those reported in precious studies.

**Determination of Salt Optima and Salt Tolerance of Isolates**

As a first step in determining if any of the isolates were novel halotolerant organisms, the growth of each of the 955 isolates was characterized in media containing varying concentrations of salt (0, 5, 10, 15, 20, 25% NaCl). Pure cultures were inoculated into liquid media. Turbidity was evaluated every other day for approximately 24-30 days, using a 4-point scale, in which cultures showing no signs of growth were assigned a value of zero and cultures that appeared to have reached maximum turbidity were assigned a value of four. On the final day, the optical density (OD) was recorded for each isolate at each concentration. The salt concentration at which the highest turbidity was reached in the shortest amount of time was determined to be the isolate’s optimum salt concentration for growth. In the event that there was more than one concentration in which the highest turbidity was achieved, and the amount of time it took to reach this turbidity was separated by four days or less, the isolate would be considered to have a wider optimal range. Salt growth range was defined as the concentrations that the isolates grow well in (a value of 2 or better on the 4-point scale). The range would consist of the optimum or optima, as well as any other concentrations in which the isolate either reached the highest turbidity but took a greater amount of time, or that in which the isolate reached the next highest turbidity. Representative graphs are presented in the text, as well as in their entirety in Appendix A.

Once the isolate was identified to a specific species, it was then broken down further into a subspecies, if necessary, based on either colony characteristics (color or
morphology), salt tolerance (optimum growth concentration or concentration range), or both.

**Strains, Media and Growth Conditions**

Strains were grown in R2 medium [composition per liter medium: 0.5 g each proteose peptone, casamino acids, yeast extract, dextrose, soluble starch, sodium pyruvate, 0.3g potassium phosphate (dibasic), and 0.024 g magnesium sulfate] [67], at room temperature (approximately 18 degrees Celsius), without aeration for 7-30 days, unless otherwise specified. For solid medium, 2% agar was added. All media contained a salt concentration ranging from 0 to 25% (w/v) NaCl. Both liquid cultures and plates were grown until ample turbidity or colonies were visible. Pure cultures were stored in 25% glycerol at -80 degrees Celsius. When applicable, cultures were serially diluted and appropriate dilutions were spread onto solid media to obtain a final concentration of 300-500 colony-forming units per plate, or 10 μl of the dilution was spotted onto media in square plates.

**Electron Beam Irradiation and Dosimetry**

A pulsed 20 MeV S-band medical grade LINAC located at the Idaho State University Idaho Accelerator Center, in Pocatello, ID was used for irradiation experiments. The accelerator delivered electrons at a repetition rate of 60 Hz with a pulse-width of 2 μs. Average dose rate was 30 Gy/s. For irradiation, 200 μl of stationary phase culture was placed into a 0.2 mL thin-walled PCR tube, which was held in a
custom electrically conductive plastic holder (Fig. 3). The holders, designed for even charge distribution, were placed 2.5 m from the beam port to ensure uniformity (Fig. 4).

![Image](image1.png)

**FIG. 3.** Custom electrically conductive sample holder used for irradiation [68].

![Image](image2.png)

**FIG. 4.** The setup of the LINAC. Note the sample holder and the beam port of the LINAC (both circled in red) [68].
The beam uniformity was within 10% of the peak dose over a 10 cm diameter circular area, which fully covered the samples in the holder. Dose variations between samples were less than 1% as the samples were located on isocontours of dose. GEX B3 radiochromic thin-film dosimeters were placed behind the tubes in the holder, and a GEX Corporation (Centennial, CO) thin-film dosimetry system was used to measure the total dose delivered. Dosimetry uncertainty for all irradiations was assumed to be 10% or less [69, 70].

Approximate doses of 500, 1000, 2000, 4000, 6000, 8000 and 10000 Gy were used to determine the max dose. Since we did not know anything about the radiation-resistance of the organisms, we chose a range of doses in order to make sure that that we would get significant data from each isolate and not just those that survived high doses. Since this data was to be used simply to get an idea of the radiation-resistance, more in-depth radiation experiments did not need to be performed.
Chapter 3

Results

Determining Salt optima and tolerance

In order to establish a link between salt tolerance and radiation tolerance, isolates with the ability to grow either in high salt or over a wide range of salt concentrations were irradiated in order to determine if this salt tolerance also conferred radiation tolerance. The first step toward being able to test the salt tolerance of individual organisms was obtaining pure cultures. The mixed Antarctic cultures were purified until individual isolates were obtained. In all, 955 isolates were derived from the 76 mixed enrichment cultures.

After the isolates were identified by 16S rRNA sequencing, they were grouped according to their identities. The colony morphology and color, as well as the salt growth range and salt optima were examined for each isolate to determine what isolates were consistent with one another and which were different. Tables were created for the various organisms. Graphs depicting the salt growth of all isolates were created. Select graphs for various isolates/organisms are referenced in the discussion; the remaining graphs were placed in Appendix A. The salt growth range as well as colony color and morphology for each organism was obtained from other studies and compared to what was seen in this study. If the salt growth range and colony characteristics were consistent with the literature, the isolate was determined to be that organism. If the salt growth range or colony characteristics were inconsistent with the literature, but the 16S rRNA identified the isolate as a particular organism, then the isolate was determined to likely be a subspecies. This was the case with 257 isolates (covering 77 different species).
Actinobacteria

*Clavibacter michiganensis*

Fifteen isolates were identified as *Clavibacter michiganensis*. All grew optimally in medium with no additional salt (Figure 5); however, there were distinct differences in both colony characteristics and salt growth ranges, which further divided this species into three subgroups (Table 1). Since the time that this organism was first identified, there have been new subspecies identified through 16S and 23S rRNA intergenic sequence comparisons, which differ markedly from the original isolate in terms of colony morphology and color, as well as lipid content and DNA G+C [71-73]. Based on colony color alone, both subgroups 1 and 2 are likely *C. michiganensis ssp. michiganensis*, and the narrow salt growth tolerance is consistent with this assignment, despite the slight difference in range between subgroups 1 and 2 [74, 75]. Colony color is consistent with subgroup 3 being *ssp. nebraskensis*; however, definitive assignment will require more extensive testing [76].

![FIG. 5. Growth of three *Clavibacter michiganensis* isolates in various salt concentrations. Growth was monitored for 30 days and turbidity evaluated on a 4 point scale. (A) isolate 300, representing those isolates in sub-group 1; (B) isolate 849, representing those isolates in sub-group 2; (C) isolate](image-url)
Twenty isolates were identified as belonging to three species of the genus *Kocuria* (*K. palustris*, *K. rhizophila*, and *K. rosea*) (Table 2). Kovács *et al.* found that *Kocuria rhizophila* grew in up to 7% NaCl, but not over 10% NaCl [77]. All of the *K. rhizophila* isolates tested in this study had higher salt growth ranges, showing significant growth in medium containing up to 25% NaCl. Although the salt growth range was the same for the *K. rhizophila* isolates, the optima were different, so the isolates were divided into 3 subgroups. As shown in Figure 6, the optimum for sub-group 1 isolates was clearly 0% (Panel A), while the optimum for the sub-group 2 isolates was 5% NaCl (Panel B). The isolates in sub-group 3 were much different in that they did not have a clear optimum, and grew equally well in all of the concentrations tested (Panel C). This information combined with that of the reported growth range suggested that at least one of the sub-groups, if not all, may define a new subspecies.

### Table 1. Salt Tolerance and Colony Characteristics of Isolates Identified as *Clavibacter michiganensis*

<table>
<thead>
<tr>
<th>Sub-group</th>
<th>Salt Tolerance</th>
<th>Colony Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optimum Growth (%)</td>
<td>Range of Growth (%) *</td>
<td>Color</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>optimum only</td>
<td>yellow/orange</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0-5</td>
<td>yellow</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0-20</td>
<td>peach</td>
</tr>
</tbody>
</table>

* *isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.*

*Kocuria sp.*

Twenty isolates were identified as belonging to three species of the genus *Kocuria* (*K. palustris*, *K. rhizophila*, and *K. rosea*) (Table 2). Kovács *et al.* found that *Kocuria rhizophila* grew in up to 7% NaCl, but not over 10% NaCl [77]. All of the *K. rhizophila* isolates tested in this study had higher salt growth ranges, showing significant growth in medium containing up to 25% NaCl. Although the salt growth range was the same for the *K. rhizophila* isolates, the optima were different, so the isolates were divided into 3 subgroups. As shown in Figure 6, the optimum for sub-group 1 isolates was clearly 0% (Panel A), while the optimum for the sub-group 2 isolates was 5% NaCl (Panel B). The isolates in sub-group 3 were much different in that they did not have a clear optimum, and grew equally well in all of the concentrations tested (Panel C). This information combined with that of the reported growth range suggested that at least one of the sub-groups, if not all, may define a new subspecies.
<table>
<thead>
<tr>
<th>Species</th>
<th>Sub-group</th>
<th>Optimum Growth (% NaCl)</th>
<th>Range of Growth (% NaCl)*</th>
<th>Color</th>
<th>Morphology</th>
<th>Identification Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. palustris</em></td>
<td>0</td>
<td>0</td>
<td>0-5</td>
<td>cream</td>
<td>circular, shiny, opaque</td>
<td>291, 551, 658</td>
</tr>
<tr>
<td><em>K. rhizophila</em></td>
<td>1</td>
<td>0</td>
<td>0-25</td>
<td>dark yellow</td>
<td>circular, opaque, dull</td>
<td>492, 689</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>0-25</td>
<td>light yellow</td>
<td>circular, opaque, shiny</td>
<td>696, 851, 759, 868</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>None</td>
<td>0-25</td>
<td>light yellow</td>
<td>circular, opaque, shiny</td>
<td>869</td>
</tr>
<tr>
<td><em>K. rosea</em></td>
<td>1</td>
<td>0</td>
<td>0-5</td>
<td>cream</td>
<td>circular, opaque, shiny</td>
<td>465, 802</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>0-25</td>
<td>peach/pink</td>
<td>circular, opaque, shiny</td>
<td>692</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0/5/10</td>
<td>0-20</td>
<td>peach/pink</td>
<td>circular, opaque, shiny</td>
<td>669, 824</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>0-10</td>
<td>peach/pink</td>
<td>circular</td>
<td>944</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5/10</td>
<td>0-25</td>
<td>light yellow</td>
<td>circular, opaque, shiny</td>
<td>632, 640</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>10</td>
<td>0-10</td>
<td>peach/pink</td>
<td>circular, shiny</td>
<td>511, 590</td>
</tr>
</tbody>
</table>

*K*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.
Mayilraj et al. tested the growth of *Kocuria rosea* in various salt concentrations, ranging from 0-10% NaCl and found it was only able to grow in 0-5% NaCl [78]. This was consistent only with those isolates in sub-group 1, as the remaining isolates all grew in media with 10% NaCl or more. The peach/pink colony color of those isolates in sub-groups 2, 3, 4, and 6 was found to be consistent with that described in previous studies, while the cream and yellow of isolates in sub-groups 1 and 5, (respectively) was not [78-81]. Neither the salt growth ranges for the isolates in sub-groups 2-6 nor the colony color described for those in sub-group 1 was consistent with what was reported in the literature.
Microbacterium sp.

Forty-eight isolates were identified as belonging to the *Microbacterium* genus. Those isolates were further divided into eight species (*M. esteraromaticum, M. foliorum, M. hydrocarbonoxydans, M. lacticum, M. laevaniformans, M. oxydans, M. paraoxydans, and M. schleiferi*). Isolates identified as *Microbacterium sp.* had somewhat limited growth in salt, with the optima being 5% NaCl or less (Table 3). Wu et al., and Behrendt et al. reported the salt growth range for *Microbacterium foliorum* as being up to 5% but less than 6.5% NaCl [82, 83]. Although subgroup 1 and 2 isolates had the same range as that reported, the isolate in subgroup 3 had a broader salt tolerance range, showing significant growth in medium containing 10%.

In 1998, Takeuchi and Hatano reported the salt growth range of *Microbacterium laevaniformans* as 0-5% NaCl, as no growth was observed in 6.5% NaCl [84]. The salt growth range for isolate 324 (sub-group 2) was consistent with that reported, while the range for isolate 347 (sub-group 1) was more limited than that reported, only growing significantly when in medium containing no additional salt.

Schippers et al. (2005) tested the salt growth range of *Microbacterium oxydans* in media containing 0, 2, and 6.5% NaCl, and found growth in both 0 and 2%, but not in 6.5% NaCl [85]. The salt growth ranges for those isolates in sub-groups 1 and 3 were consistent with that reported, while the range for the isolates in sub-groups 2 and 4 was not, as growth was shown in media that exceeded the stated 6.5% NaCl.
<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Salt Tolerance</th>
<th>Colony Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species</strong></td>
<td><strong>Optimum Growth (% NaCl)</strong></td>
<td><strong>Range of Growth (%NaCl)</strong>*</td>
<td><strong>Color</strong></td>
</tr>
<tr>
<td>M. esteraromaticum</td>
<td>0/5</td>
<td>0-5</td>
<td>yellow</td>
</tr>
<tr>
<td>M. foliorum</td>
<td>1</td>
<td>0 optimum only</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0/5</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0/5</td>
<td>0-10</td>
</tr>
<tr>
<td>M. hydrocarbonoxydans</td>
<td>0</td>
<td>0-5</td>
<td>yellow</td>
</tr>
<tr>
<td>M. lacticum</td>
<td>0</td>
<td>0-5</td>
<td>yellow/white</td>
</tr>
<tr>
<td>M. laevaniformans</td>
<td>1</td>
<td>0 optimum only</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>0-5</td>
</tr>
<tr>
<td>M. oxydans</td>
<td>1</td>
<td>0</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0-10</td>
<td>yellow</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0/5</td>
<td>0-15</td>
</tr>
<tr>
<td>M. paraoxydans</td>
<td>0/5</td>
<td>0-5</td>
<td>yellow</td>
</tr>
<tr>
<td>M. schleiferi</td>
<td>0</td>
<td>0</td>
<td>0-5</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.
Micrococcus sp.

Forty-six different isolates were identified as belonging to the genus Micrococcus, representing five different species (M. antarcticus, M. flavus, M. luteus, M. thailandicus, and M. yunnanensis) (Table 4). The salt growth range of Micrococcus flavus was reported by Chen et al. as 0% NaCl only; however, there were only two different salt concentrations tested in that experiment: 0 and 7% NaCl [86]. Sub-group 1 isolates were the only ones that had the same reported growth of 0% (Figure 7A), while the remaining isolates all grew in 5% NaCl or greater (Figure 7B). In fact, those isolates in sub-groups 3 and 4 grew in up to 25% NaCl (Figure 7C and D).
<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Salt Tolerance</th>
<th>Colony Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optimum Growth (% NaCl)</td>
<td>Range of Growth (% NaCl)*</td>
<td>Color</td>
</tr>
<tr>
<td><strong>M. antarcticus</strong></td>
<td>0/5</td>
<td>0-10</td>
<td>yellow</td>
</tr>
<tr>
<td><strong>M. flavus</strong></td>
<td>1</td>
<td>0</td>
<td>optimum only</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>0-25</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0/5</td>
<td>0-25</td>
</tr>
<tr>
<td><strong>M. luteus</strong></td>
<td>1</td>
<td>0</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>0-10</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>0-20</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>0-25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0/5/10</td>
<td>0-10</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5</td>
<td>0-10</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>10</td>
<td>0-20</td>
</tr>
<tr>
<td><strong>M. thailandicus</strong></td>
<td>0</td>
<td>0-5</td>
<td>yellow</td>
</tr>
<tr>
<td><strong>M. yunnanensis</strong></td>
<td>1</td>
<td>0</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>0-10</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>0-15</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>10</td>
<td>0-15</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.
The ranges of growth in salt-containing media for those isolates identified as *M. luteus* and *M. yunnanensis* were also compared against previous studies. Wieser et al. tested the range of growth of *Micrococcus luteus* in salt concentrations ranging from 0-10% NaCl, and saw growth in all [87]. Salem et al. reported an *M. luteus* strain grew in media containing 0-20% NaCl, when tested over a range of 0-50% NaCl [88]. As those isolates in sub-group 1 did not grow in 10% NaCl, they were inconsistent with the literature, while the isolates in sub-groups 3 and 7 were consistent with the strain identified by Salem. The isolates in sub-groups 2, 5, and 6 are likely the same as those reported by Wieser while the sub-group 1 and 4 isolates are possibly a new subspecies, due to their lack of growth in medium over 5% NaCl, and their growth in 25% NaCl, respectively. Zhao et al. reported the salt growth range for *Micrococcus yunnanensis* as 0-7% NaCl (there was no testing at

FIG. 7. Growth of four *Micrococcus flavus* isolates in various salt concentrations. Growth was monitored for 30 days and turbidity evaluated on a 4 point scale. (A) isolate 154, representing those isolates in sub-group 1 (see text); (B) isolate 54; representing those isolates in sub-group 2; (C) isolate 318; representing those isolates in sub-group 3; (D) isolate 362; representing those isolates in sub-group 4.
concentrations greater than 7% NaCl) [89]. The isolates in this study were consistent with that reported, and are likely *M. yunnanensis*.

**Rhodococcus sp.**

There were four *Rhodococcus* species identified (*R. corynebacterioides*, *R. equi*, *R. fascians*, and *R. kroppenstedtii*); the salt tolerances and colony characteristics are listed for those organisms in Table 5. Mayilraj et al. tested the growth of *Rhodococcus corynebacterioides* in media at two different salt concentrations: 0% and 7% NaCl, and found no growth in 7% NaCl [90]. The isolates in all six of the sub-groups, therefore, had salt growth ranges that were inconsistent with the literature as the growth capabilities extended beyond that reported.

Gesheva et al. found that *Rhodococcus fascians* grew in media containing 9% NaCl or less, while previous isolates were reported to grow in 7% NaCl or less [91]. Sub-group 1 isolates had the same range as that reported for Gesheva’s strain, while sub-group 2 isolates had a greater growth range. The orange colony color of all of the isolates in this study was similar to that of the typical *Rhodococcus fascians* organism, not the Gesheva’s strain, which was described as cream or pale sandy [91].
The salt growth range of 0-10% NaCl reported for *Rhodococcus kroppenstedtii* by Mayilraj, et al., was different than the isolates identified in this study as *R. kroppenstedtii*, as the isolates in sub-group 1 did not grow in 10% NaCl (Figure 8A), while the sub-group 2 isolates grew in 15 and 20% (Figure 8B). In addition, all isolates in both subgroups had tan colonies which was inconsistent with that of the orange-red color reported by Mayilraj et al. [90].
Arthrobacter sp., Curtobacterium sp., Rathayibacter sp., and Williamsia sp.

The remaining organisms classified as Actinobacteria (consisting of 16 genera, and 24 species) were all grouped together and are presented in Table 6. The *Arthrobacter agilis* isolates in sub-group 1 had an optimum of 0% NaCl which was identical to that reported by Koch et al., and are likely the same organism [92]. The remaining sub-groups were inconsistent with that reported, as they grew optimally in 5% NaCl and greater.

In 1989, Dunleavy tested the range of growth of *Curtobacterium luteum* in various salt concentrations ranging from 0-10% NaCl, and found the organism was able to grow in media containing 0 and 5% NaCl, but not 10% NaCl [93]. This reported range was consistent with what was seen for *Curtobacterium luteum* isolates in sub-group 1, but not for those in sub-group 2, as they had significant growth in media containing both 10% and 15% NaCl.

![Graph](image)

FIG. 8. Growth of two *Rhodococcus kroppenstedtii* isolates in various salt concentrations. Growth was monitored for 30 days and turbidity evaluated on a 4 point scale. (A) isolate 220 (B) isolate 607.
Dorofeeva et al. reported that *Rathayibacter festucae* grew only in 0% NaCl, although 5% NaCl was also tested [94]. *Rathayibacter festucae* Isolate 562 had growth and colony characteristics consistent with that organism, while 866 grew in 10% NaCl, and had a different colony color than the yellow described by Dorofeeva [94].
<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Genus</th>
<th>Species</th>
<th>Sub-group</th>
<th>Optimum Growth (% NaCl)</th>
<th>Range of Growth (% NaCl)*</th>
<th>Color</th>
<th>Morphology</th>
<th>Identification Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agrococcus</td>
<td>jenensis</td>
<td></td>
<td>0/5</td>
<td>0-5</td>
<td>cream</td>
<td>circular</td>
<td>537</td>
</tr>
<tr>
<td></td>
<td>Agromyces</td>
<td>terreus</td>
<td></td>
<td>0</td>
<td>0-5</td>
<td>cream</td>
<td>circular</td>
<td>106, 151</td>
</tr>
<tr>
<td></td>
<td>Arthrobacter</td>
<td>agilis</td>
<td>1</td>
<td>0</td>
<td>optimum only</td>
<td>pink</td>
<td>circular, opaque</td>
<td>172, 239, 750, 950</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>0-5</td>
<td></td>
<td></td>
<td>679</td>
</tr>
<tr>
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<td>optimum only</td>
<td></td>
<td></td>
<td>504</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>10</td>
<td>optimum only</td>
<td></td>
<td></td>
<td>618</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aurescens</td>
<td>0</td>
<td>optimum only</td>
<td>cream</td>
<td></td>
<td>circular</td>
<td>896</td>
</tr>
<tr>
<td></td>
<td></td>
<td>gandavensis</td>
<td>0</td>
<td>0-10</td>
<td>yellow</td>
<td></td>
<td>circular</td>
<td>406</td>
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<td></td>
<td></td>
<td>oxydans</td>
<td>0</td>
<td>0-5</td>
<td>yellow</td>
<td></td>
<td>circular</td>
<td>443</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sulfonivorans</td>
<td>0</td>
<td>0-5</td>
<td>cream</td>
<td></td>
<td>irregular</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Brachybacterium</td>
<td>nesterenkovi</td>
<td>5</td>
<td>0-10</td>
<td>cream</td>
<td></td>
<td>circular</td>
<td>952</td>
</tr>
<tr>
<td></td>
<td></td>
<td>paraconglomeratum</td>
<td>5</td>
<td>0-10</td>
<td>tan</td>
<td></td>
<td>circular</td>
<td>884</td>
</tr>
<tr>
<td></td>
<td>Brevibacterium</td>
<td>epidermidis</td>
<td>0/5/10</td>
<td>0-20</td>
<td>orange</td>
<td></td>
<td>circular</td>
<td>953</td>
</tr>
<tr>
<td></td>
<td>Corynebacterium</td>
<td>lipophiloflavum</td>
<td>0</td>
<td>0-5</td>
<td>pink</td>
<td></td>
<td>circular</td>
<td>839</td>
</tr>
<tr>
<td></td>
<td>Curtobacterium</td>
<td>luteum</td>
<td>1</td>
<td>0/5</td>
<td>0-5</td>
<td>cream</td>
<td>circular</td>
<td>734</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>0-15</td>
<td></td>
<td></td>
<td>348</td>
</tr>
<tr>
<td></td>
<td>Frigoribacterium</td>
<td>faeni</td>
<td>0</td>
<td>0-5</td>
<td>orange</td>
<td></td>
<td>irregular</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Gordonia</td>
<td>aichiensis</td>
<td>10</td>
<td>5-15</td>
<td>orange</td>
<td></td>
<td>irregular</td>
<td>599</td>
</tr>
<tr>
<td></td>
<td>Janibacter</td>
<td>melonis</td>
<td>1</td>
<td>0</td>
<td>0-5</td>
<td>yellow</td>
<td>circular</td>
<td>431, 619</td>
</tr>
<tr>
<td></td>
<td>Kytococcus</td>
<td>sedentarius</td>
<td>0</td>
<td>optimum only</td>
<td>cream</td>
<td></td>
<td>irregular</td>
<td>261, 262</td>
</tr>
<tr>
<td></td>
<td>Nocardioides</td>
<td>alkalitolerans</td>
<td>0</td>
<td>0-10</td>
<td>white</td>
<td></td>
<td>irregular</td>
<td>317</td>
</tr>
<tr>
<td></td>
<td></td>
<td>maritimus</td>
<td>0</td>
<td>0-5</td>
<td>white</td>
<td></td>
<td>circular</td>
<td>316</td>
</tr>
<tr>
<td></td>
<td>Rathayibacter</td>
<td>cariciis</td>
<td>0</td>
<td>optimum only</td>
<td>orange</td>
<td></td>
<td>circular</td>
<td>224, 346</td>
</tr>
<tr>
<td></td>
<td></td>
<td>festucae</td>
<td>1</td>
<td>0</td>
<td>optimum only</td>
<td>orange</td>
<td>circular, opaque</td>
<td>562</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>0-10</td>
<td>yellow</td>
<td>circular</td>
<td>866</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tritici</td>
<td>0</td>
<td>0-5</td>
<td>yellow</td>
<td></td>
<td>circular</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Rothia</td>
<td>terrae</td>
<td>0</td>
<td>0-10</td>
<td>white</td>
<td></td>
<td>circular</td>
<td>383</td>
</tr>
<tr>
<td></td>
<td>Sanguibacter</td>
<td>suarezii</td>
<td>0</td>
<td>0-5</td>
<td>yellow</td>
<td></td>
<td>circular, opaque</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>Williamsia</td>
<td>marianensis</td>
<td>1</td>
<td>0</td>
<td>0-25</td>
<td>pink</td>
<td>circular, opaque</td>
<td>636, 852</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td>yellow</td>
<td></td>
<td>circular, opaque</td>
<td>788</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.
Pathom-aree et al. found that *Williamsia marianensis* grew in 0-7% NaCl [95]. The salt growth range for all of the isolates identified as *W. marianensis* was consistent with that reported; however, they had a wider range, and grew in all of the concentrations tested (Figure 9). The colony colors, however, were not consistent among the sub-groups, nor were they similar to the orange color reported by Pathom-aree [95].

![Fig. 9. Growth of *Williamsia marianensis* isolate 636 in various salt concentrations. Growth was monitored for 30 days and turbidity evaluated on a 4 point scale.](image)

**Alphaproteobacteria**

There were sixteen isolates identified that were classified as Alphaproteobacteria, representing six genera (*Agrobacterium, Brevundimonas, Mesorhizobium, Paracoccus, Rhizobium, and Sphingomonas*) (Table 7). The salt growth range of *Brevundimonas kwangchunensis* was determined by Yoon et al. to be 0-2% NaCl, with no growth shown above that concentration. The growth range for those isolates in sub-group 1 was consistent with that reported (Figure 10A), while the growth range for sub-group 2 isolates was
inconsistent, as growth was clearly demonstrated in media containing 5% NaCl (Figure 10B).

**TABLE 7. Salt Tolerance and Colony Characteristics of Isolates Identified as Alphaproteobacteria**

<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Species</th>
<th>Sub-group</th>
<th>Optimum Growth (% NaCl)</th>
<th>Range of Growth (% NaCl)*</th>
<th>Species</th>
<th>Sub-group</th>
<th>Optimum Growth (% NaCl)</th>
<th>Range of Growth (% NaCl)*</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Agrobacterium</em></td>
<td>tumefaciens</td>
<td>0</td>
<td>0-25</td>
<td>cream</td>
<td>circular</td>
<td>363,373,396</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Brevundimonas</em></td>
<td>kwangchunensis</td>
<td>1</td>
<td>0</td>
<td>optimum only</td>
<td>cream</td>
<td>circular</td>
<td>283,351</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0-5</td>
<td>yellow/cream</td>
<td>circular</td>
<td>148</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Mesorhizobium</em></td>
<td>mediterranea</td>
<td>1</td>
<td>0</td>
<td>optimum only</td>
<td>yellow/cream</td>
<td>32,62,89</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Paracoccus</em></td>
<td>solventivorans</td>
<td>0</td>
<td>0-5</td>
<td>yellow/cream</td>
<td>circular</td>
<td>15,390</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhizobium</em></td>
<td>huautlense</td>
<td>0</td>
<td>0-5</td>
<td>cream</td>
<td>circular</td>
<td>327</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sphingomonas</em></td>
<td>faeni</td>
<td>0</td>
<td>0-5</td>
<td>cream</td>
<td>circular</td>
<td>295,320</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.

**FIG. 10. Growth of two *Brevundimonas kwangchunensis* isolates in various salt concentrations. Growth was monitored for 30 days and turbidity evaluated on a 4 point scale. (A) isolate 283, representing those isolates in sub-group 1; (B) isolate 148.**
The salt growth range listed for the *Brevundimonas mediterranea* sub-group 2 isolates was consistent with the range of 0-5% NaCl, stated by Fritz et al. (2005), while the growth range for the isolates in sub-group 1 was inconsistent, as there was no growth in media containing 5% NaCl [96].

**Betaproteobacteria**

There were sixteen isolates classified as Betaproteobacteria, representing the *Masillia, Naxibacter, and Variovorax* genera (Table 8). Weon et al. tested the ability of *Masillia niabensis* to grow in various salt concentrations ranging from 0 to 5% NaCl, and determined the salt growth range to be limited to 0-1% NaCl [97]. The ability to grow in 0% was consistent with the isolates in sub-group 1, but not those isolates in sub-groups 2-4 as there was significant growth in higher salt concentrations (Figure 11A-D).

FIG. 11. Growth of four *Masillia niabensis* isolates in various salt concentrations. Growth was monitored for 30 days and turbidity evaluated on a 4 point scale. (A) isolate 191, representing those isolates in sub-group 1; (B) isolate 660; (C) isolate 756; (D) isolate 659.
Gammaproteobacteria

There were seven different genera identified belonging to the Gammaproteobacteria phylum: *Acinetobacter, Enhydrobacter, Moraxella, Pantoea, Pectobacterium, Pseudomonas* and *Psychrobacter* (for *Acinetobacter, Enhydrobacter, Moraxella, Pantoea, and Pectobacterium* species, refer to Table 9). Due to the number of isolates and species identified as *Pseudomonas* and *Psychrobacter*, they were placed into three separate tables. The *Pseudomonas* isolates with circular colonies were placed into one table (Table 10), while those with irregular colonies were placed into another (Table 11). All of the *Psychrobacter* isolates were placed into one table (Table 12). Bouvet and
Grimont reported that *Acinetobacter johnsonii* grew in 0-10 % NaCl [98]. As such, the salt growth range for those isolates in sub-group 2 was found to be consistent with the stated range (Figure 12B), while the ranges for the isolates in sub-groups 1 and 3 were not, as sub-group 1 isolates clearly only grew in 0%, and sub-group 3 isolates grew in all concentrations tested (Figure 12A and C).

### TABLE 9. Salt Tolerance and Colony Characteristics of Isolates Identified as Gammaproteobacteria

<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Salt Tolerance</th>
<th>Colony Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genus</strong></td>
<td><strong>Species</strong></td>
<td><strong>Sub-group</strong></td>
<td><strong>Optimum Growth (% NaCl)</strong></td>
</tr>
<tr>
<td>Acinetobacter</td>
<td>johnsonii</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Acinetobacter</td>
<td>lwoffii</td>
<td>0</td>
<td>optimum only</td>
</tr>
<tr>
<td>Enhydrobacter</td>
<td>aerosaccus</td>
<td>0</td>
<td>0-5</td>
</tr>
<tr>
<td>Pantoea</td>
<td>agglomerans</td>
<td>0/5/10</td>
<td>0-10</td>
</tr>
<tr>
<td>Pectobacterium</td>
<td>carotovorum</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>5/10</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.
<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Salt Tolerance</th>
<th>Colony Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Species</td>
<td>Subgroup</td>
<td>Optimum Growth (% NaCl)</td>
</tr>
<tr>
<td></td>
<td>P. brenneri</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>P. frederiksbergensis</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>P. guineae</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0/5</td>
</tr>
<tr>
<td></td>
<td>P. mandelii</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>P. oryzihabitans</td>
<td>0/5</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td>P. veronii</td>
<td>1</td>
<td>0</td>
</tr>
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<td></td>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>0/5/10</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.
### TABLE 11. Salt Tolerance and Colony Characteristics of Isolates Identified as Belonging to the Genus *Pseudomonas* (Irregular Colony Morphology)

<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Salt Tolerance</th>
<th>Colony Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species</strong></td>
<td><strong>Sub-group</strong></td>
<td><strong>Optimum Growth (% NaCl)</strong></td>
<td><strong>Range of Growth (% NaCl)</strong>*</td>
</tr>
<tr>
<td><em>P. migulae</em></td>
<td>1</td>
<td>0</td>
<td>optimum only</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>0-10</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>0-25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0/5/10</td>
<td>0-15</td>
</tr>
<tr>
<td><em>P. peli</em></td>
<td>0</td>
<td>0</td>
<td>0-5</td>
</tr>
<tr>
<td><em>P. trivialis</em></td>
<td>0</td>
<td>0</td>
<td>0-5</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.

### TABLE 12. Salt Tolerance and Colony Characteristics of Isolates Identified as Belonging to the Genus *Psychrobacter*

<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Salt Tolerance</th>
<th>Colony Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species</strong></td>
<td><strong>Sub-group</strong></td>
<td><strong>Optimum Growth (% NaCl)</strong></td>
<td><strong>Range of Growth (% NaCl)</strong>*</td>
</tr>
<tr>
<td><em>P. alimentarius</em></td>
<td>0/5</td>
<td>0-15</td>
<td>cream</td>
</tr>
<tr>
<td><em>P. aquaticus</em></td>
<td>0/5</td>
<td>0-5</td>
<td>cream</td>
</tr>
<tr>
<td><em>P. cryohalolentis</em></td>
<td>1</td>
<td>0/5</td>
<td>0-25</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0/5/10</td>
<td>0-10</td>
</tr>
<tr>
<td><em>P. glacincola</em></td>
<td>1</td>
<td>0/5</td>
<td>0-10</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>0-10</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.
The salt concentration of *Moraxella osloensis* in media containing different salt concentrations was tested by Vandamme et al., and was found to be limited to 0-1.5% NaCl [99]. Isolates in sub-group 1 had growth ranges consistent with that stated, while those isolates in sub-group 2 were not consistent as they grew in all of the concentrations tested.

FIG. 12. Growth of three *Acinetobacter johnsonii* isolates in various salt concentrations. Growth was monitored for 30 days and turbidity evaluated on a 4 point scale. (A) isolate 119, representing those isolates in sub-group 1; (B) isolate 104, representing those isolates in sub-group 2; (C) isolate 662, representing those isolates in sub-group 3.
The salt growth range for *Pectobacterium carotovorum* isolates in sub-group 2 was consistent with that described by Gardan et al., in which the salt growth range was 0-5% NaCl (Figure 13B) [100]. The salt growth ranges for isolates in sub-groups 1 and 3 were not consistent, as sub-group 1 isolates only grew in 0%, and sub-group 3 isolates grew better in 10% than they did in 0% NaCl (Figure 13A and C).

![Figure 13](image)

**FIG. 13.** Growth of three *Pectobacterium carotovorum* isolates in various salt concentrations. Growth was monitored for 30 days and turbidity evaluated on a 4 point scale. (A) isolate 553, representing those isolates in the first group; (B) isolate 863, representing those isolates in the second group; (C) isolate 615.

*Pseudomonas sp.*

Seven of the ten *Pseudomonas* species (*P. fluorescens*, *P. frederiksbergensis*, *P. mandelii*, *P. oryzihabitans*, *P. peli*, *P. trivialis*, and *P. veronii*) identified in this study are presented in Table 10; because of the number of isolates associated with them, the remaining three species (*P. brenneri*, *P. guineae*, and *P. migulae*) are in Table 11. In
Andersen et al. found that *Pseudomonas frederiksbergensis* grew in 0-2% NaCl but not in 4 or 6% NaCl [101]. The salt growth range for the isolate in sub-group 1 was consistent with that reported, while that for the isolate in sub-group 2 was very different (Figure 14A) as there was significant growth in both 5 and 10% NaCl (Figure 14B).

The salt growth range for the *Pseudomonas mandelii* isolates in sub-group 2 was consistent with that reported by Rhodes et al. (0-5% NaCl), while the range for those isolates in sub-group 1 was different, as substantial growth in 5% NaCl was not demonstrated [102]. Since there was no growth in any concentration greater than 5% NaCl, and there was some growth in 5%, it would seem possible that there was perhaps a problem with the 5% NaCl medium being more concentrated than it should have been, and the growth was underrepresented. However, the three isolates comprising sub-group 1 were not tested at the same time, but in different batches, with different 5% NaCl.
medium, making it likely that these organisms genuinely do not grow well in medium containing 5% NaCl.

*Pseudomonas veronii* was determined by Ivanova et al. to have a salt growth range of less than 5% NaCl, as there was no growth in 5% NaCl or more [103]. Therefore, the salt growth ranges for all of the isolates in the sub-groups were inconsistent with that found by Ivanova as they grew in media containing 5% NaCl or greater, and in the case of sub-group 3 isolates, even grew in 25% NaCl.

In 2001 Baida et al. found that *Pseudomonas brenneri* grew in 0-5% NaCl, but no higher; therefore, only the sub-group 1 isolates had salt growth ranges consistent with that in the study [104]. Isolates in the other sub-groups grew in media containing 5% NaCl or more. All of the *Pseudomonas guinea* isolates had a salt growth range (0-5% NaCl) consistent with that reported by Bozal et al. [105].

Isolates identified as *Pseudomonas migulae* were placed into five subgroups based on their range of growth in salt. Verhille et al. found that the highest concentration at which *Pseudomonas migulae* grew was 0.8% NaCl [106]. Those isolates in sub-group 1 were the only ones with salt growth ranges consistent with that reported (Figure 15A), as the isolates in the remaining sub-groups all grew in media containing 5% NaCl or greater (Figure 15B-C), and in the case of those isolates in sub-groups 4 and 5, there was growth in all of the concentrations of media tested (Figure 15D and E).
There were four Psychrobacter species identified (P. alimentarius, P. aquaticus, P. cryohalolentis, and P. glacincola) all of which were moderately salt-tolerant (Table 12). At 0-10% NaCl, the salt growth range for the Psychrobacter cryohalolentis isolates in sub-group 2 was the same as that described in a study by Bakermans et al. [107]; however, the sub-group 1 isolates were not similar, as they grew in all of our tested concentrations.

The isolates in this study identified as Psychrobacter glacincola grew in 0-10% salt. Since the isolates did not grow in 15% NaCl and 12% NaCl was not tested, the growth range for the isolates was consistent with the 0-12% NaCl reported by Bozal et al.
there were no isolates identified as *Psychrobacter glacincola* that were not consistent with the literature in terms of growth in salt and colony color and morphology.

**Bacteroidetes/Chlorobi**

There were nine isolates identified that were classified as Bacteroidetes/Chlorobi; four genera (five species) were represented (*Chryseobacterium*, *Dyadobacter*, *Flavobacterium*, and *Hymenobacter*) (Table 13). The salt growth range listed by Buczolits, et al. for *Hymenobacter aerophilus* was 0-2% NaCl, as no growth was observed in media containing an additional 5 or 10% NaCl [109]. The salt growth range listed for those isolates in sub-group 1 was consistent with that reported while the range for the isolate in sub-group 2 was not, as there was growth in 5% NaCl. The red colony color of the isolates in sub-group 1 was also consistent with that determined in the previous study, while the white colony color of the isolate in sub-group 2 was not [109].

<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Salt Tolerance</th>
<th>Colony Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Genus</strong></td>
<td><strong>Species</strong></td>
<td><strong>Sub-group</strong></td>
<td><strong>Optimum Growth (% NaCl)</strong></td>
</tr>
<tr>
<td><em>Chryseobacterium</em></td>
<td><em>soldanellicola</em></td>
<td>0</td>
<td>optimum only</td>
</tr>
<tr>
<td><em>Dyadobacter</em></td>
<td><em>koreensis</em></td>
<td>0</td>
<td>optimum only</td>
</tr>
<tr>
<td><em>Flavobacterium</em></td>
<td><em>degerlachei</em></td>
<td>0</td>
<td>optimum only</td>
</tr>
<tr>
<td><em>Hymenobacter</em></td>
<td><em>aerophilus</em></td>
<td>1</td>
<td>optimum only</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td><em>roseosalivarius</em></td>
<td>1</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>5-10</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.
**Firmicutes**

Among the many isolates identified as Firmicutes, eight genera were represented (Brevibacillus, Paenibacillus, Planococcus, Planomicrobium, Terribacillus, Bacillus, and Exiguobacterium) (refer to Table 14 for Brevibacillus, Paenibacillus, Planococcus, Planomicrobium and Terribacillus). Due to the large number of isolates identified as Bacillus and Exiguobacterium, they were broken down into several individual tables. There are 5 Bacillus species in Table 15, Bacillus subtilis isolates in Table 16, Bacillus pumilus isolates in Table 17, Bacillus megaterium isolates in table 18, and 3 different Exiguobacterium species in Table 19. In 2002, Dasman et al., reported that Paenibacillus glycanalyticus grew in 0% but not 5% NaCl [110]. Of the eight sub-groups of Paenibacillus glycanalyticus, only those isolates in sub-group 1 had a salt growth range consistent with that found by Dasman [110]; the remaining sub-groups grew in media containing 5% NaCl or more.
A large number of the isolates were identified as the genus *Bacillus* (Table 15), which was not a surprise, as *Bacillus* is quite resilient. Since they are spore-forming organisms, *Bacillus* species are incredibly resilient, and are often found in extreme environments, such as those with very high and low temperatures, desiccated environments, and areas of high ultraviolet radiation [79, 80].
The growth range of *Bacillus cereus* in various salt concentrations was investigated in numerous studies and determined to be 0-7% NaCl [111-113]. Even though the salt optima were different for the isolates identified as *B. cereus* in this study, the salt growth range was 0-5% NaCl for all.

**TABLE 15. Salt Tolerance and Colony Characteristics of Isolates Identified as Belonging to the Genus *Bacillus***

<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Salt Tolerance</th>
<th>Colony Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Species</td>
<td>Sub-group</td>
<td>Optimum Growth (% NaCl)</td>
<td>Range of Growth (% NaCl)*</td>
</tr>
<tr>
<td>B. altitudinis</td>
<td>5</td>
<td>0-10</td>
<td>yellow</td>
</tr>
<tr>
<td>B. amyloliquefaciens</td>
<td>5</td>
<td>5-10</td>
<td>yellow</td>
</tr>
<tr>
<td>B. cereus</td>
<td>0</td>
<td>0-5</td>
<td>white</td>
</tr>
<tr>
<td></td>
<td>0/5</td>
<td>0-5</td>
<td>white/cream</td>
</tr>
<tr>
<td>B. mycoides</td>
<td>0</td>
<td>optimum only</td>
<td>white</td>
</tr>
<tr>
<td>B. simplex</td>
<td>0/5</td>
<td>0-5</td>
<td>cream</td>
</tr>
</tbody>
</table>

*Isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.

Isolates identified as *Bacillus subtilis* were split into four subgroups based on their growth ranges and optimum growth. Only those isolates in subgroup 3 with a growth range of 0-10% NaCl were consistent with that reported by O’Donnell et al.[114]. The other subgroups only grew in 0% NaCl (subgroup 1), 10% NaCl (subgroup 4) or only up to 5% NaCl (subgroup 2).
Of those isolates identified as belonging to the *Bacillus* genus, a large number were identified as *Bacillus pumilus* and classified into 10 subgroups based on optimum salt concentration and range of growth in salt (Table 17). The salt growth ranges for those isolates in sub-groups 6 and 9 were the only ones that were consistent with the 0-10% NaCl reported by O’Donnell et al. [114]. Those isolates in the remaining subgroups had ranges that were inconsistent with that reported, as they were too limited (subgroups 1, 2, 4, 5, and 7), or too broad (subgroups 3, 8, and 10).

### TABLE 16. Salt Tolerance and Colony Characteristics of Isolates Identified as *Bacillus subtilis*

<table>
<thead>
<tr>
<th>Sub-group</th>
<th>Optimum Growth (% NaCl)</th>
<th>Range of Growth (% NaCl)*</th>
<th>Color</th>
<th>Morphology</th>
<th>Identification Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>optimum only</td>
<td>cream</td>
<td>irregular</td>
<td>573, 664, 666, 806, 815, 816, 818</td>
</tr>
<tr>
<td>2</td>
<td>0/5</td>
<td>0-5</td>
<td></td>
<td></td>
<td>670, 796, 817, 894</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>0-10</td>
<td></td>
<td></td>
<td>777, 898</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>10</td>
<td></td>
<td></td>
<td>614, 897</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optimal, or isolate grows very quickly, but does not reach maximum turbidity.*
The salt tolerance and colony characteristics for those isolates identified as *Bacillus megaterium* are presented in Table 18. *Bacillus megaterium* was found by Täubel et al., to grow in 0-5% NaCl, but not 10% NaCl [115]. The isolates in sub-groups 1 and 2 had growth consistent with that reported, while those in sub-groups 3 and 4 did not, as there was growth above 5% NaCl. Though the salt growth ranges for these two sub-groups are the same, the optima are not.
Exiguobacterium sp.

Of the 31 isolates identified as *Exiguobacterium*, three species were identified (*E. oxidotolerans*, *E. sibiricum*, and *E. undae*) (Table 19). The salt growth range of 0-5% NaCl for the *Exiguobacterium oxidotolerans* sub-group 2 isolates was consistent with that determined by Yumoto et al. [116]. Isolates in sub-group 1 were only able to grow substantially in media containing 5% NaCl, and not in that containing 0% NaCl.

The salt growth ranges for the *Exiguobacterium undae* isolates in sub-groups 2 and 3 were both consistent with the 0-5.8% NaCl described by Chaturvedi, et al. where growth in media containing salt concentrations up to the 5.8 % NaCl was tested [117]. The salt growth range for sub-group 1 isolates was not similar as there was no substantial growth in the medium containing 0% NaCl.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Salt Tolerance</th>
<th>Colony Characteristics</th>
<th>Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-group</td>
<td>Optimum Growth (% NaCl)</td>
<td>Range of Growth (%NaCl)*</td>
<td>Color</td>
</tr>
<tr>
<td>1</td>
<td>0/5</td>
<td>0-5</td>
<td>cream</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>0-5</td>
<td>cream</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>0-10</td>
<td>cream</td>
</tr>
<tr>
<td>4</td>
<td>5/10</td>
<td>0-10</td>
<td>cream</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.
TABLE 19. Salt Tolerance and Colony Characteristics of Isolates Identified as Belonging to the Genus *Exiguobacterium*

<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Salt Tolerance</th>
<th>Colony Characteristics</th>
<th>Isolate Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species</strong></td>
<td><strong>Sub-group</strong></td>
<td><strong>Optimum Growth (% NaCl)</strong></td>
<td><strong>Range of Growth (% NaCl)</strong>*</td>
</tr>
<tr>
<td><strong>E. oxidotolerans</strong></td>
<td>1 5</td>
<td>optimum only</td>
<td>orange</td>
</tr>
<tr>
<td></td>
<td>2 5</td>
<td>0-5</td>
<td>orange</td>
</tr>
<tr>
<td><strong>E. sibiricum</strong></td>
<td>0/5</td>
<td>0-10</td>
<td>orange</td>
</tr>
<tr>
<td><strong>E. undae</strong></td>
<td>1 5</td>
<td>optimum only</td>
<td>creamy yellow</td>
</tr>
<tr>
<td></td>
<td>2 5</td>
<td>0-5</td>
<td>creamy yellow</td>
</tr>
<tr>
<td></td>
<td>3 5</td>
<td>0-10</td>
<td>creamy yellow</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.

**Maximum Radiation Dose**

A select group of isolates with unique salt characteristics were irradiated with electrons to determine their maximum dose, or highest dose at which there were visible colonies following irradiation. The salt growth optima, ranges, and levels of radiation-resistance for the selected isolates is presented in Table 20. All of the organisms that were able to grow in at least 20% NaCl had either moderate or high levels of radiation resistance. The data provided enough information to suggest that there is a potential for
establishing a correlation between salt-tolerance and radiation-resistance, and that further testing should be done.

Using radiation-tolerance as a second variable proved to be valuable as it allowed us to demonstrate further differences even within these groups of potential new organisms. We found that resistance levels for these isolates were incongruous with one another. As seen in Table 20, the two *Bacillus pumilus* isolates from sub-group 7 that were irradiated showed vastly different tolerances. One isolate was radiation-resistant, the other was not. The five sub-group 1 isolates identified as *Pseudomonas veronii* served as another example since they had tolerances ranging from low to high.
**TABLE 20. Salt and Radiation Tolerance of Select Isolates Identified as Potential New Subspecies**

<table>
<thead>
<tr>
<th>Organism Identity</th>
<th>Salt Tolerance</th>
<th>Radiation Tolerance</th>
<th>Isolate Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus</td>
<td>Species</td>
<td>Sub-group</td>
<td>Optimum Growth (% NaCl)</td>
</tr>
<tr>
<td>Bacillus pumilus</td>
<td>3</td>
<td>0</td>
<td>0-25</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5</td>
<td>optimum only</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5</td>
<td>5-10</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5</td>
<td>0-25</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5/10</td>
<td>0-25</td>
</tr>
<tr>
<td>Brevundimonas kwangchunensis</td>
<td>2</td>
<td>0</td>
<td>0-5</td>
</tr>
<tr>
<td>Brevundimonas mediterranea</td>
<td>1</td>
<td>0</td>
<td>optimum only</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>optimum only</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
<td>optimum only</td>
</tr>
<tr>
<td>Kocuria rosea</td>
<td>2</td>
<td>0</td>
<td>0-25</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0/5/10</td>
<td>0-25</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5/10</td>
<td>0-25</td>
</tr>
<tr>
<td>Microbacterium foliorum</td>
<td>3</td>
<td>0</td>
<td>0-10</td>
</tr>
<tr>
<td>Microbacterium oxydans</td>
<td>2</td>
<td>0</td>
<td>0-10</td>
</tr>
<tr>
<td>Micrococcus flavus</td>
<td>2</td>
<td>0</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-5</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0</td>
<td>0-25</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-25</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-25</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0/5</td>
<td>0-25</td>
</tr>
<tr>
<td>Micrococcus luteus</td>
<td>1</td>
<td>0</td>
<td>0-5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-5</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-5</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-5</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0</td>
<td>0-25</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-25</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-25</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-25</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

*isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.

**Level of radiation tolerance, rather than LD50 value. The level is based on maximum dose survived. Levels are defined as follows: LOW= 0-2,000 Gy, MODERATE= 2,100-4,000 Gy, HIGH= 4,100-6,000 Gy, VERY HIGH= 6,100-10,000 Gy
<table>
<thead>
<tr>
<th>Genus</th>
<th>Species</th>
<th>Subgroup</th>
<th>Optimum Growth (% NaCl)</th>
<th>Range of Growth (%NaCl)*</th>
<th>Level of Tolerance**</th>
<th>Isolate Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas brenneri</td>
<td>2</td>
<td>0</td>
<td>0-10</td>
<td>Low</td>
<td>Isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-10</td>
<td>Low</td>
<td>Low</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-10</td>
<td>Low</td>
<td>Low</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-10</td>
<td>Low</td>
<td>Low</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-10</td>
<td>Low</td>
<td>Low</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-10</td>
<td>Low</td>
<td>Low</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0/5</td>
<td>0-15</td>
<td>Low</td>
<td>Isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0/5</td>
<td>0-15</td>
<td>Low</td>
<td>Low</td>
<td>917</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0/5</td>
<td>0-15</td>
<td>Low</td>
<td>Low</td>
<td>923</td>
<td></td>
</tr>
<tr>
<td>Pseudomonas migulae</td>
<td>2</td>
<td>0</td>
<td>0-5</td>
<td>Low</td>
<td>Isolate either reaches highest turbidity but requires more time than with optima, or isolate grows very quickly, but does not reach maximum turbidity.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-5</td>
<td>Low</td>
<td>Low</td>
<td>171</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-5</td>
<td>Low</td>
<td>Low</td>
<td>176</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-5</td>
<td>Low</td>
<td>Low</td>
<td>266</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-5</td>
<td>Low</td>
<td>Low</td>
<td>269</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-5</td>
<td>Low</td>
<td>Low</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0-5</td>
<td>Low</td>
<td>Low</td>
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<td>0-5</td>
<td>Very High</td>
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<td>Very High</td>
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Chapter 4

Discussion

The overall goal of this research was to determine if salt-tolerance is directly linked to radiation-resistance, and if salinity screening could be used to indirectly screen organisms for radiation-resistance. In general, the organisms that were able to grow over a wider range of salt concentrations were more likely to be able to survive moderate or high doses of radiation. Further exploration of the metabolic commonalities amongst the organisms would be useful to elucidate any common radiation tolerance mechanisms. Those organisms that were able to withstand moderate or high levels of radiation but were unable to grow in the higher salt concentrations may have unique methods of radiation tolerance or may have acquired their mechanisms via horizontal gene transfer.

The second part of this research was to identify new organisms with unique salt-tolerances. This portion proved to be much more fruitful as there were numerous isolates that grew well in high salt and are believed to be new subspecies. Not only were there isolates that grew well in high salt, but also some that grew equally well in all of the concentrations tested. These particular isolates would be ideal for future experiments that focus on repair mechanisms.

No common characteristics could be linked to the isolates that were able to grow well in higher salt concentrations. There appeared to be no correlation with colony color or morphology since isolates identified as the same organism with identical colony characteristics demonstrated vastly different salt tolerances. This is not surprising, however, as the small subunit rRNA gene changes very slowly in comparison with the
rest of the genome. The *Micrococcus flavus* isolates provided a great example of this discrepancy. Referring back to Table 4, all of the isolates identified as *M. flavus* had the same colony characteristics, yet the salt growth ranges varied from 0% only (sub-group 1 isolates) to 0-25% NaCl (sub-group 4 isolates). There was also no apparent correlation between the colony characteristics and radiation-tolerance either, as those isolates in sub-group 2 with the exact same colony and salt growth characteristics had very different resistance levels. For example, isolate 54 tolerated high levels of radiation, while isolate 205 tolerated only low levels.

Being able to determine the identity of the organisms and further divide them into subgroups allowed us to be able to examine how the different subgroups (with difference salt tolerances) held up during our radiation experiments. The data was not as clear-cut as we were anticipating; however, some conclusions can be inferred by examining the information presented in Table 20. For instance, ignoring the identity of the isolate, and simply focusing on salt growth characteristic, all of the isolates that had growth ranges of 0-25% NaCl had either moderate (2,100-4,000 Gy) or high (4,100-6,000 Gy) tolerances to radiation. None of the isolates tested with a growth range of 0-25% NaCl had a low tolerance where they could only survive doses of 2,000 Gy or less. The same was seen with those isolates that grew in 0-20% NaCl. There were 2 isolates irradiated that had that particular growth range in salt and both showed moderate resistance to radiation (surviving up to 4,000 Gy). It seems that having a wide range of growth in various salt concentrations is a good indicator that the organism is likely to be able to withstand moderate or high levels of radiation. Therefore, using salinity to screen for organisms that grow well in a wide range of salt concentrations (such as 0-25% NaCl) may allow for
finding organisms that are also tolerant to radiation, and may perhaps have unique mechanisms for their tolerances.

Since many organisms had varying tolerances to salt and radiation, it was difficult to compare organisms at the species level; instead, the subgroups of the organisms were examined and provided better insight into how salt-tolerance and radiation-resistance may be linked. There were seven different isolates identified as *Bacillus pumilus* that were irradiated. Looking at the salt growth ranges and radiation tolerance levels, it appeared that there was a correlation as those isolates that had wider or higher salt growth ranges were also able to tolerate higher doses of radiation. However, isolate 70 was inconsistent with others since it was able to grow in 5-10% NaCl but was not very resistant to radiation (low level of radiation tolerance). Perhaps there was an issue with the culture and if the experiment was repeated for that isolate, the radiation tolerance would be greater, which would be more consistent with the other isolates identified as that organism. Or, it is possible that this particular strain has a mutation in the pathway for radiation damage repair that make it more susceptible.

Three *Brevundimonas mediterranea* isolates were irradiated, with varying results. Two of the isolates had a moderate tolerance to radiation while the third had a high tolerance. All three of the isolates had identical salt growth ranges and salt optima (0% NaCl). All three also had identical colony characteristics. There is no clear difference between the isolates to warrant the difference in the tolerance to radiation. A similar situation occurred with isolates identified as *Kocuria rosea*, *Micrococcus flavus*, *Micrococcus luteus*, *Pseudomonas veronii*, *Rhodococcus corynebacterioides*, and *Rhodococcus fascians*. In each situation, there were multiple isolates that appeared to be
identical based on their salt growth ranges, salt optima, and colony characteristics, yet one or more would show higher or lower tolerance to radiation than the others. Of particular interest were the isolates identified as *Rhodococcus corynebacterioides* and *Rhodococcus fascians*. There were 2 isolates identified as *Rhodococcus corynebacterioides* that were irradiated. One had a low tolerance to radiation (could only survive doses under 2,000 Gy) while the other, seemingly identical isolate had a very high tolerance to radiation (could survive doses from 6,100 – 10,000 Gy). The same was seen with *Rhodococcus fascians*. Four out of the five isolates had a low tolerance to radiation while the fifth was very tolerant. It is possible that these organisms either developed novel resistance mechanisms or possibly acquired them via horizontal gene transfer. It would be very interesting to do genomic sequencing/testing to see the differences between the isolates and possibly isolate what genetic difference is making the one subspecies so much more resistant to radiation.

Another aspect of this research was demonstrating the speed at which speciation occurs and how using 16S rRNA sequencing, while helpful, does not begin to allow one to predict specific attributes of the organism (including colony characteristics and salt-tolerance). Some of the isolates identified as the same organism had vastly different tolerances or preferences in terms of salt. There were numerous situations in which some of the isolates identified as an organism would only be able to grow in media containing no additional salt while some of the other isolates that were identified as the same organism would either be able to grow in all salt concentrations tested or even exclusively in high salt. In all, there were 257 isolates, comprising 77 different sub-groups that we believe to be potential new subspecies. There were two organisms with
known subspecies, but since no salt or colony characteristic data was provided in those reports, we were unable to determine if the isolates in this study were similar to those reported. In order to determine if these isolates are new organisms, further investigating would need to be done.

Since isolates identified as the same organism were indistinguishable from one another by 16S rRNA sequencing, yet astonishingly different in terms of salt-tolerance, it showed that the organism’s genes and proteins are changing and adapting to provide tolerance to salt at a faster rate than the 16S ribosome sequence is changing. This has major implications for other analyses where 16s sequencing is used to identify community structure, as the particular environment may have allowed divergent evolution within a species that renders the individuals completely different from the commonly accepted type species.
References

17. Dobson, S. and P. Franzmann, *Unification of the Genera Deleya (Baumann et al. 1983), Halomonas (Vreeland et al. 1980), and Halovibrio (Fendrich 1988) and


83. Behrendt, U., A. Ulrich, and P. Schumann, Description of Microbacterium foliorum sp. nov. and Microbacterium phyllosphaerae sp. nov., isolated from the phyllosphere of grasses and the surface litter after mulching the sward, and reclassification of Aureobacterium resistent (Funke et al. 1998) as


Appendix

*Agrobacterium tumefaciens*  *Agrococcus jenensis*

**Isolate 363**  **Isolate 537**

**Isolate 373**

**Isolate 396**  **Isolate 106**

**Isolate 151**

---

**Legend:**  
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Arthrobacter agilis

Isolate 172

Isolate 679

Isolate 239

Isolate 750

Isolate 504

Isolate 950

Isolate 618

Growth

Day

R2, R2 = 5%, R2 = 10%, R2 = 15%, R2 = 20%, R2 = 25%
**Bacillus altitudinis**

- **Isolate 3**
- **Isolate 5**
- **Isolate 540**
- **Isolate 955**
- **Isolate 575**

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Bacillus amyloliquefaciens

Isolate 755

- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Bacillus cereus

Isolate 30

Isolate 375

Isolate 61

Isolate 393

Isolate 259

Isolate 462

Isolate 367

Isolate 480

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Bacillus cereus

**Isolate 514**

**Isolate 520**

**Isolate 548**

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Bacillus megaterium

Isolate 573

Isolate 777

Isolate 614

Isolate 796

Isolate 664

Isolate 806

Isolate 666

Isolate 815

Isolate 670

Isolate 816

Growth vs. Day

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Bacillus pumilus

Isolate 19

Isolate 66

Isolate 38

Isolate 70

Isolate 43

Isolate 144

Isolate 52

Isolate 184

Isolate 58

Isolate 193

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Bacillus pumilus

Isolate 484

Isolate 542

Isolate 495

Isolate 544

Isolate 496

Isolate 553

Isolate 498

Isolate 557

Isolate 519

Isolate 583

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Bacillus pumilus

Isolate 834

Isolate 861

Isolate 837

Isolate 870

Isolate 840

Isolate 877

Isolate 848

Isolate 885

Isolate 859

Isolate 887

Growth
Day

0 4 8 12 16 20 24 28

0 4 8 12 16 20 24 28

0 4 8 12 16 20 24 28

0 4 8 12 16 20 24 28

R2, R2 + 5%, R2 + 10%, R2 + 15%, R2 + 20%, R2 + 25%
Bacillus subtilis

Isolate 254

Isolate 272

Isolate 255

Isolate 284

Isolate 256

Isolate 288

Isolate 257

Isolate 395

Isolate 264

Isolate 450

Growth

0 4 8 12 16 20 24
Day

0 4 8 12 16 20 24
Day

0 4 8 12 16 20 24
Day

0 4 8 12 16 20 24
Day

0 4 8 12 16 20 24
Day

0 4 8 12 16 20 24
Day

0 4 8 12 16 20 24
Day

0 4 8 12 16 20 24
Day

R2  
R2 + 5%  
R2 + 10%  
R2 + 15%  
R2 + 20%  
R2 + 25%  

94
Bacillus subtilis

Isolate 487

Isolate 648

Isolate 512

Isolate 693

Isolate 545

Isolate 865

Isolate 563

Day

Growth

Day

Growth

Day

Growth

Day

Growth

Day

Growth

Day

Growth

Day
Chryseobacterium soldanellicola

Isolate 56

<table>
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- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Clavibacter michiganensis
Dyadobacter koreensis

Isolate 99

Isolate 823

Enhydrobacter aerosaccus

Isolate 694

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Exiguobacterium oxidotolerans

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<th>8</th>
<th>1.2</th>
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<td>0.8</td>
<td>1</td>
<td>2</td>
<td>4</td>
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</table>

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Exiguobacterium oxidotolerans

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Exiguobacterium sibiricum

Isolate 638

Growth

Day

R2
R2 + 5%
R2 + 10%
R2 + 15%
R2 + 20%
R2 + 25%
Exiguobacterium undae

Isolate 567

Isolate 790

Isolate 634

Isolate 855

Isolate 752

Isolate 857

Isolate 753

Isolate 902

Isolate 780

Isolate 903

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Kocuria palustris

Isolate 291

Isolate 551

Isolate 658

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
*Kocuria rosea*

- **Isolate 465**
  - Growth
  - Days: 0, 4, 8, 12, 16, 20, 24

- **Isolate 669**
  - Growth
  - Days: 0, 4, 8, 12, 16, 20, 24

- **Isolate 511**
  - Growth
  - Days: 0, 4, 8, 12, 16, 20, 24

- **Isolate 692**
  - Growth
  - Days: 0, 4, 8, 12, 16, 20, 24

- **Isolate 590**
  - Growth
  - Days: 0, 4, 8, 12, 16, 20, 24

- **Isolate 802**
  - Growth
  - Days: 0, 4, 8, 12, 16, 20, 24

- **Isolate 632**
  - Growth
  - Days: 0, 4, 8, 12, 16, 20, 24

- **Isolate 824**
  - Growth
  - Days: 0, 4, 8, 12, 16, 20, 24

- **Isolate 640**
  - Growth
  - Days: 0, 4, 8, 12, 16, 20, 24

- **Isolate 944**
  - Growth
  - Days: 0, 4, 8, 12, 16, 20, 24

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
*Klyococcus sedentarius*

**Isolate 261**

**Isolate 262**

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Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Massilianiabensis

Isolate 191

Isolate 756

Isolate 630

Isolate 771

Isolate 660

Isolate 811

Isolate 747

Isolate 846

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%

Day
Microbacterium esteraromaticum

Isolate 774

Day

Growth

R2
R2 + 5%
R2 + 10%
R2 + 15%
R2 + 20%
R2 + 25%
Microbacterium foliorum

- **Isolate 95**
- **Isolate 213**
- **Isolate 103**
- **Isolate 226**
- **Isolate 147**
- **Isolate 245**
- **Isolate 209**
- **Isolate 275**
- **Isolate 210**
- **Isolate 287**

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
**Microbacterium foliorum**

- **Isolate 491**
- **Isolate 516**
- **Isolate 518**
- **Isolate 762**

**Microbacterium lacticum**

- **Isolate 529**
Microbacterium oxydans
Microbacterium oxydans

Isolate 319

Isolate 605

Isolate 455

Isolate 704

Isolate 527

Isolate 853

Growth vs Day

R2, R2 + 5%, R2 + 10%, R2 + 15%, R2 + 20%, R2 + 25%
Micrococcus flavus

Isolate 9

Isolate 203

Isolate 49

Isolate 205

Isolate 54

Isolate 315

Isolate 154

Isolate 318

Isolate 175

Isolate 362

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Micrococcus flavus

Isolate 467

Isolate 554

Isolate 558

Isolate 909

Growth

Day

0 4 8 12 16 20 24

0 2 4

R2 R2 + 5% R2 + 10%

R2 + 15% R2 + 20% R2 + 25%
Micrococcus luteus

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<tr>
<td>297</td>
<td><img src="image8.png" alt="Graph" /></td>
</tr>
<tr>
<td>230</td>
<td><img src="image9.png" alt="Graph" /></td>
</tr>
<tr>
<td>298</td>
<td><img src="image10.png" alt="Graph" /></td>
</tr>
</tbody>
</table>

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Micrococcus luteus

Isolate 299

Isolate 560

Isolate 329

Isolate 646

Isolate 350

Isolate 743

Isolate 434

Isolate 949

Isolate 534

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Micrococcus thailandicus

Isolate 108

Growth

Day

R2
R2 + 5%
R2 + 10%
R2 + 15%
R2 + 20%
R2 + 25%
Micrococcus vinnemensis
Micrococcus yumanensis

Isolate 847

Growth over time for different R2 concentrations.
Moraxella osloensis

<table>
<thead>
<tr>
<th>Isolate 63</th>
<th>Isolate 229</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Graph" /></td>
<td><img src="image2" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isolate 107</th>
<th>Isolate 323</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image3" alt="Graph" /></td>
<td><img src="image4" alt="Graph" /></td>
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</table>

<table>
<thead>
<tr>
<th>Isolate 116</th>
<th>Isolate 326</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image5" alt="Graph" /></td>
<td><img src="image6" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isolate 123</th>
<th>Isolate 391</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image7" alt="Graph" /></td>
<td><img src="image8" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isolate 130</th>
<th>Isolate 591</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image9" alt="Graph" /></td>
<td><img src="image10" alt="Graph" /></td>
</tr>
</tbody>
</table>

Legend:
- Blue = R2
- Red = R2 + 5%
- Green = R2 + 10%
- Purple = R2 + 15%
- Cyan = R2 + 20%
- Orange = R2 + 25%
**Moraxella osloensis**

- **Isolate 598**

**Naxibacter haematophilus**

- **Isolate 758**

**Naxibacter indica**

- **Isolate 644**

<table>
<thead>
<tr>
<th>Growth</th>
<th>Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>1.5</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>2.5</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>3.5</td>
<td>28</td>
</tr>
</tbody>
</table>

- **Isolate 657**

- **Isolate 710**

- **Isolate 659**

The graphs show the growth of different isolates over a period of 28 days, with various dilutions indicated by different colors:

- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Pectobacterium carotovorum

Isolate 468

Isolate 813

Isolate 522

Isolate 828

Isolate 533

Isolate 831

Isolate 615

Isolate 863

Isolate 652

Isolate 872

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Planococcus psychrotoleratus  Planomicrobiurn glaciei

Isolate 574

Isolate 526

Isolate 645

Isolate 651

Isolate 723

Growth vs. Day

Growth vs. Day

Growth vs. Day

Growth vs. Day

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Pseudomonas bremeri

<table>
<thead>
<tr>
<th>Isolate 16</th>
<th>Isolate 97</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Graph" /></td>
<td><img src="image2" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isolate 73</th>
<th>Isolate 98</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image3" alt="Graph" /></td>
<td><img src="image4" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isolate 81</th>
<th>Isolate 167</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image5" alt="Graph" /></td>
<td><img src="image6" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isolate 92</th>
<th>Isolate 186</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image7" alt="Graph" /></td>
<td><img src="image8" alt="Graph" /></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Isolate 96</th>
<th>Isolate 232</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image9" alt="Graph" /></td>
<td><img src="image10" alt="Graph" /></td>
</tr>
</tbody>
</table>

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Pseudomonas bremeri

Isolate 234

Isolate 388

Isolate 242

Isolate 515

Isolate 247

Isolate 917

Isolate 248

Isolate 923

Isolate 309

Isolate 928

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Pseudomonas fluorescens

Isolate 11

Isolate 33

Isolate 12

Isolate 36

Isolate 13

Isolate 45

Isolate 14

Isolate 65

Isolate 21

Isolate 74

R2, R2 + 5%, R2 + 10%,
R2 + 15%, R2 + 20%, R2 + 25%
Pseudomonas fluorescens

Isolate 163

Isolate 178

Isolate 387

Isolate 404

Isolate 410

Legend:

- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
*Pseudomonas frederiksbergensis*

**Isolate 486**

**Isolate 699**

---

**Legend:**
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Pseudomonas guinea

Graphs showing growth over days for different isolates.

Isolate 204

Isolate 451

Isolate 279

Isolate 528

Isolate 292

Isolate 716

Isolate 334

Isolate 864

Isolate 441

Isolate 886

Key:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Pseudomonas guinea

Isolate 901

Pseudomonas mendelii

Isolate 6

Isolate 401

Isolate 23

Isolate 577

Isolate 24

Isolate 581

Growth vs. Day

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Pseudomonas migulae

Isolate 82

Isolate 170

Isolate 87

Isolate 171

Isolate 88

Isolate 176

Isolate 90

Isolate 183

Isolate 169

Isolate 263

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Pseudomonas migulae

Isolate 663

Isolate 765

Isolate 665

Isolate 766

Isolate 705

Isolate 769

Isolate 712

Isolate 781

Isolate 751

Isolate 782

Legend:
- \(R2\)
- \(R2 + 5\%\)
- \(R2 + 10\%\)
- \(R2 + 15\%\)
- \(R2 + 20\%\)
- \(R2 + 25\%\)
**Pseudomonas migulae**

<table>
<thead>
<tr>
<th>Isolate 785</th>
<th>Isolate 910</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="#" alt="Growth graph" /></td>
<td><img src="#" alt="Growth graph" /></td>
</tr>
<tr>
<td>Isolate 800</td>
<td>Isolate 912</td>
</tr>
<tr>
<td><img src="#" alt="Growth graph" /></td>
<td><img src="#" alt="Growth graph" /></td>
</tr>
<tr>
<td>Isolate 830</td>
<td>Isolate 918</td>
</tr>
<tr>
<td><img src="#" alt="Growth graph" /></td>
<td><img src="#" alt="Growth graph" /></td>
</tr>
<tr>
<td>Isolate 841</td>
<td>Isolate 924</td>
</tr>
<tr>
<td><img src="#" alt="Growth graph" /></td>
<td><img src="#" alt="Growth graph" /></td>
</tr>
<tr>
<td>Isolate 844</td>
<td>Isolate 925</td>
</tr>
<tr>
<td><img src="#" alt="Growth graph" /></td>
<td><img src="#" alt="Growth graph" /></td>
</tr>
</tbody>
</table>

Legend:

- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
<table>
<thead>
<tr>
<th>Isolate 926</th>
<th>Isolate 464</th>
</tr>
</thead>
</table>
| ![Graph](image1)
| ![Graph](image2) |
| Isolate 927 | Isolate 466 |
| ![Graph](image3)
| ![Graph](image4) |
| Isolate 929 | Isolate 538 |
| ![Graph](image5)
| ![Graph](image6) |
| Isolate 550 |           |
| ![Graph](image7)
| ![Graph](image8) |

**Legend:**
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Pseudomonas veronii

<table>
<thead>
<tr>
<th>Isolate 35</th>
<th>Isolate 152</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Isolate 55</th>
<th>Isolate 409</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Isolate 60</th>
<th>Isolate 499</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Isolate 71</th>
<th>Isolate 919</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Isolate 139</th>
<th>Isolate 930</th>
</tr>
</thead>
</table>

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Psychrobacter cryohalolentis

Isolate 8

Isolate 370

Isolate 17

Isolate 394

Isolate 281

Isolate 403

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Psychrobacter glacincola

Isolate 78

Isolate 244

Isolate 113

Isolate 294

Isolate 149

Isolate 301

Isolate 231

Isolate 546

Isolate 235

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%

Day
Rhodococcus corynebacterioides

Isolate 219

Isolate 539

Isolate 260

Isolate 543

Isolate 330

Isolate 601

Isolate 459

Isolate 612

Isolate 535

Isolate 633

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%

Growth
Day
Rhodococcus fascians

Isolate 22

Isolate 276

Isolate 57

Isolate 311

Isolate 211

Isolate 342

Isolate 214

Isolate 345

Isolate 228

Isolate 349

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Rhodococcus fascians

![Growth curves for Isolates 385, 745, 386, 787, 489, and 862](image)

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
Staphylococcus epidermidis

Isolate 4

Isolate 265

Isolate 135

Isolate 325

Isolate 190

Isolate 332

Isolate 202

Isolate 335

Isolate 308

Isolate 339

R2  R2 + 5%  R2 + 10%  R2 + 15%  R2 + 20%  R2 + 25%
Staphylococcus epidermidis

Isolate 343

Isolate 549

Isolate 415

Isolate 600

Isolate 428

Isolate 661

Isolate 461

Isolate 682

Isolate 513

Isolate 873

Legend:
- R2
- R2 + 5%
- R2 + 10%
- R2 + 15%
- R2 + 20%
- R2 + 25%
*Staphylococcus epidermidis*

**Isolate 876**

**Isolate 889**

**Isolate 905**

![Graphs showing growth over days for different isolates.](image)
Staphylococcus pasteuri