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ENHANCING THE ENVIRONMENTAL SUSTAINABILITY OF THE PRODUCTION AND DEGRADATION OF PHA POLYMERS

by

Joshua R. Harris

A thesis
submitted in partial fulfillment
of the requirements for
Master of Science Degree
State University of New York
College of Environmental Science and Forestry
Syracuse, NY
May, 2018

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Acknowledgements

First and foremost, I would like to give thanks to my advisor Christopher Nomura for allowing me the opportunity to be a part of his research group, both as an undergraduate student and for my master's degree. I remember first meeting Chris in 2011 and asking to join his lab the very next day. Since then he has been a major influence in my life as a mentor, a role-model, and a friend. He has helped guide me in the realms of science, and sometimes life, and I give him a lot of credit for helping direct the trajectory of my career as scientist. I would also like to thank my other research mentor Benjamin Lundgren for the excellent hands on training and day-to-day guidance he has given me over the past several years. I have a lot of respect for Ben's patience and willingness in his direction on how to work and write in a research laboratory at a professional level. In addition, I would like to thank some of the other post-docs that were part of the Nomura Research Group; Ata Pinto and Zaara Sarwar. I am very grateful to have been a part of this lab during the time that it had such phenomenal researchers so willing to help an aspiring scientist such as myself.

Many thanks to my other steering committee members Gregory Boyer and Lee Newman. Dr. Newman was also my undergraduate advisor and her openness as a person and dedication as a scientist continue to be an inspiration. I would like to thank the Department of Chemistry here at SUNY-ESF as well as the university as a whole. I am proud to have had the privilege to earn my BS and MS degrees at such a wonderful and conscious institution.

During my time here as a student I have formed many meaningful and lasting relationships with other students, especially my close friends Joseph Shoytush and Christopher Thomas. I am lucky to have had such rad buds that were willing to take the leap into graduate school with me at the

same time and also willing to play frisbee in the quad when I needed a break from research (among other extracurricular activities). Thanks to some of the other members of the department Ryan Scheel and Robert Moesch and a special thanks to Joy Logan with whom I have had many long and meaningful conversations about science and the importance of this career path.

Lastly, I would like to thank my parents for their continued encouragement to pursue whatever makes me happy. They don’t always understand exactly what I am doing, or why, but they have never stopped showing interest and unrelenting support for my choices in life. I consider myself to be exceedingly fortunate to have such a wonderful mother and father and I try to express that gratitude as often as possible.

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Abstract

J.R. Harris, Enhancing the Environmental Sustainability of the Production and Degradation of PHA Polymers, 109 pages, 21 figures, 4 tables, 2018.

The negative impacts caused by traditional plastics to natural ecosystems are becoming increasingly drastic. One solution to this issue is greater implementation of polyhydroxyalkanoates (PHAs), which are biodegradable plastics naturally produced by bacteria. The primary objective of this work was to exemplify and add to the body of research being conducted to further progress the PHA industry as a whole. Economic feasibility is a major barrier preventing broader usage of PHAs, therefore a study was performed to create a novel method for engineering *Halomonas* bacteria, a highly attractive genus for its potential in lowering PHA production costs. To ensure environmental compatibility, research was conducted to investigate the natural biodegradation of PHA polymers and determine how some naturally present microorganisms metabolize components of these materials. The insights provided by these studies will further promote our ability to expand PHA technologies while maintaining a focus on environmental sustainability.

*Keywords: Polyhydroxyalkanoates, Halomonas, electroporation, Pseudomonas aeruginosa, (*R*)-3-Hydroxybutyrate, environmental sustainability, biodegradable plastics*

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Chapter 1. Introduction and Background

1.1 The 'Age of Plastics'

1.1.1 Global Plastic Production

In 1909, in front of the New York chapter of the American Chemical Society, Leo Hendrick Baekeland publicly announced the invention of Bakelite, the first commercially produced synthetic plastic (American Chemical Society, 1993). This historic discovery ushered in what can only be known as the 'Age of Plastics' and, over the past hundred years, the industrial world has been revolutionized by the increasing presence and necessity of synthetically made polymers that permeate almost all aspects of our everyday lives. Although the original synthesis of Bakelite occurred at the turn of the 20th century, it wasn't until the 1940s and 1950s that commercial plastic production began to exceed 1 million tons annually (Thompson et al., 2009). In 1993, global plastic production was reported at over 107 million tons. In only seven years, at the dawn of the new millennium, global production rose over 36% and annual plastic production peaked at 146 million tons (Shah et al., 2008). Fast forward another 13 years and we can see an increase in manufacturing of over 100% to 299 million tons in 2013 (Gourmelon, 2015). Even with conservative estimates, reports have been released that predict annual plastic production will continue to rise at least 3-5% annually, and for many developing nations, such as Pakistan, plastic manufacturing is growing at annual rates as high as 15% (Gourmelon, 2015; Sabir, 2004).

From the 1950s until as late as 2008, manufacturing of plastic has grown at an exponential rate that has only begun to level off (Figure 1.1). Regardless of any recent decreases in the rate of growth of the plastic industry, it is highly likely that production of these materials will be maintained on the level of hundreds of millions of tons per year for the foreseeable

future. Clearly, plastics are here to stay and, as more countries usher in their own industrial ages, the importance and integration of plastic materials into global society will continue to grow.

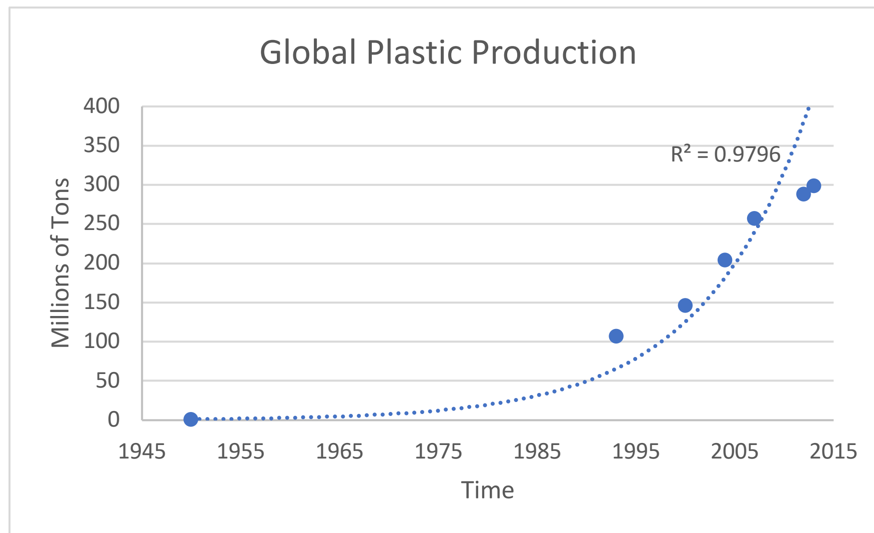

Figure 1.1 Annual global plastic production in metric tons since 1950. Line of best fit is exponential curve with R^2 value of 0.98. Exponential phase appears to begin to level off around 2010, but continues to increase (Shah et al., 2008; Gourmelon, 2015; Plastics Europe, 2017).

1.1.2 Plastics in Society

Considering all the amazing benefits that have come from this ‘Age of Plastic’, it is easy to understand why global production is growing so rapidly. Items ranging from shoes and toothbrushes to telephones and spacecraft have all been revolutionized by the expansion of the plastic industry. These materials have enabled enormous enhancements to our quality of life and the expansion of technology. The integration of plastics to our everyday life has become so commonplace it’s almost easy to overlook. For example, many of the clothing items we wear include materials such as nylon and polyester, which are completely composed of synthetic plastic fibers. Additionally, replacing heavier metal parts with lightweight plastics in vehicles and airplanes has improved the efficiency of travel and has actually reduced the amount of CO_2 emissions per vehicle (Andrady & Neal, 2009). Without including the aspects of plastic litter and waste, in many cases the development of plastic materials that can be recycled or replace less

efficient parts in preexisting commodity items represents an environmental benefit to having increased amounts of plastic in society. When we finally consider the development of computer technologies such as cell phones and laptops made possible by plastic, it becomes impossible to ignore the incredible influence these materials have on human civilization.

In terms of public health, plastics have provided substantial benefits in both the medical field and in everyday life. Using plastic piping such as PVC in both industrialized and developing nations has directly lead to a reduction in lead and other heavy metal exposure (Levin et al., 2008). In the medical field, plastics have revolutionized the ability for surgical procedures to be performed in aseptic conditions (Andrady & Neal, 2009). Sterility is just the tip of the iceberg of ways that plastics have changed the way we practice medicine; applications ranging from artificial heart valves, a variety of prosthetics, hearing aids, plastic pill capsules, and even artificial corneas are just a few of the medical breakthroughs enabled by plastic materials (Sholz et al., 2011). Additionally, the use of plastics in the packaging of meats and other perishables has not only enhanced the shelf-life of many foods but has also greatly reduced the prevalence of food-borne illnesses (Mullan, 2002). Unfortunately, technological breakthroughs of this scale are often riddled with drawbacks and for plastics there are two major disadvantages. The first is the necessity of fossil fuels to create the majority of plastics used today. The second drawback can be observed at the other end of the cycle when plastics outlive their use in our society yet persist and accumulate in our environment.

1.1.3 Fossil Fuels and Plastics

Over the past century, the monumental rise in the presence and use of petroleum-based plastic materials in global society has been consequently and inversely shadowed by a decline in the available reserves of oil on this planet. In a 2006 article on the World Energy Outlook, it was

estimated that oil reserves will only last for about 45 more years, natural gas will be depleted in 64 years, and coal will be available for another 160 years (International Energy Agency, 2006). Overall, the majority of plastic materials produced from fossil fuel feedstocks comes from crude oil and natural gas with 4% of global oil production going towards these materials and 33% of all U.S. natural gas going towards the industrial sector of which plastic production is included (U.S. Energy Information Administration, 2017; British Plastics Federation, 2008).

The effect of dwindling fossil fuels reserves, especially crude oil and natural gas, has a two-fold effect on the plastic industry. The first and most immediate effect is that, despite the overall small percentage of harvested crude oil and natural gas that is used for plastic production, increases in costs of these raw materials will directly lead to an increase in the cost of plastic and eventually create an economically unsustainable model for using these fossil fuels as the primary feedstock. Additionally, considering that these resources are at their core nonrenewable, once crude oil and natural gas run out, the option for making plastics from these fossil fuels will become virtually non-existent.

Considering the previously discussed data regarding the longevity of energy reserves on this planet, one option to satisfy to the demand for fossil fuel-based plastics throughout the next century will be to establish coal as the primary raw feedstock for the production of plastic materials. Intuitively this makes sense considering coal represents 55% of all remaining non-renewable fuels and research has already begun on how to integrate coal as a plastic feedstock on an industrial scale (Shafiee & Topal, 2009; Schobert & Song, 2002). Although global coal reserves may be our saving grace for diminishing supplies of oil and natural gas, the use of coal still fails to address the issue at the core and eventually coal will also run-out. Therefore, the future of plastic will undoubtedly depend on the development of methods that utilize renewable

energy and renewable feedstocks for these materials. The growing need to find renewable resources to make plastic materials is predicated by the limited supply of fossil fuels, but the lack of resources for making these materials is an issue that ultimately pales in comparison to the negative environmental impact fossil fuel-based plastics are already having on natural ecosystems and the environment.

1.1.4 Accumulation of Plastic in the Environment

Plastics are highly desired for their ability to resist degradation and deterioration, especially against natural forces such as uv exposure, moisture, oxidation, and biological organisms (Lucas et al., 2008). Unfortunately, the same properties that make plastics useful during their shelf-life are causing unforeseen consequences in the natural environment when these materials are discarded. For some applications, such as in permanent structures, a plastic that never degrades is extremely useful and, if it lives up to its reputation, it should never end up in landfills or the environment. But, as many are aware, there are multitudes of plastic items that are used once, or maybe several times, then discarded to never be used again.

For example, consider the fate of a common plastic grocery bag, just like those found in almost every grocery and department store around the world. In many cases, these bags can be made, packaged, shipped, and used on a timescale of weeks to months. After the single use of this item, it will likely be thrown in the trash and go to a landfill. Typically, these plastic bags are made from of either low-density polyethylene (LDPE) or high-density polyethylene (HDPE), both of which are thermoplastics composed of ethylene monomers (Figure 1.2). In 2014, the EPA estimated that 5.6% of all LDPE used for making plastic bags was recycled, indicating that 94.4% of this plastic material ends up in either landfills or as litter in the environment (U.S. Environmental Protection Agency, 2014). One study found that even in ideal conditions for

biodegradation (LDPE cultivated in a nutrient solution inoculated with cultivated soil microbes and aerated continuously at 25°C in the dark) it took over two years for the plastic to release only 0.1% of its mass in the form of CO_2 (Albertsson, 1980). Extrapolating this data implies that this same mass of LDPE would take up to 2000 years to completely degrade. When one considers how many millions of tons of plastic are been created every year, it becomes very easy to understand that plastic wastes in the environment are increasing at a much greater rate than they are decomposing, and this accumulation is not negligible. The best evidence for illustrating the sheer magnitude of plastic-waste buildup is the Pacific Trash Gyre; a diffuse soup of floating plastic in the Northern Pacific that is estimated to contain at least 100,000 tons of floating plastic debris (Eriksen et al., 2014).

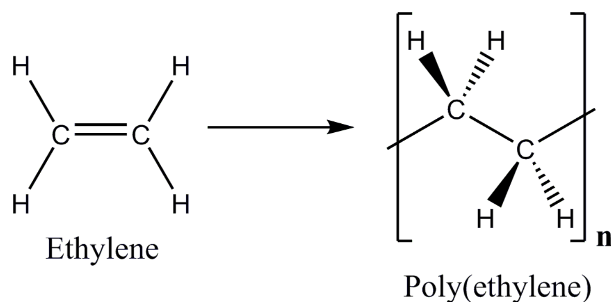

Figure 1.2 Chemical structures of ethylene and poly(ethylene). Both LDPE and HDPE are composed of same basic polymeric structure and differences in the materials are a result of the degree of cross-linking (Purdue University)

1.1.5 Chemical Structure of Plastics

Data shows that the accumulation of plastics in our natural ecosystems, especially oceans, is continuously growing in distribution and abundance (Rochman, 2015). As previously stated, the majority of these materials are created from petroleum, natural gas, and coal. These feedstocks are the end-product of millions of years of natural decay of once-living organisms and are essentially, themselves, natural products of the environment (Wolchover, 2011). Although it appears that traditionally-made plastics seem to be somewhat natural in their origins, the

chemical processes used to make them and the resulting molecular structures are unlike anything else seen in nature and are thus considered to be synthetic products. It is the synthetic nature of these materials that make them so well suited to resist environmental forces and, as a result, persist in ecosystems well beyond their intended usage.

The chemical configuration of plastic is what determines its physical properties and is the major consideration for understanding why nature is ill-suited to decompose these items. On many common plastic items in the United States, there is a number known as a resin identification code that describes what material the plastic is made from and what the restrictions are for recycling these materials. The numbers are listed as either 1-7 and although this does not encompass all plastic materials that can be found in society, it includes the most common and widely dispersed of plastic items [Figure 1.3] (Sustainable Packaging Coalition, 2017). Despite the variation in monomeric structure of these materials, there is a unifying theme amongst all of them; they are polymers, which can be defined as large macromolecules that are essentially composed of repeating structural units. It is in the chemical process of polymerization that transforms what was once a natural product into a synthetic form that can resist the natural forces that degrade almost all other materials.

As is evidenced by Figure 1.3, these plastic polymers all consist of repeating monomer units and are typically composed of a backbone structure containing hydrocarbons. Many of these polymers also contain side-chain groups, which are highly variable and can range from a simple hydrogen as in polyethylene to the benzene ring in polystyrene and can even include non-organic halides such as chlorine, as is the case with polyvinyl chloride. In plastics labeled as #2-#6, the backbone is composed entirely of carbon-carbon bonds with a CH_2-CHR repeating moiety, whereas polyethylene terephthalate (#1) and polycarbonate (#7) contain an ester-bond or

carbonate bond, respectively, and at least one aromatic hydrocarbon ring in their backbone structure resulting in unique properties including increased relative rigidity (Venkatachalam et al., 2012). The backbone structure of a given polymer is a major determinant of its chemical and physical characteristics and is a defining feature of many plastic materials.

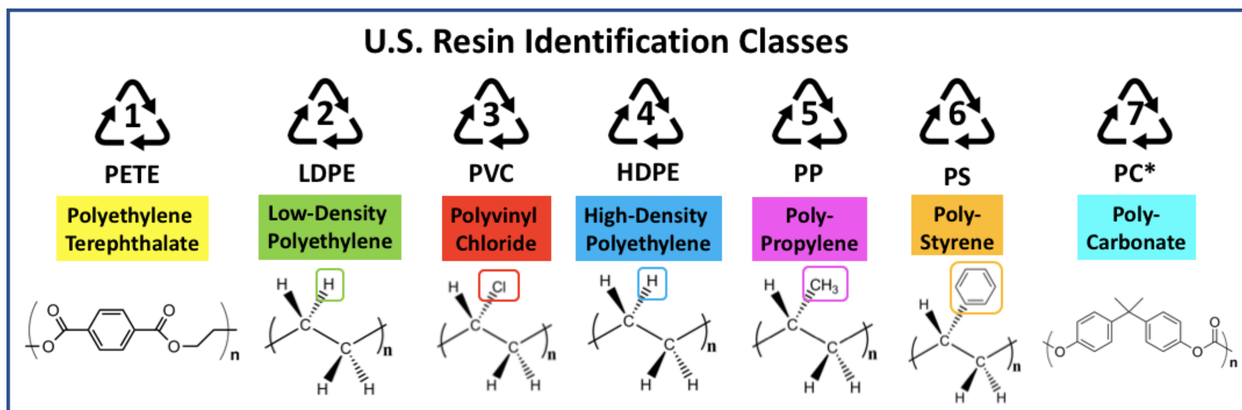

Figure 1.3 Classification of plastics for recycling purposes in United States. Monomer units of primary polymeric backbone are illustrated. Degrees of branching, cross-linking, crystallinity/amorphousness, and other modifications are not depicted. Plastics 2-6 are all derivatives of the polyethylene backbone that primarily differ in their side chain composition. **Class 7 is technically categorized as 'other plastics' and includes polycarbonate (PC) as well as all other recyclable plastics that do not fit within the classes 1-6* (Sustainable Packaging Coalition, 2017).

Considering the straight chain backbone of a polymer to be a 1-dimensional structure, there can also exist two- and three-dimensional configurations based upon potential branching, cross-linking, and the overall crystallinity or amorphousness of the molded plastic (American Chemistry Council, 2006). Polymeric branching occurs when groups of units branch off from the main polymer chain either from side groups or directly off the backbone to form extensions of the polymer in multiple directions. Similarly, cross-linking is the covalent or ionic attachment of two or more polymer chains to one another and can greatly alter the physical properties of the plastic especially in regard to stiffness, viscosity, and its ability to be remolded (Purdue University). During the formation and solidification of molded plastics, it is possible for linear polymers to fold and align into secondary and even tertiary crystalline structures depending on the process of synthesis as well as the polymer's backbone and side-group composition (Carraher

& Seymour, 2003). Ultimately, the degree of branching, crosslinking, and overall crystal and amorphous molecular arrangement are additional components that can greatly alter the physical properties of a plastic and is highly influential in the material's ability to resist degradative forces.

1.1.6 Degradation of Synthetic Plastics

When a plastic is discarded, including into landfills and oceans, the subsequent degradation of the material occurs through a combination of biotic and abiotic mechanisms depending on the durability and composition of the plastic and the environment in which it is disposed. Abiotic factors that contribute to the breakdown of plastics include; mechanical, which is the combination of shear, tensile, and compressive forces; light, which exploits the photosensitivity of some materials and causes deterioration through photoionization and thermal radiation; and chemical, which is one of the most powerful abiotic degradative forces and includes oxidation from atmospheric O_2, hydrolysis by H_2O, and degradation caused by pollutants present in the atmosphere and certain agrochemical runoffs (Lucas et al., 2008). In conjunction with these abiotic factors, microorganisms present in the environment such as bacteria, fungi, algae, protozoa, and even lichens can all contribute to the biodegradation of plastic macromolecules (Wallström et al., 2005). Overall, it is a combination of all these degradative forces, biotic and abiotic, that lead to the breakdown of a plastic polymer. Ultimately, however, complete conversation of plastic macromolecules from their original form into chemical constituents indistinguishable from naturally present compounds is dependent upon the microorganisms in the environment that are capable of the biodeterioration, biofragmentation, and assimilation steps required for true biodegradation to occur (Lucas et al., 2008).

The European Union standard for defining a material as biodegradable requires that 90% of the material breaks down into CO_2, water, and minerals, within 6 months, through the biological action of naturally occurring microorganisms (Breulmann et al., 2009). Although somewhat stringent, this definition can be broadly applied to quintessentially describe biodegradation as the breakdown of a material, not into smaller and smaller pieces which can be ascribed as disintegration, but rather chemically into natural components through biological activity into a form that is not biologically toxic. Therefore, the basis for classifying a substance to be biodegradable hinges on the natural availability of microorganisms that contain the prerequisite chemicals and enzymes capable of catalyzing the decomposition of these substances into a form that can be naturally assimilated and metabolized.

The development of synthetic plastics and their introduction into the environment is a very recent occurrence relative to the evolutionary scale of life on earth (Mojzsis et al., 1996). The mechanisms of evolution dictate that given enough time, a substance containing high-energy covalent bonds, such as those found in polymers, will eventually spawn the adaptation of organisms and enzymes capable of sequestering this energy into a useable form. However, it is not just one organism and one enzyme that is responsible for the biodegradation of a material, but rather a synergistic effect of all the microorganisms present (Karlsson & Albertsson, 1998). Therefore, for the biodegradation of each different type of synthetic polymer present in the environment to occur would require the collective adaptation of a myriad of microorganisms followed by global dispersion of these organisms, and that process would have to be repeated for each different type of plastic present in the environment. Simply stated, the build-up of synthetic plastic litter is far outpacing the ability of life to adapt, which means synthetic plastic litter in the environment is likely to persist for thousands of years (Albertsson, 1980).

This issue is compounded by the facts that many plastics have been designed to strongly resist degradative forces of any kind (Lucas et al., 2008). In addition to their synthetic backbone, the durability of these materials is a result of a combination of branching, cross-linking and their 3-dimensional crystalline structure as well as the incorporation of additives such as antioxidants, flame retardants, and plasticizers (Banerjee et al., 2014). Even in the presence of enzymes capable of catalyzing scission of a polymer backbone, polymer crystallinity can greatly hinder the ability of an enzyme's active site to properly orient the substrate leading to greatly reduced rates of catalysis (Iwata & Yoshiharu, 1999). Essentially a paradox has been created; the same properties that make synthetic plastics highly useful in society have made them highly toxic in the environment and the true impacts of this pollution has only begun to be realized.

1.1.7 Toxicity of Petroleum-Based Plastics

One of the most well-known cases of plastic-related toxicity is in regard to bisphenol-A (BPA), a primary reagent in polycarbonate production and a known mimic of estrogen when ingested by mammals [Figure 1.4] (Sonnenschein & Soto, 1998). Studies have shown that during production of polycarbonate (PC), trace amounts of unreacted BPA become incorporated into the plastic and later leach out during use (Wu et al., 2010). The use of PC as a primary component in food-packaging, water bottles, and even baby bottles has led to a direct consumption of BPA by large populations of people around the globe, especially children and infants (Talsness et al., 2009). Experiments with mice have shown that exposure to low-doses of BPA, similar to the levels ingested by many children, has been linked to adverse health effects including endocrine disruptions, early onset of puberty in females, neural and behavioral alterations, and precancerous lesions in the prostrate and mammary glands (Shelby, 2008). Even after banning

the use of BPA in many plastic products, studies have shown that some of the BPA-free PC replacements still leach xenobiotic chemicals that exhibit estrogenic activity (Bittner et al, 2014).

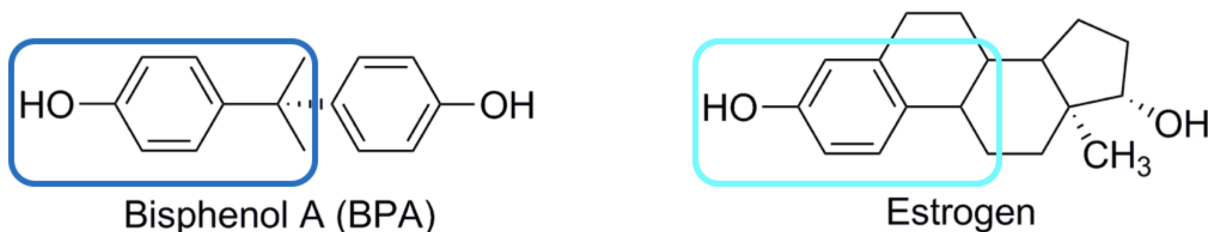

Figure 1.4 Chemical structures of bisphenol A (BPA) and estrogen. The boxed area of each molecule highlights structural similarities which enable BPA to act as a substitute for estrogen and mimic its properties in the endocrine system.

The infiltration of plastics into our infrastructure and environment have become so widespread that even if people avoided direct use of these products, exposure to and ingestion of plastic materials and byproducts would still occur. A study performed in 2017 has found that many commercially-sold food-grade table salts from around the globe contain trace amounts of polymers such as polyethylene and polypropylene incorporated into the crystal structure (Karami et al., 2017). There is also evidence that shows the presence of volatile organic compounds such as aromatic hydrocarbons and terpenoids in many sources of drinking water that flow through plastic pipes made out of HDPE (Skjevrak et al., 2003). Additionally, it is also believed that up to 90% of the tap water in the United States is contaminated with plastic microfibers (Tyree & Morrison, 2018). The unintended consumption of plastic materials has become so commonplace that it is no longer a question of if it is happening, but rather how toxic are the effects.

In many cases, the monomer of the plastic polymer itself is toxic, as is the case for PVC, polyurethane, polycarbonate, and polystyrene, whereas in other cases, the polymers may be relatively inert (polyethylene and polypropylene), but additives in these materials such as brominated and fluorinated compounds as well as lead heat stabilizers pose extreme hazards to

living organisms (Rochman, 2015). Considering that a vast amount of plastic litter eventually ends up in the marine environment, more and more studies have begun to focus on the toxic effects of these materials on marine ecosystems. The plasticizer di-2-ethylhexyl phthalate found in PVC has been shown to cause endocrine disruptions in fish that lead to oocyte development inhibition (Kim et al., 2002). There has also been a correlation between the presence of brominated flame retardants in fish samples caught off coastal artic waters and immunotoxicity, hemotoxictiy, and neurotoxicity in these fish (de Wit, 2002).

The continued discovery of plastic debris in our environment and even our own bodies poses some serious questions regarding the long-term health effects of plastic accumulation in humans and other organisms, especially those found in the marine ecosystems. To date, there is believed to be a total of 5 large-scale trash gyres in the oceans throughout the world that have become essentially floating landfills where much of the world's plastic litter accumulates (Pierre-Louis, 2017). There is also ever-increasing evidence showing that chemicals present in synthetic plastics are toxic and our environment is not suited to be able to process and eliminate this waste any time in the near future.

1.1.8 Moving Forward in the Plastic Age

The bottom line is that we have become absolutely dependent on plastics and the benefits they provide to all aspects of industrialized life. To reduce our use of plastics in society and the inherent advantages provided by a plastic life is somewhat analogous to a digression back to the stone age and simply does not appear to be a viable option. Therefore, the only feasible way to move forward cannot be described in terms of reducing plastic use and instead lies in the development of more efficient and environmentally sustainable ways to produce, reuse, and dispose of plastic materials.

Enhanced overall sustainability in the plastic industry starts with improving our ability to recycle the plastics already in use and come up with better methods of disposal to prevent further accumulation in the oceans. In addition to these shifts, there must be continued and increasing efforts towards creating more environmentally sustainable methods of manufacturing plastics. In order to circumvent the inherent shortcomings of traditionally-made plastic materials, three goals must be accomplished to create an environmentally sustainable plastic industry: 1) find a way to produce these or similar materials using methodologies that do not depend on petroleum or other nonrenewable fossil fuels; 2) create alternatives to the toxic monomers and additives used in many synthetic plastics; 3) design polymers that are biodegradable and will not persist in the environment for thousands of years. Although plastic production will never be truly carbon neutral in the sense that there will always be energy costs and waste generated from industrial production methods, the assimilation of biodegradable plastics into our society to replace petro-plastics represents a big leap towards using a material that has much less of a negative impact on the environment. Fortunately, many scientists have seen this writing on the walls and the expansion of biodegradable plastic technology has become an area of intense research and development. A method of particular interest capable of alleviating some of our dependence on environmentally unsustainable plastic production is to use bacteria to create a class of plastic-like polymers known as polyhydroxyalkanoates or PHAs.

1.2 The Biodegradable Plastic Revolution

1.2.1 Birth of an Industry

The initial discovery of biodegradable plastics came in 1920s by a French scientist by the name of Maurice Lemoigne who was studying *Bacillus megaterium* at the Institut Pasteur

(Fridovich-Keil, 2008). In his inceptive finding, Lemoigne was able to prove that a polymer of 3-hydroxybutyric acid could be produced by *B. megaterium* under anaerobic conditions. Like many great innovations, however, necessity is the prerequisite of invention and the field of bio-friendly plastics is no exception to this rule. Lemoigne's discovery went largely unnoticed until the oil crisis in 1970s created a large enough impetus to start exploring alternatives to traditionally-produced petrochemicals. During this time, the UK firm ICI formulated a method for using the Gram-negative bacterium *Alcaligenes latus* to produce the biodegradable polymer poly-3-hydroxybutyrate (P3HB), but high production costs and other barriers prevented further exploration of these methodologies (Howells, 1982). Then in 1990 a German hair care company by the name of Wella AG released the first fully biodegradable consumer product, a shampoo bottle, made of polyhydroxyalkanoates (PHAs) of which P3HB is included (Reis & Neves, 2008).

Although still in the nascent stages compared to petroleum-based plastics, the biodegradable plastic industry is gaining some serious momentum and is projected to grow in value from $2.0 billion USD in 2015 to $3.4 billion USD in 2020 (Rohan 2015). In the industry as a whole, there exists several broad categories of biodegradable plastics based on the chemical nature and origin of the material. These include poly(lactic acid) and poly(butylene succinate) of which are produced through bio-chemosynthetic methods, starch polymers and cellulose derivatives which are modified natural polymers, and of course PHAs which are manufactured entirely by biosynthetic processes (Mohanty et al., 2000). Although PHAs are not the most widespread of biodegradable plastics on the market, they have to potential to be one of the most environmentally sustainable due to their ability to be manufactured using totally renewable precursors such as biomass derived plant oils, starches and sugars (Sudesh & Iwata, 2008). The

promise that PHAs offer as completely biodegradable polymers produced through biosynthetic routes with little to no synthetic intervention using fully renewable feedstocks is, in many ways, an archetypical example of a completely environmentally sustainable plastic.

1.2.2 Biosynthesis of Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are a diverse group of intracellularly produced biopolymers naturally present in a variety of Gram-negative and Gram-positive bacteria [Figure 1.5] (Tian et al., 2009). Evolutionarily, PHAs have been used by bacteria for what can essentially be analogized as a 'fat reserve' or a method of storing excess carbon and energy as an intracellular inclusion macromolecule while under nutrient limiting conditions that would otherwise restrict the further growth and proliferation of the organism (Nomura & Taguchi, 2006). Bacteria can tap into this reserve to survive if in conditions where a supply of energy is limited, or if they were to find a more nutrient rich environment, it can use this stored energy to grow and multiply. The organization of PHAs within bacteria has been described as an organelle-like structure in which both polymer and enzymatically active proteins coalesce to form hydrophobic granules that have the ability to not only store and release energy depending on the metabolic state of the cell, but also act as an electron sink when nutritional elements such as nitrogen, sulfur, or phosphorous are limited (Uchino et al., 2007; Muhammadi et al., 2015).

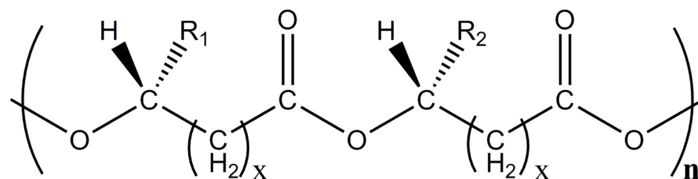

Figure 1.5 General structure of polyhydroxyalkanoates. R_1/R_2 can include a range of diverse substituents of which alkyl groups of C1-C12 are the most common. X denotes a range of 1-3 hydrocarbon substituents that can exist within the backbone structure, although X=1 is by far the most commonly present configuration, and 'n' refers to the polymer size which is highly variable and can range from hundreds to tens of thousands of units (Muhammadi et al., 2015).

Like many biopolymers, the biosynthesis of PHAs is dependent upon the availability of the monomer units of which they are composed. Natural PHA polymers can exist as either *homo*-polymers, which consist of a single type of monomer throughout the entire macromolecule, or *co*-polymers, which are a combination of two or more different types of monomers within the same polymer. The exact arrangement of monomers along the copolymer chain is dependent, and sometimes unique, to the specific species of bacteria synthesizing the polymer as well as the nutrients and metabolites available (Mantzaris, 2002). An illustration of the diversity of monomers capable of being incorporated into PHA polymers can be seen in Figure 1.6. Of these monomers, 3-hydroxybutyric acid (3HB), which as a homopolymer is known as poly-3-hydroxybutyrate (P3HB or PHB), is one of the simplest and best understood in terms of biosynthesis (Fridovich-Keil, 2008).

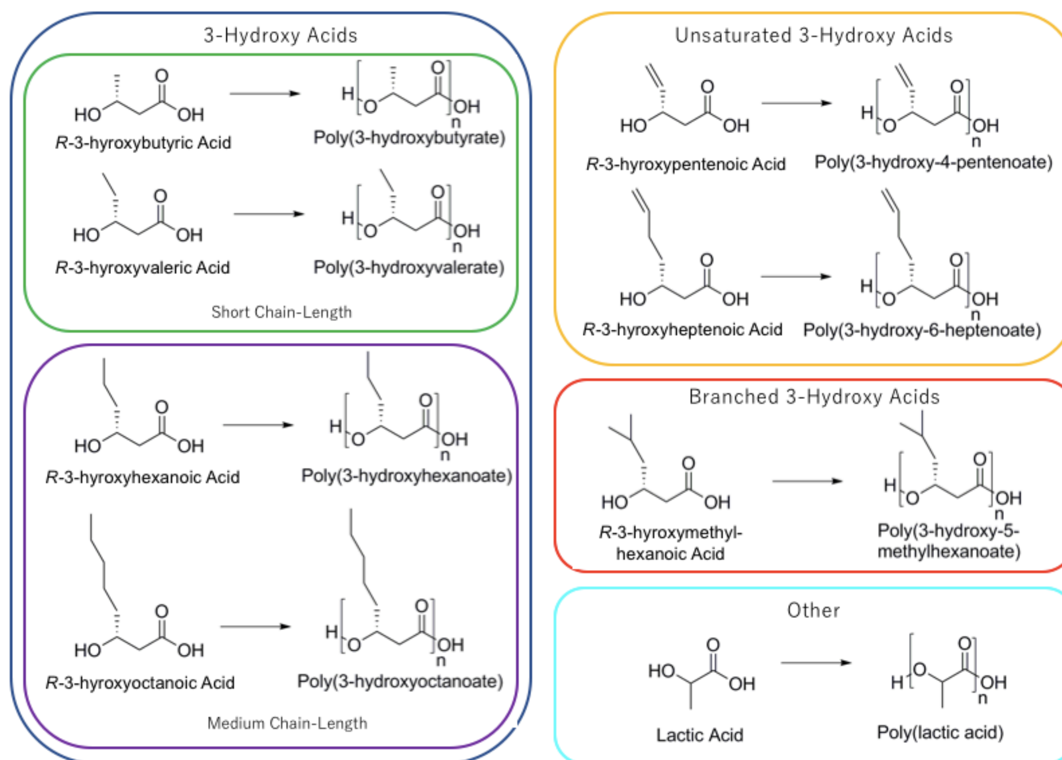

Figure 1.6 Structure of selected poly[(*R*)-3-hydroxyalkanoates] (PHAs), some of which are referenced in this chapter and others that are included to illustrate the diversity of this class of macromolecules. Many of these monomers can also exist as copolymers with one another. Poly(lactic acid) is not technically a PHA but is capable of forming copolymers with poly(3-hydroxybutyrate) [Reddy et al., 2003].

The three-step biosynthesis of PHB is a fairly common pathway found among many PHA producing bacteria and begins with two molecules of acetyl-CoA, a downstream product derived from glycolysis and a major precursor of the tricarboxylic acid cycle [Figure 1.7] (Lu et al., 2009). A condensation of these two molecules is catalyzed by β -ketothiolase to form acetoacetyl-CoA followed by a reduction step by an NADPH-dependent acetoacetyl-CoA reductase to create the monomeric substrate 3-hydroxybutyrl-CoA. The final step in this synthesis is polymerization by PHA synthase to form the PHB polymer. In many organisms, PHA synthase has a broad substrate specificity capable of catalyzing the polymerization of a variety of hydroxyalkanoic acid – coenzyme A thioesters (Rehm & Steinbüchel, 1999).

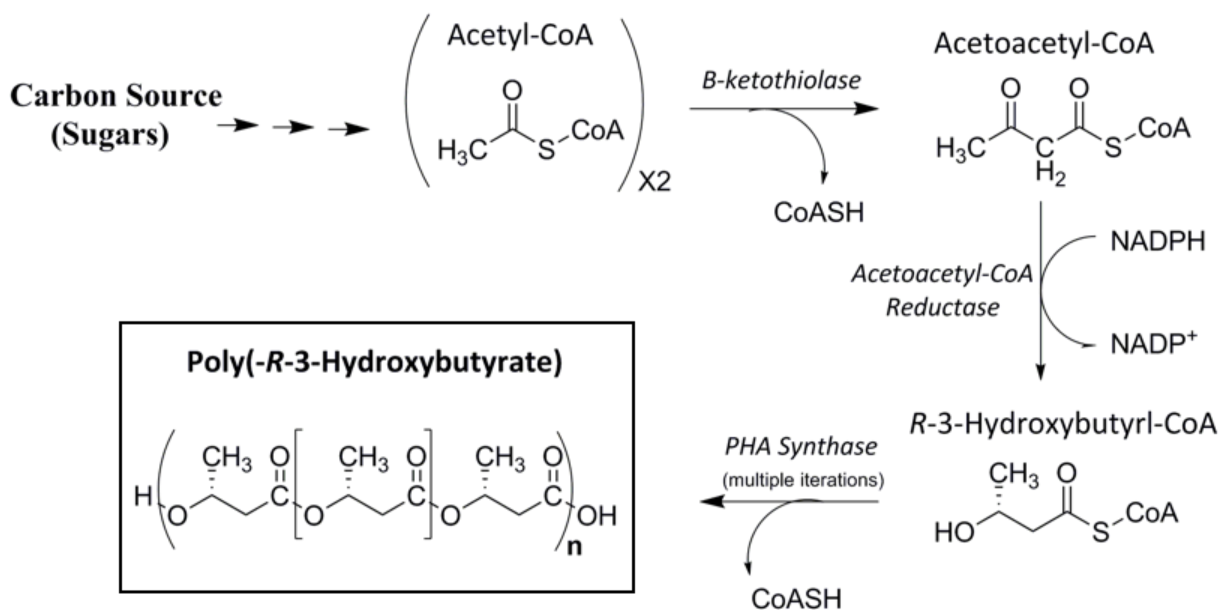

Figure 1.7 Common pathway for the biosynthesis of poly(3-hydroxybutyrate) from sugars catabolized via glycolysis. The three-step pathway consists of the successive catalytic actions of β -ketothiolase, acetoacetyl-CoA reductase, and PHA synthase to generate a single monomeric unit of PHB from two molecules of acetyl-CoA. Variations in enzymatic structure exists from species to species.

In a number of *Pseudomonas* species including *P. oleovorans*, *P. aeruginosa*, *P. putida*, and *P. fluorescens*, production of PHB does not occur naturally, but the biosynthesis of PHAs composed of medium-chain-length (C_6-C_{12}) 3-hydroxy fatty acids has been observed (Huisman,

1989). In these metabolic pathways, 3-hydroxyacyl-CoA substrates are generated as a shunted product of fatty acid metabolism and polymerized by the native PHA synthases found in these organisms. The generation of these substrates can occur through fatty acid biosynthesis or the degradation of fatty acids provided by β-oxidation (Philip, 2007). In the former pathway, molecules such as glucose, fructose, and sucrose can be used for the synthesis of fatty acids, allowing for inexpensive, simple sugars to be the primary source of carbon for PHA production.

With the discovery of a multitude of different bacteria capable of synthesizing a high diversity of PHA polymers and the continued advancement of genetic recombination technologies, it has been possible to engineer different organisms to produce enhanced amounts and highly variable versions of these macromolecules. The high versatility that bacteria exhibit in their PHA metabolism has allowed scientists to take non-native PHA producers, such as *E. coli*, and design completely novel synthesis pathways composed of an amalgamation of genes from multiple species (Wang et al., 2012). Further expansion of this technology has led to the development of engineered PHA synthase enzymes, based on those found in natural systems, that have been optimized to produce higher yields and/or unique compositions of copolymers. Additionally, supplementary pathways are being engineered into PHA producing bacteria that allows for a highly diverse range of initial carbon sources, including woody biomass, to be used as PHA precursor (Keenan et al., 2006). This highly iterative process of designing genes and metabolic pathways followed by experiments altering environmental conditions and carbon and nutrient sources has led to the creation of PHAs with physical and chemical properties that closely mimic those of many petroleum-based plastics (Chen et al., 2001).

1.2.3 Mechanical Properties of PHAs

As with any type of plastic polymer, the overall physical characteristics are a reflection of

the monomer constituents and their 3-dimensional organization. As previously described, PHAs can exist as either homopolymers of the same monomer or copolymers containing a combination of different monomers. In addition to this distinction, PHA polymers can generally be categorized into 3 different classes based on the size of the monomeric side chains: 1) short chain-length (SCL) PHAs consist of side chains of 3-5 carbons long; 2) medium chain-length (MCL) PHAs have chain lengths ranging from 6-14 carbons; and 3) long chain-length (LCL) PHAs, which include all monomers of chain length greater than 14 carbons (Zinn et al., 2001). Copolymers containing different length monomers can be further modified by altering the relative proportion of one monomer to another, which often times results in enhanced tensile and elastic properties (Li et al., 2016).

For example, the SCL homopolymer PHB has a high crystallinity and plastics made purely of PHB are very brittle, whereas a random copolymer that contains both 3-hydroxybutyrate and 3-hydroxyvalerate to form poly(3HB-*co*-3HV) results in a material that is stronger, more pliable, and easier to mold than PHB (Holmes, 1985). It has also been observed that copolymer mixtures of a high percentage of SCL monomers and a low percentage of MCL monomers yield a material with properties similar to polypropylene (Abe & Doi, 2002). Furthermore, it is also possible to create copolymers that contain both PHA and polylactic acid (PLA) using an engineered PHA synthase capable of incorporating both hydroxyacyl-CoA and lactyl-CoA into the same polymer (Taguchi et al., 2008). This seminal study has created new avenues of exploration for developing biodegradable plastics with an even greater range of properties and uses. A table listing some common PHAs and their material properties can be found in Table 1.1.

Table 1.1 Physical properties of common PHAs and the bulk commodity plastics polypropylene and polyurethane for comparative purposes.

Polymer	Melting Temperature (°C)	Glass Transition Temperature (°C)	Tensile Strength (MPa)	Tensile Modulus (GPa)	Elongation to Break (%)
P(3HB)	177	4	40	3.5	6
P(4HB)	60	-50	104	0.149	1000
P(3HB-co16%-4HB)	152	-8	26	N.D.	444
P(3HB-co20%-3V)	145	-1	32	1.2	50
P(3HB-co17%-3HHx)	120	-2	20	0.173	850
P(3HO-co12%-3HHx)	61	-35	9	0.008	380
Polypropylene	176	-10	38	1.7	400
Polyurethane	195	20	38	0.004	550

P(3HB): Poly-3-hydroxybutyrate; P(4HB): Poly-4-hydroxybutyrate; P(3HB-co16%-4HB): Poly-3-hydroxybutyrate-co-16% 4-hydroxybutyrate; P(3HB-co20%-3HV): Poly-3-hydroxybutyrate-co-20% 3-hydroxyvalerate; P(3HB-co17%-3HHx): Poly-3-hydroxybutyrate-co-17% 3-hydroxyhexanoate; P(3HO-co12%-3HHx): Poly-3-hydroxyoctanoate-co-12% 3-hydroxyhexanoate. N.D.: Not Determined. Data shown adapted from Williams et al., 1999.

Beyond the attributes bestowed on PHAs purely through biologically engineered routes, it is also possible to perform post-biosynthetic modifications to further alter the mechanical and chemical characteristics of the polymer. Possible methods of PHA modification include graft or block copolymerization, cross-linking, chlorination, and carboxylic acid functionalization (Hazer & Steinbüchel, 2007). These techniques have expanded the repertoire of PHA applications, especially in the fields of medicine where PHAs have been developed for both drug delivery systems, medical implants, and scaffold design (Nigmatullin, 2015). A study conducted by Levine et al., using thiolene click chemistry to catalyze the cross-linking reaction of poly[(*R*)-3-hydroxybutyrate-co-(*R*)-3-hydroxy-10-undecenoate] created of a novel material with properties that exhibited little cytotoxicity towards human cells and a high potential for use as a scaffold in tissue-engineering (Levine et al., 2015). Biocompatibility is a very attractive trait in the continued development of PHAs for biomedical applications and is an example of an even greater feature of these polymers; biodegradability.

1.2.4 PHA Biodegradation

The inherent ability of many PHAs to naturally degrade in the environment is a major force behind the interest in commercially developing and mass producing these polymers. Due to their origins as natural products of bacteria, PHA degradation is typically accomplished through the enzymatic activities of microorganisms present in soil, compost, and marine sediment environments (Anderson & Dawes, 1990). As a result of their large size, many PHAs must first be degraded extracellularly into dimers and monomers of hydroxyacids before they can be transported into a cell where they are metabolized as energy [Figure 1.8] (Numata et al., 2009). In the case of *Ralstonia pickettii* T1 (formerly *Alcaligenes faecalis*), degradation of an assortment of PHAs including PHB, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate), poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate), poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) and poly(3-hydroxybutyrate-*co*-6-hydroxyhexanoate) was shown to be accomplished by a combination of extracellular enzymes including a PHB depolymerase with endo-type hydrolase activity and a distinct PHB hydrolase with a high affinity for oligomeric esters (Iwata et al., 1999; Shirakura et al., 1983). Often times, the substrate specificities of PHA depolymerase enzymes operate within a narrow range of acceptable substrates, of which one consequence is the need for substrate-specific degradation studies for each new type of PHA synthesized (Jaeger et al, 1995).

In the well-studied PHA depolymerase of *Pseudomonas stutzeri*, the enzymatic structure was shown to have a catalytic domain connected by a linker region to two putative substrate-binding domains (SBD) [Ohura et al., 1989]. It was shown that the cadherin-like linker domain of this enzyme is necessary for maintaining optimal distance between the catalytic domain and SBD and, furthermore, substrate binding to the surface of water insoluble polyester materials is

vital in the initiation of PHB degradation (Uefuji et al., 1997). Another important factor of PHA degradation is the overall structure of the substrate, where it has been shown that amorphous polyester degrades much quicker than its crystalline counterpart and the rates of enzymatic degradation of crystalline PHAs are determined by a variety of factors including reaction conditions and the overall density of ester bonds at the surface of the material, all which influence the ability of a depolymerase to bind substrate (Iwata et al., 1999). Lastly, it was found that polymer composition can play an important role in the rate of PHA degradation. For example, Doi and colleagues demonstrated that copolymers containing 3HB and monomers of 4-hydroxybutyrate biodegrade at a rate (2 weeks) roughly five times faster than 3HB homopolymer (10 weeks) under similar environmental conditions (Doi et al., 1989).

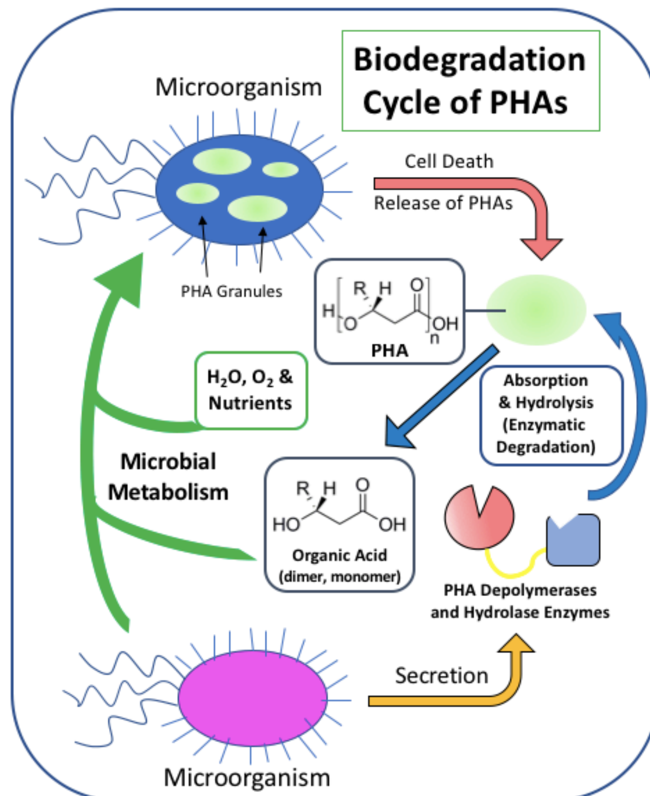

Figure 1.8 General cycle of PHA synthesis and biodegradation in the environment. Microorganisms that create reserves of intracellular PHA may eventually die and release polymer into the environment. Other organisms of the same or different species, many of which are also PHA producers, release extracellular depolymerase and hydrolase enzymes that break PHAs down into dimer and monomer units. These free organic acids are then taken into the cell where they can be used as energy and potentially serve as a carbon and/or energy source for the creation of more PHA molecules.

The variation in biodegradation of PHAs based on polymer structure and composition has certain environmental implications in terms of applicability of different polymers depending on their intended use as well as implications in the use of these materials for biomedical purposes. For example, in mammalian systems PHB is degraded to its monomeric unit 3-hydroxybutyrate, which already exists as an endogenous ketone body. Therefore, the absorbable and non-toxic nature of PHB makes it an appropriate candidate for non-permanent applications such as sutures and meshes (Brigham & Sinskey, 2012). The overall biocompatibility of PHAs is specific to the monomer composition and type of environment (tissue) it is placed in, but generally, polymers containing 3HB, 4HB, 3HV, 3HHx, and 3HO (Table 1) have been shown to have low toxicity and high biocompatibility in a number of *in vivo* studies using mammalian models (Reusch, 2000; Laborit, 1964; Gürsel et al., 2001; Marois et al., 1999). The medical applications of the listed polymers, either as homo- or co-polymers, has included stents, bone tissue scaffold, nerve repair, blood vessels, heart valves, wound dressing, vascular grafts, and as vectors for drug delivery systems and the applications of each type of polymer is partially dependent on the rate at which the body can degrade and metabolize the material. (Mei et al., 2006). The inherent bio-friendly features of PHAs has led to the development of a whole industry of biomedical applications and has further established the growing role of PHAs in society.

1.2.5 Advancements in the PHA Industry

The implementation of PHAs as commodity plastics in society is a multifaceted issue that depends not only on the research and development of new polymers, but also relies heavily on technological and economic factors. In an extensive review by Chen, the steps required for the industrial production of a PHA polymer via fermentation were outlined as follows; 1) strain development, which includes rapid cell growth to high cell densities, high PHA to cell weight

ratio, simple substrate requirements, and controllable PHA weight and structure; 2) shake flask studies to optimize growth and product pH, temperature, and substrates, as well as any required downstream processing steps; 3) lab and pilot fermenter studies necessary to optimize and develop the growth conditions at an industrial scale; and lastly 4) implementation of scaled-up production which requires that cell growth and product formation occurs rapidly with low oxygen demand, inducible cell lysis, high substrate to PHA yield, and the ability to use simple and mixed substrates (Chen, 2009). This process outlined, although simplified, illustrates some of the barriers facing large-scale PHA production especially in terms of being able to offer a final product that is competitively priced compared to common petrochemicals. Despite these barriers, the PHA industry continues to grow and expand.

One avenue of plastic production that has already created economically sustainable PHA-based companies is related to single use and disposable plastic items. Companies such as Metabolix, P&G, and Biomers have been able to produce and sell PHA items such as shopping bags, utensils, medical surgical garments, razors, female hygiene products, and compostable food wrappers (Clarinval & Halleux, 2005; Mikova & Chodák, 2006). The unique and highly modifiable properties of PHAs has also led to use in printing and the photography industry, textile industry for use as fibers, and biofuels (Li et al., 2009; Zhang et al., 2009; Martin et al., 1999). As previously described, the development of PHAs for biomedical purposes also represents a highly promising application especially considering it circumvents some of the current issues in producing these materials at a scale and cost that allows reasonable competition with petroleum-based plastics.

Due to the current and ongoing energy crisis and the well-understood diminishing supplies of fossil fuels, greater emphasis is being placed on creating an overall economically

sustainable industry for PHAs. Efforts at all levels of production are being made to optimize and reduce costs, such as the use of waste water from food, dairy, and distillery industries as the primary source of H_2O, thus reducing the high costs of fresh water needed for many large-batch fermentations (Khardenavis et al., 2007). Research is also being conducted on how to engineer metabolic pathways that allow bacteria to use components of plant biomass such as xylose to serve as a primary carbon source for PHA production (Salamanca-Cardona at al., 2014). Advancements in molecular biology have also allowed for transgenic plants, like engineered sugar cane, to be used to make PHAs, which represents a highly exciting development in the industry due to the ability of plants to harness the energy of the sun and carbon from atmospheric CO_2 to generate macromolecules (Petrasovits at al., 2012).

1.2.6 Modern Plastic Society

In summary, the development of plastic materials has granted amazing improvements to quality of life and have become an integral part of society. Unfortunately, the current plastic industry, based on the use of fossil fuels as feedstock, is not sustainable and will lead eventually to rising costs and lowering availabilities of the items that have become so vital in day-to-day life. Adding to this issue is the growing body of knowledge that indicates many common plastics in use today are having devastating effects on the environment and, in some cases, are highly toxic to human beings. As the use of plastics continues to expand globally, the negative effects on the environment that plastic litter creates and the infiltration of chemicals into our own bodies is undoubtedly going to increase. The time to evolve as a global society that uses modernized plastics is upon us.

The development and implementation of polymer materials that can be created using renewable methods and will naturally biodegrade in the environment with little consequence is

vital for the continued growth and health of the human population. Polyhydroxyalkanoates can help alleviate the current plastic epidemic and it has become the goal of many researchers to further progress PHA technology and industry. The next chapter of this work will focus on the development of a novel molecular engineering technique that has the potential to expand the sustainability of the PHA industry. Specifically, a method for genetic recombination was created for the halophilic, PHA-producing bacteria *Halomonas* sp. O-1; a genus of bacteria receiving increased attention for industrial PHA-production due to their ability to withstand harsh environmental conditions and grow in readily available ocean water. The third chapter will then discuss the other side of the cycle, specifically PHA biodegradation, and investigates the genetic components required for catabolism of (*R*)-3-hydroxybutyrate (3HB) in the ubiquitous soil bacterium *Pseudomonas aeruginosa*. This work as a whole is aimed to represent some of the necessary steps and research required for the further development and implementation of PHA technology into society.

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Chapter 2. A rapid and efficient electroporation method for transformation of *Halomonas* sp. O-1

Abstract

Halomonas sp. O-1 is a halophilic bacterium with a high potential for industrial application due to its natural ability to produce polyhydroxyalkanoates (PHAs) using seawater-based media, an economically beneficial alternative to freshwater-based media. However, a major barrier preventing industrial scale implementation of this organism is a lack of molecular methodologies capable of readily transforming members of the *Halomonas* genus. Currently, the only reliable method used for introducing DNA into *Halomonas* spp. is bacterial conjugation, a somewhat tedious and time-consuming technique compared to electroporation-based methodologies. Here we describe a rapid and reproducible method for the electroporation of *Halomonas* sp. O-1 with plasmid DNA. Electrocompetent cells were generated by growing *Halomonas* sp. O-1 in a yeast extract-tryptone medium with a final salinity of 3.5%, pH of 7.5, followed by several washes using 300 mM sucrose. Results show that plasmids containing chloramphenicol (Cm^R) and gentamicin (Gm^R) resistance cassettes are suitable antibiotic selection markers for transformation and yields of 10^4 transformants per μg of DNA were obtained. This method is simple to perform and the materials used are readily available in most research labs. Additionally, this plasmid-based transformation procedure has the potential to be adapted for a number of applications including the creation of recombinant stains and the generation of deletion mutants of *Halomonas* spp.

The work presented in this chapter is a slight adaptation of a published article by J.R. Harris et al. (2016) and can be found in the *Journal of Microbiological Methods*, Issue 129, pages 127–132

***Note;** The author, JR Harris, was the primary contributor to this study and responsible for the execution of all experiments and the preparation of the manuscript. Co-author contribution included the provision of the* Halomonas *sp. O-1 strain, experimental design, and guidance on manuscript preparation.*

2.1 Introduction

2.1.1 *Halomonas*; Halophilic PHA Producers

Halophiles are a class of extremophiles characterized by their ability to thrive in high salt environments that are typically toxic to most other forms of life (Oren, 2008). There has been increasing interest in the use of these microorganisms for various biotechnology and industrial applications due to the overall enzymatic stability required for survival in such harsh environments and a decreased risk of contamination during processing as a result of these conditions (Aguilar, 1996). The genus of Gram-negative halophilic bacteria known as *Halomonas* was first established in 1980 with the discovery and morphological characterization of *Halomonas elongata* (Vreeland et al., 1980). Since then, isolates of this genus have been identified in a myriad of habitats including coastal sea waters (Ilham et al., 2014), salt-mines (Cardinali-Rezende et al., 2016), saline lakes (Zhang et al., 2016), salt-secreting leaves (Finkel et al., 2011), industrial brine (Carlson et al., 2016), heavy metal polluted shores (Jain et al., 2016), rust found on the sunken RMS *Titanic* (Sánchez-Porro et al., 2010), and even as part of the human skin microbiome (Cosseau et al., 2016). New members of the *Halomonas* genus are being identified at an extraordinary rate and the habitats in which they thrive are equally astounding.

Polyhydroxyalkanoates (PHAs) are a class of diverse polyesters that are naturally produced by a variety of microorganisms to serve as intracellular carbon storage molecules (Anderson and Dawes, 1990). PHAs have attracted great interest as biodegradable replacements for petroleum-based plastics (Lu, et al. 2009) and as biomaterials for biomedical applications (Levine, et al. 2015). In addition to the inherent environmentally friendly properties of PHA-based polymers, studies have also shown that bacteria can be engineered to make these materials from a variety of renewable resources and waste products such as woody biomass and biodiesel

glycerol, thus enhancing overall sustainability (Pan et al., 2012; Salamanca-Cardona et al., 2014; Zhu et al., 2010). However, major barriers that exist for wide-scale industrial application and integration of these processes include the large amount of freshwater needed to sustain PHA production and the associated energy costs to sterilize medium for bacterial growth (Chen, 2012).

A number of *Halomonas* isolates and recombinant strains have been shown to be capable of producing a variety of PHAs, some at levels that exceed 80% cell dry weight (Ilham et al., 2014; Quillaguamán et al., 2005; Tan et al., 2011; Yue et al., 2014). *Halomonas* spp. have been gaining heightened attention as attractive production strains for these materials because of their capacity to produce PHAs in a continuous and open process that does not require fresh water or sterilized media (Tan et al., 2011; Yue et al., 2014). A major limitation for further implementation at an industrial-scale is an overall lack of molecular methodologies to genetically manipulate these microorganisms to produce PHA derivatives at levels that can compete with synthetic processes (Chen, 2012; Fu et al., 2014).

2.1.2 Molecular Methods for Engineering *Halomonas* bacteria

Currently, the most common and primary method for genetically modifying *Halomonas* spp. is via bacterial conjugation, a process first established in this genus in 1995 (Vargas et al., 1995; Li et al., 2016). The process of bacterial conjugation can be described as a unidirectional, conservative transfer of DNA, typically as a plasmid or transposon, through cell-to-cell contact between two bacterial cells (Sørensen et al., 2005). This process of 'bacterial mating' requires the formation of a conjugative pili necessary to provide a physical channel between the two cells in which the exchange of genetic material occurs (Llosa et al., 2002). In order to generate the bacterial conjugation machinery, a number of genes and proteins collectively known as *Type IV*

Secretion Systems [T4SS] must be expressed and constructed (Ding et al., 2003). T4SS are naturally present in a variety of bacteria and in addition to horizontal gene transfer are also capable of transferring proteins, as well as participate in virulence and infection of bacterial and non-bacterial hosts (Cascales and Christie, 2003). In molecular biology, T4SS have been an extremely useful tool for genetically engineering both bacteria and plants, however, proper function involves a complex orchestration of cellular functions to occur in both the donor and recipient for successful gene transfers to occur (van Elsas and Bailey, 2002). Additionally, development of a donor strain requires successful expression of two separate plasmids, one for the T4SS and another containing the gene(s) to be transferred, thus creating a somewhat tedious and labor-intensive process that is often times difficult to reliably reproduce (Wang et al., 2015).

An alternative method for genetically engineering bacteria is through the use of a process known as electroporation; a technique in which short pulses of high electric fields are used to perforate the outer membranes of a cell without killing it in order to create transient pores large enough for ions and other non-permeable molecules to pass through (Rolong et al., 2018). The creation of a field using electrical potential is accomplished by suspending a non-electrolytic cell solution between two electrodes followed by the application of a voltage pulse via a stored charge (Dower, 1990). There exists an inverse relationship between cell size and voltage required to properly permeabalize a cell's membrane, therefore, unlike mammalian cells, the small size of bacteria cells necessitate a relatively large voltage (1000-7000V) and pulse duration (1-5 ms) for a successful electroporation to occur (Dower et al., 1988). Through the generation of transient pores in the outer membranes it is possible to facilitate the uptake of foreign DNA into bacterial cells. This phenomenon occurs, not by diffusion as many initially suspected, but rather through the migration of polyanion DNA macromolecules towards the positive electrode and the

concomitant orientation of the open cell pore towards the negative electrode which allows the field to drive the genetic material towards the center of the cell (Gehl, 2003). Once uptake of exogenous DNA has occurred, it is then possible for the cell's native machinery to assimilate and express the genes present in the DNA (Figure 2.1).

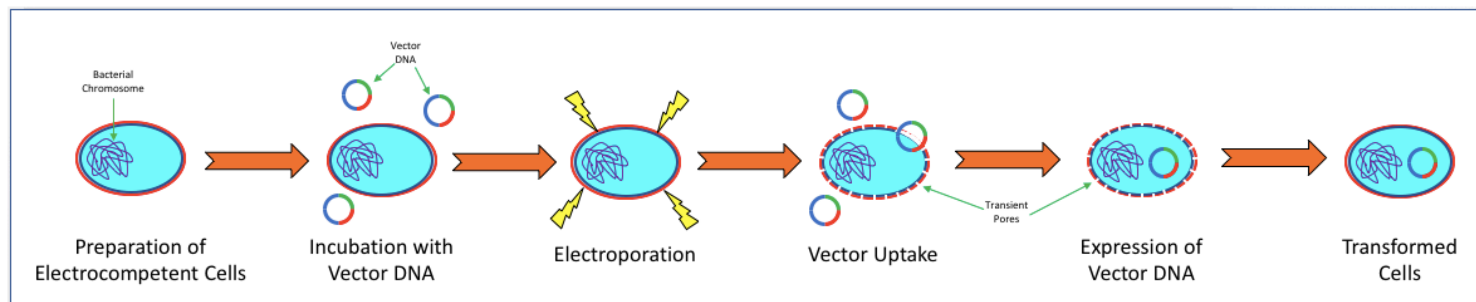

Figure 2.1 General outline of bacterial electroporation. Prepared electrocompetent cells are incubated with vector DNA followed by addition to an electporation cuvette and application of an electrical charge within an electrporator apparatus. Through the formation of transient pores, vector DNA migrates into cells depending on electric field orientation. The plasma membrane then reseals and expression of the newly introduced DNA begins.

Establishing an electroporation protocol for a specific organism opens the door for the application of a myriad of methods for genetic engineering including expression of foreign plasmids, homologous recombination, creation of recombinant biosynthetic pathways, and gene knockout procedures (Warming et al., 2005). Although these methods can potentially be accomplished using conjugation, electroporation greatly enhances the relative ease, efficiency, and reproducibility of these techniques. In several notable examples directly related to PHA production in bacteria, electroporation and gene knockout methodologies were utilized to create recombinant strains of *Escherichia coli*, a non-native PHA producer, that were capable of producing large amounts of polyhydroxybutyrate (90% cell dry weight) as well as a variety of PHA copolymers from simple carbon sources (Nikel et al., 2006; Scheel et al., 2016; Wang et al., 2012). These results were accomplished through a combination of procedures that synergistically resulted in enhanced PHA production such as: i) introduction of novel PHA synthesis pathways

using a collection of genes from native PHA producers; ii) removal of genes that inhibit PHA production, such as those involved in β-oxidation; and iii) transforming additional genes encoding catabolic enzymes that expand the repertoire of carbon sources that can be used to make PHAs. To date, there are two instances in which a *Halomonas* spp. is said to have been electroporated, but in both cases there is little description of the methodology used or the efficacy of transformation (Azachi et al., 1996; Burch et al., 2013). Through the establishment of a reproducible electroporation protocol for *Halomonas* species, it becomes possible to apply the same engineering techniques that have been designed for *E. coli* to generate large amounts and diverse varieties of PHA plastics.

2.1.3 Objectives of this Study

The goal of this study was to develop an electroporation method for the transformation of *Halomonas* spp. that was relatively simple and reliable but had the capacity to greatly expand the molecular mechanisms that could be used to engineer this genus for enhanced industrial PHA production. To create this method, we used *Halomonas* sp. O-1, an isolate from the coast of Japan that shares 98% homology with *Halomonas* sp. MAN K22 (Ilham et al., 2014). *Halomonas* is typically grown in high-ionic strength media (5% NaCl), which can complicate electroporation due to the non-ionic wash buffers commonly employed for encouraging electrocompetency (Prasanna and Panda, 1997). A recent review of the increasing prevalent role of *Halomonas* spp. in industrial biotechnology has cited the preparation of electrocompetent cells as a major barrier preventing transformation of these organisms (Yin et al., 2015). In addition to the generation of electrocompetent cells, a successful electroporation protocol must also consider plasmid size, selectable markers, device parameters, and recovery and screening conditions. Here we describe a simple and rapid electroporation method that reliably and reproducibly allows for

the creation of viable electrocompetent cells of *Halomonas* sp. O-1 capable of upta king broad-host range plasmid DNA containing antibiotic selectable markers.

2.2 Materials and Methods

2.2.1 Bacterial Strains, Plasmids, and Plasmid Preparations

The bacterial strains and plasmids used in this study are listed in Table 1. For plasmid maintenance, *E. coli* Top 10 was grown in Lennox broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) supplemented with a final concentration of 25 µg/mL chloramphenicol, 20 µg/mL gentamicin, or 10 µg/mL tetracycline. Plasmids were purified using the Wizard Plus SV Miniprep DNA Purification System (Promega) with the manufacturer's protocol.

Table 2.1 Bacterial Strains and Plasmids

Bacterial strains and plasmids	Descriptions	Sources
Strains		
Halomonas sp. O-1	Wild-type *Halomonas* strain isolated from seawater collected from the Fukuoka Prefecture of Japan	Ilham et al., 2014
E. coli Top 10	*E. coli* strain used for cloning and plasmid maintenance purposes	Invitrogen
Plasmids		
pBBR1MCS	Broad-host range plasmid, chloramphenicol resistance (Cm^R) marker	Kovach et al., 1994
pBBR1MCS-3	Broad-host range plasmid, tetracycline resistance (Tet^R) marker	Kovach et al., 1995
pBBR1MCS-5	Broad-host range plasmid, gentamicin resistance (Gm^R) marker	Kovach et al., 1995

2.2.2 Optimization of Halomonas Growth Conditions for Electroporation

Halomonas sp. O-1 was grown in 16 x 100 mm culture tubes containing 2 mL of yeast extract-tryptone (YT) medium (10 g/L tryptone, 5 g/L yeast extract) supplemented with variable concentrations of NaCl (1%, 3.5%, 5%, 10%, 15%, or 20% [w/v]). Cultures were grown at 30°C on a rotary shaker set to 250 RPM. The cell densities (growth) of the cultures were determined by measuring their absorbances at 600 nm (OD_{600}) at 5 h post inoculation. All subsequent culturing of *Halomonas* sp. O-1 was performed using YT medium supplemented to a final concentration of 3.5% NaCl, pH of 7.5, and will be referred to as *Halomonas* Growth Medium [HGM].

Growth analysis of *Halomonas* sp. O-1 in HGM was performed by first creating seed cultures, which consisted of 16 x 100 mm culture tubes containing 2 mL of HGM inoculated with single colonies of *Halomonas* sp. O-1 plated on HGM. Seed cultures were incubated at 30°C on a rotary shaker set to 250 RPM for 16 h and used to inoculate 50 mL of media (in a 500 mL baffled shake flask) with 0.5% v/v (0.25 mL) cell culture followed by incubation at 30°C, 250 RPM for a total of 12 h. Growth was assayed by measuring cell densities at 600 nm (OD_{600}) every 1.5 h for the duration of the growth period.

2.2.3 Antibiotic Screening

Antibiotic screening was accomplished by seeding untransformed colonies of *Halomonas* sp. O-1 into 16 x 100 mm culture tubes containing 2 mL of HGM supplemented with the following antibiotics at variable concentrations: ampicillin (50–200 μg mL^{-1}), carbenicillin (50–200 μg mL^{-1}), chloramphenicol (2−10 μg mL^{-1}), gentamicin (5−20 μg mL^{-1}), kanamycin (25−100 μg mL^{-1}), and tetracycline (5−15 μg mL^{-1}). Inoculated cultures were grown at 30°C, 250 RPM and assayed for OD_{600} at 24 and 48 h post inoculation.

2.2.4 Preparation of Electrocompetent Cells

Halomonas sp. O-1 cells were made electrocompetent by adapting a method originally devised for *Pseudomonas aeruginosa* (Smith and Iglewski, 1989). Cell cultures (50 mL) were grown in HGM at 30°C, 250 RPM to mid log phase (OD_{600} = 0.500), then harvested in 25 mL aliquots by centrifugation at 5,000 $\times$ *g* for 10 minutes at 23°C. Supernatant was decanted and the cells were gently washed using an equal volume (25 mL) of room temperature 300 mM sucrose. Note that all wash steps were performed at room temperature and resuspension of cell pellets was performed very delicately using wide-bore pipette tips to prevent cell shearing. Resuspended cells were collected by centrifugation at 5,000 $\times$ *g* for 10 minutes at 23°C and then washed using 0.5 culture volumes (12.5 mL) of 300 mM sucrose followed by a final centrifugation and resuspension using 0.01% (0.25 mL) culture volumes of 300 mM sucrose. Electrocompetent cells were then pooled and 100 µL aliquots were immediately combined with vector DNA and applied to cuvettes for electroporation (see below).

2.2.5 Electroporation of *Halomonas* sp. O-1

Electroporation was carried out using an ECM 399 Electroporation System (BTX Harvard Apparatus, voltage range of 60–2500V, fixed capacitance of 36 µF, fixed resistance of 150 Ω). Aliquots (100 uL) of electrocompetent cells were transformed with a variable amount of plasmid DNA in 2 mm gap parallel electrode cuvettes (Fisherbrand). Controls for all electroporation experiments consisted of cell aliquots without added DNA. Initial experiments were performed using a variable voltage ranging from 1.5–2.3 kV and a constant concentration (100 ng) of plasmid DNA. Subsequent experiments were performed at a constant 2.1 kV with plasmid DNA ranging from 10–500 ng. Directly following electroporation, cells were quantitatively transferred from the electroporation cuvette to a sterile 16 x 100 mm culture tube

containing 1 mL of HGM, which was then incubated for 1 h at 30°C, 250 RPM. Positive transformants were selected for by plating recovered cells (100–200 µL) directly onto HGM plates containing the appropriate antibiotic. Plated cells were incubated at 30°C for 24 h followed by colony formation unit (CFU) counts to quantify transformation efficiencies.

2.2.6 Cell Viability Assays

Cells were measured for viability during each wash step of the electroporation procedure. Cell suspensions were diluted (10^6-fold) in sterile 5% saline solution to a level that would yield single colonies. Diluted cells were plated onto HGM plates and incubated at 30°C for 24 h followed by CFU counts. Aliquots of washed cells were taken throughout each step of the wash procedure and were diluted, plated, and incubated following the same methods described for cell culture. To ensure consistency, the reduced buffer volumes of subsequent wash steps were taken into consideration to maintain constant rates of dilution for all samples.

2.3 Results

2.3.1 Development of a Low Ionic Strength Growth Medium

In order to make *Halomonas* sp. O-1 electrocompetent, we needed to develop a growth medium with a relatively low ionic strength that could still achieve adequate cell densities. Due to the non-ionic wash buffer used during the electrocompetency procedure, it was believed that developing a low-ionic strength growth medium would induce a phenotype in *Halomonas* that enhanced its ability to survive the low-ionic conditions experienced during the wash steps. Therefore, *Halomonas* sp. O-1 was grown in yeast extract-tryptone (YT) medium supplemented with NaCl to various concentrations (1, 3.5, 5, 10, 15 and 20% [w/v]). As shown in Figure 2.2, final NaCl concentrations of 3.5 and 5% yielded similar cell densities (OD_{600} of ~1.2–1.4) after

5h of growth. These cell densities were significantly greater than those observed for cultures supplemented to 1, 10, 15 and 20% NaCl. Ultimately, the developed medium, termed *Halomonas* Growth Medium [HGM], consisted of the typical components found in YT medium supplemented to a final NaCl concentration of 3.5%, pH 7.5, and was capable of satisfying the need for a relatively low ionic strength media while maintaining adequate cell densities. A growth assay of *Halomonas* sp. O-1 in baffled shake flasks containing HGM was also performed to determine when early, mid, late, and stationary phase growth occurs (Figure 2.3). Results of this experiment indicate that mid-phase growth occurs between 3 and 6 h post-inoculation with corresponding OD_{600} measurements of ~0.1–0.5 , respectively.

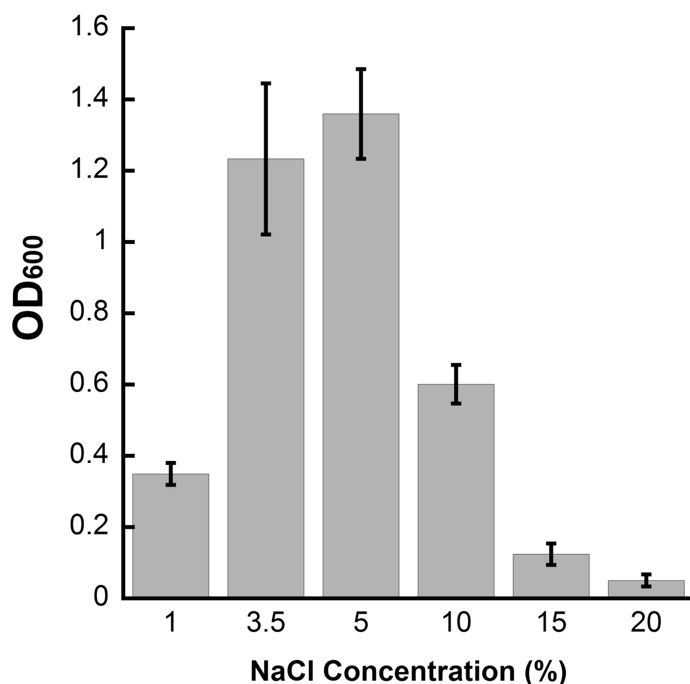

Figure 2.2 Growth of *Halomonas* sp. O-1 in YT medium supplemented to various concentrations of NaCl. *Halomonas* sp O-1 was grown in YT medium supplemented to 1, 3.5, 5, 10, 15 and 20% NaCl at 30°C in a rotary shaker at 250 RPM. At 5 h post inoculation, OD_{600} was measured for each culture. Experiments were performed in triplicate, and data points represent mean values with error bars representing standard deviation.

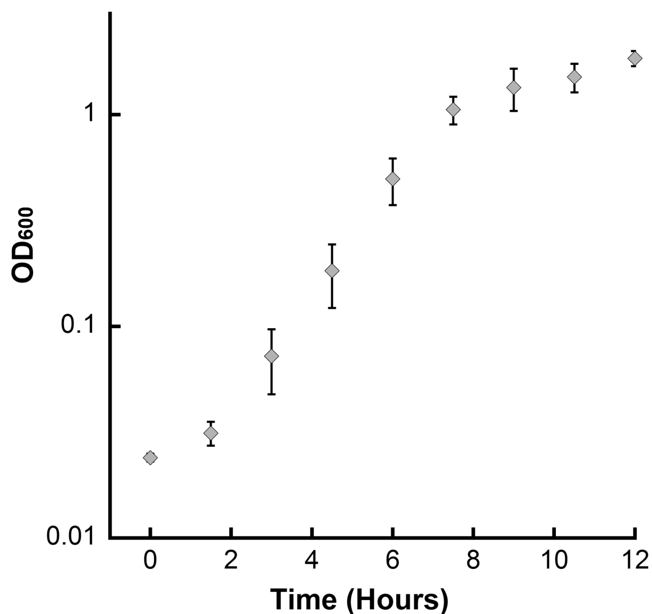

Figure 2.3 Growth profile of *Halomonas* sp. O-1 in HGM. Baffled shake flasks containing 50 mL HGM were inoculated with 0.5% (v/v) seed culture and grown at 30°C in a rotary shaker at 250 RPM for a total of 12 h. Measurements of cell density at 600 nm were taken initially and every 1.5 h for entirety of growth period. Experiments were performed in triplicate, and data points represent mean values with error bars representing standard deviation.

2.3.2 Antibiotic screening

Untransformed *Halomonas* sp. O-1 was screened for antibiotic sensitivity to determine which resistance markers could be used for plasmid selection (Table 2). Growth was not observed for *Halomonas* sp. O-1 in HGM supplemented with chloramphenicol (10 μg mL^{-1}), gentamicin (30 μg mL^{-1}), and tetracycline (15 μg mL^{-1}). However, cloudy or turbid growth was found for cultures grown in media supplemented with kanamycin (100 μg mL^{-1}), ampicillin (200 μg mL^{-1}), and carbenicillin (200 μg mL^{-1}), suggesting that *Halomonas* sp. O-1 is resistant to these antibiotics.

Table 2.2 Antibiotic sensitivity of *Halomonas* sp. O-1.

Antibiotic	Concentration ($\mu g\ mL^{-1}$)	Growth Phenotype 24 h	Growth Phenotype 48 h
Chloramphenicol	2	+	+
	5	-	+
	10	-	-
Kanamycin	25	+	+
	50	-	+
	100	-	+
Tetracycline	5	-	+
	10	-	+
	15	-	-
Ampicillin	50	+	+
	100	+	+
	200	+	+
Carbenicillin	50	+	+
	100	+	+
	200	+	+
Gentamicin	5	+	+
	15	-	+
	30	-	-

All experiments were performed in duplicate in liquid HGM containing antibiotic at the indicated concentrations. Cultures were grown at 30°C, 250 RPM shaking for the duration of the assay. Cultures were recorded as having either turbid growth (+) or zero growth (-).

2.3.3 Preparation and Viability of Electrocompetent Cells

Based on a procedure devised for *Pseudomonas aeruginosa*, we prepared electrocompetent cells of *Halomonas* sp. O-1 using a 300 mM sucrose solution as the sole wash buffer (Smith and Iglewski, 1989). To determine the effect of the wash procedure on cell viability, colony formation unit (CFU) plate assays were performed on initial cell cultures and throughout the wash procedure (Figure 2.4). Results revealed only a 20% decrease in overall cell viability when the non-ionic 300 mM sucrose buffer was used as a wash solution, therefore, it was concluded that the relatively low loss of cell viability was a worthwhile sacrifice for creating

a concentrated cell solution with high resistivity capable of being electroporated without a high potential for arcing. Additionally, centrifugation and wash steps performed at room temperature (23°C) were found to enhance the homogeneity of cell resuspensions compared to procedures performed between 4°C and 10°C.

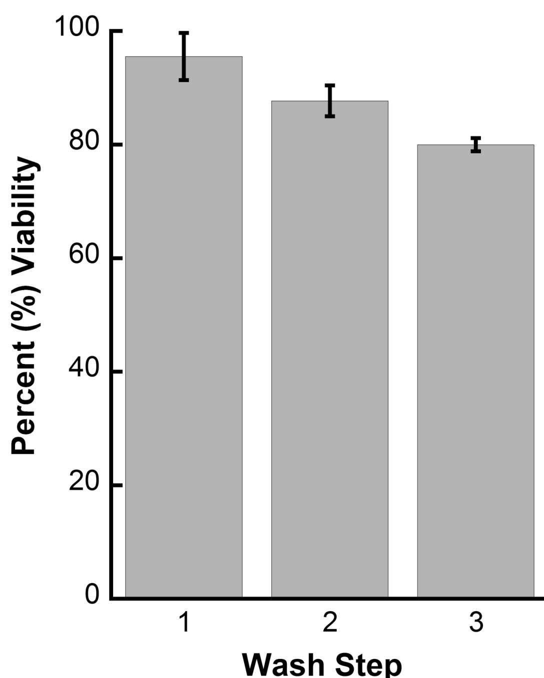

Figure 2.4 Effect of wash procedure on viability of *Halomonas* sp. O-1. Viability was calculated as percentages of surviving cells (CFUs) relative to the initial CFUs (set at 100%). Experiments were performed in triplicate, and data points represent mean values with error bars representing standard deviation.

2.3.4 Electroporation of Halomonas sp. O-1

Table 1 lists the vectors that were used for electroporation of *Halomonas* sp. O-1, all of which are pBBR1MCS derivatives; a relatively small (<5.3 kb), broad-host, and mobilizable suite of plasmids containing multiple cloning sites within the *lacZα* gene and several antibiotic resistant variants (Kovach et al., 1994; Kovach et al., 1995). The antibiotics chosen for selection were chloramphenicol, tetracycline, and gentamicin (Table 2) and the corresponding vector notations are pBBR1MCS, pBBR1MCS-3, and pBBR1MCS-5, respectively (Table 1).

Optimal settings for electroporation were determined through a series of preliminary experiments that tested key parameters such as parallel electrode cuvettes with 1 and 2 mm gaps, variable volumes of electrocompetent cells, and applied voltage. Electroporations of *Halomonas* sp. O-1 with a 1 mm gap cuvette (40 μL cell volume) or a 2 mm gap cuvette (100 μL of cell volume) with a constant plasmid amount (100 ng) successfully resulted in positive transformants, although the latter resulted in enhanced transformation efficiencies. Experiments using 2 mm gap cuvettes and a range of cell volumes (40−200 μL) resulted in arcing for volumes <100 μL whereas for volumes >100 μL there was a reduction in the time constant in which charge was delivered and led to lower overall transformation efficiencies. Lastly, it was found that when using a 2 mm gap cuvette and 100 μL cell aliquots, voltages below 1.5 kV failed to deliver a charge and voltages above 2.3 kV almost invariably resulted in arcing. It should also be noted that all electroporations were optimized for and performed using a ECM 399 Electroporation System (BTX Harvard Apparatus, voltage range of 60− 500V, fixed capacitance of 36 μF, fixed resistance of 150 Ω) and use of this methodology with a different device should take into consideration the capacitance and resistance of that device in addition to the voltage settings and cuvette size. Based on results from these trial and error experiments, cuvette gap size and washed cell volume were kept constant at 2 mm and 100 μL, respectively, for all subsequent experiments. Although variations in wash buffer, cuvette size, and cell volume may still produce viable transformants, the chosen parameters were found to be optimal for the device and *Halomonas* strain being tested.

To determine the efficacy of electroporation, experiments were performed using a range of voltages (1.5−2.3 kV) and a range of plasmid concentrations (10−500 ng). It was found that the pBBR1MCS-5 (Gen^R) plasmid yielded the most consistent and highest numbers of

transformants (10^4 CFUs ug^{-1} DNA). The pBBR1MCS (Cm^R) plasmid was also suitable for electroporation in *Halomonas* sp. O-1, but with lower yields (~10^3 CFUs ug^{-1} DNA). Non-DNA electroporated controls were also plated onto media containing these antibiotics and consistently yielded no transformants. Despite the high sensitivity of *Halomonas* sp. O-1 to chloramphenicol (Table 2), transformants harboring pBBR1MCS (Cm^R) were capable of persisting on selection plates containing Cm at concentrations of 25 µg mL^{-1}, albeit at greatly reduced transformation efficiencies compared to 10 µg mL^{-1}. Lastly, we were unable to successfully transform the pBBR1MCS-3 (Tet^R) [5228 bp] vector in *Halomonas* sp. O-1 and all electroporations using this vector yielded no transformants on *HGM* selection plates containing Tet at 15 µg mL^{-1}. Because the pBBR1MCS-5 (Gen^R) showed the most promising results, all additional assays for voltage optimization and the effect of plasmid concentration on electroporation efficiency were performed with this plasmid.

Using the previously defined parameters, electroporation assays were first performed over a range of voltages at a constant vector DNA amount of 100 ng (Figure 2.5). When plotted logarithmically, there is a relatively linear increase in transformation efficiency from 10^3 CFUs ug^{-1} DNA at 1.5 kV to 10^4 CFUs ug^{-1} DNA at 2.1 kV followed by a slight decrease in efficiency at voltages above 2.1 kV. These results indicate the optimal voltage for electroporation of *Halomonas* sp. O-1 using this device was to be 2.1 kV. Transformations at voltages of 2.3 kV produced similar results to 2.1 kV, but arcing became much more common at this high potential and required electroporations to be repeated on multiple occasions. Controls containing electroporated cells without added DNA were also performed at each voltage and yielded no colonies, which indicated sustained sensitivity to gentamicin.

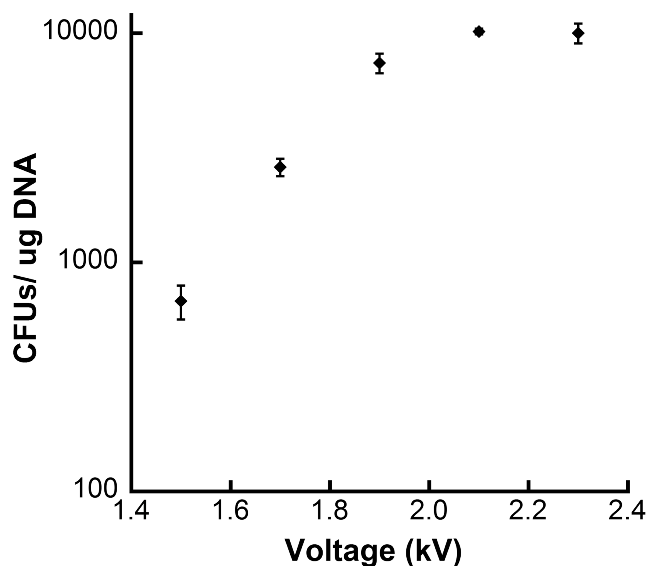

Figure 2.5 Optimization of electroporation conditions for vector DNA uptake by *Halomonas* sp. O-1. Electrocompetent cells prepared using the described wash procedure (*See Materials and Methods*) were transformed with constant 100 ng of pBBR1MCS-5 (4768 bp) [Gen^R] empty vector at a range of voltages (1.5–2.3 kV). Experiments were performed in triplicate, with data points representing mean values and error bars representing standard deviations.

Following the determination of optimal voltage, the final set of assays performed in this study sought to establish a relationship between DNA amount and the resulting number of transformants. Using a variable amount of vector DNA (pBBR1MCS-5) ranging from 10–500 ng per electroporation, all electroporations for this series were performed at the previously optimized voltage of 2.1 kV. Results show a positive, linear relationship between DNA amount and transformation efficiency with a resulting slope of ~10 transformants ng^{-1} DNA (Figure 2.6). Furthermore, non-DNA electroporated controls yielded no transformants when screened on *HGM* supplemented with gentamicin at 30 μg mL^{-1}.

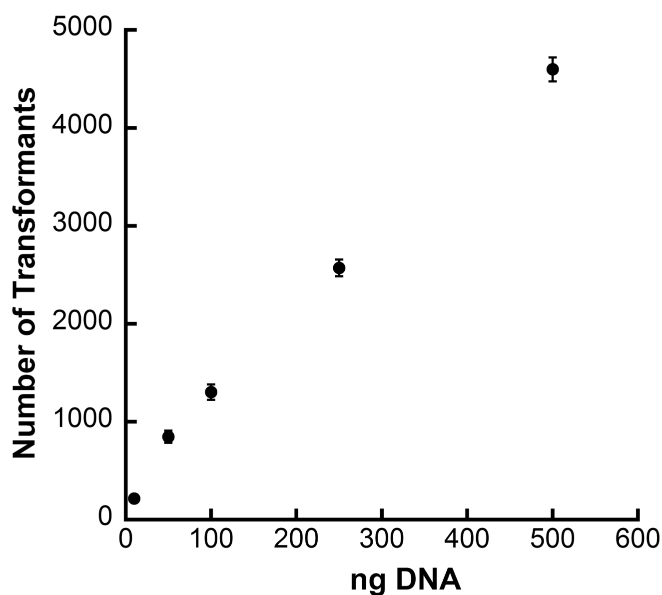

Figure 2.6 Efficiency of *Halomonas* sp. O-1 electroporation based on plasmid amount. Cells were washed using the previously described methods, then transformed with a variable amount of DNA (10–500 ng) at a constant 2.1 kV. Experiments were performed in triplicate, with data points representing mean values and error bars representing standard deviations.

2.4 Discussion

Halomonas sp. O-1 was a recently discovered seawater isolate shown to naturally produce PHAs (Ilham et al., 2014) and, similar to other members of this genus, has enormous potential for industrial scale applications (Quillaguamán et al., 2005; Tan et al., 2014; Yue et al., 2014). However, this potential for exploiting *Halomonas* spp. for industrial use has been hindered by the lack of genetic tools and techniques for manipulating these organisms (Chen, 2012). The most common method for transforming this genus has been bacterial conjugation, a somewhat tedious and time-consuming method compared to electroporation-based methods. The study presented here has provided the first detailed evidence that a *Halomonas* spp. can be made electrocompetent and is capable of being modified using plasmid DNA via electroporation.

Bacteria-based electroporation procedures typically require a non-ionic osmotic stabilizer with high resistivity as the wash buffer in order to sustain an adequate pulse duration (~5 ms) at a

high enough voltage (>1.5 kV) to permeate the cell membrane while maintaining a relatively low current to avoid excessive cell mortality (Shigekawa and Dower, 1988). A recent review has stated that the creation of viable, electrocompetent cells is the major hurdle preventing electroporation-based transformations of halophilic bacteria (Yin et al. 2015).

Due to the halophilic nature of *Halomonas* sp. O-1, in which a generally high ionic strength media is preferred for optimal growth, several obstacles had to be overcome to generate electrocompetent cells without greatly diminishing cell viability.

The media developed in this study, termed *Halomonas* Growth Media or HGM, is made from the core components tryptone, yeast extract, and NaCl with a final salt concentration of 3.5%, and was brought to a pH of 7.5. The salinity and pH of this media is based upon empirical evidence presented in this study (Figure 2.2) and also took into consideration the natural conditions of this organism and mimicked the salinity (~3.5%) and pH (7.5−8) of ocean water (Fofonoff, 1985). Although sodium (Na^+) and chloride (Cl^-) are by far the most abundant ions in seawater, other ionic components such as magnesium (Mg^{2+}), calcium (Ca^{2+}), potassium (K^+), sulfate (SO_4^{2-}), carbonate (CO_3^{2-}) are also present (Millero et al., 2008). Therefore, for *HGM* to truly mimic seawater, it would have to take the exact proportion of these ions into consideration, however, the yeast tryptone extract did contain these elements in amounts suitable to support biologic function. Future development of this methodology, or media specifically, may consider this alteration.

For the electrocompetency procedure that was devised, cells were harvested at mid-log phase growth (Figure 2.3) and used 300 mM sucrose as a non-ionic wash buffer, i.e. a buffer lacking salts and any other components with electrically conductive properties. Despite the lack of ionic strength in the wash buffer, results show that 80% cell viability was maintained

throughout the wash procedure (Figure 2.4). Taken together, it is believed that the relatively low-ionic strength of HGM compared to media containing the preferred salt concentration for this microorganism (5−15%) (Ilham et al., 2014) encouraged a phenotype that was capable of enduring the non-ionic conditions of the sucrose wash and ultimately allowed for the creation of viable, electrocompetent cells.

Other electroporation procedures concerning bacteria are often performed at low temperatures (0–4°C) and may involve a low temperature incubation of washed cells prior to electroporation to enhance plasmid uptake efficiency (Prasanna and Panda, 1997; Shigekawa and Dower, 1988). However, transformational efficiency was found to be best when electrocompetent cells were created at room temperature (23°C) and electroporations were performed immediately after the final wash step, which is similar to contemporary and rapid methods for creating electrocompetent cells of *Pseudomonas aeruginosa* (Choi et al., 2006).

A vital component for successfully screening positive transformants for the uptake of plasmid DNA is the use of antibiotic resistance markers (Schweizer, 2008). This requires the organism of interest to have susceptibility to the chosen antibiotic in order to ensure the efficacy of selection and subsequent plasmid stability in the host. Based on the results of antibiotic screening assays (Table 2), *Halomonas* sp. O-1 was found to be sensitive to the antibiotics chloramphenicol, tetracycline, and gentamicin; therefore, vectors containing resistance cassettes for the listed antibiotics were chosen for electroporation trials (Table 1). Of these vectors, pBBR1MCS (Cm^R) and pBBR1MCS-5 (Gen^R) were found to be compatible in *Halomonas* sp. O-1 and represent viable resistant cassettes for future applications in this organism and possibly other members of the genus.

Based on the success of preliminary electroporation assays using the pBBR1MCS-5 vector, experiments to optimize voltage and plasmid concentration were performed and resulted in consistent and reproducible results (Figures 2.5, 2.6) in which transformants on the scale of 10^4 μg^{-1} of plasmid DNA were obtained. Efficiency on this scale is relatively low compared to methodologies utilized in bacteria such as *E. coli* and *P. aeruginosa* where transformants are often obtained at levels higher than 10^9 μg^{-1} DNA (Calvin and Hanawalt 1988; Choi et al., 2006), however, in one of the first successful applications of electroporating plasmid DNA into intact bacterial cells performed by Powell et. al. (1988), obtaining transformants of *Streptococci* spp. on the scale of 10^4 μg^{-1} DNA was considered a success and set the stage for rapid improvement of this methodology. Potential avenues of exploration to enhance transformation efficiency may include using different strains of *Halomonas* species, different sized plasmids, plasmids that contain alternative origins of replications, as well as variations in electroporation cuvette gap size and electroporation parameters such as pulse duration (Tu et al., 2016). Additionally, based on the linear trend seen in Figure 2.6, a further increase in DNA amount to a level of 1 μg or higher may result in an increased number of transformants, but may not increase electroporation efficiency as expressed as transformants per μg of DNA.

Overall, this study represents another step in expanding the repertoire of molecular methods available to genetically engineer *Halomonas* spp. The pBBR1MCS broad host vectors were shown to be capable of uptake and replication in *Halomonas* sp. O-1 and produced transformants on the magnitude of 10^4 μg^{-1} DNA. Additionally, optimized parameters for growth, electrocompetency, and electroporation conditions were established. Recently, a constitutive promoter library with an inducible promoter system has been developed in *Halomonas* TD01 (Li et al., 2016). Using this study as a benchmark, it may be possible to

engineer novel vectors for use in *Halomonas* sp. O-1 containing inducible promoter systems to allow for precise control of recombinant gene expression. Additionally, rapid, plasmid-based gene knockout methods originally developed for use in *Pseudomonas aeuriginosa* may now be applied to *Halomonas* sp. O-1 (Choi et al., 2006). Lastly, this method can be tested in other members of the genus, particularly those already being engineered for industrial use such as *Halomonas* TD01 and *H. campaniensis* (Tan et al., 2014; Yue et al., 2014).

2.5 Conclusion

Establishment of an effective electroporation protocol for *Halomonas* species adds another powerful tool to the arsenal of molecular biology and genetic engineering techniques that have the potential to contribute to lowering the costs and barriers for producing commercial amounts of PHA plastics. Although bacterial conjugation has been a highly effective tool for designing recombinant strains of *Halomonas* and other organisms, use of electroporation as an alternative or supplementary technique can only lead to further reductions in cost and increase the ease at which new production strains can be developed. Lastly, the potential of creating industrial systems with recombinant strains of *Halomonas* in an open, continuous batch process that utilizes sea water, which circumvents the need for sterilized fresh water in batch fermentations, can greatly reduce energy costs and the carbon footprint of large-scale PHA production. Broadly, this study serves as a prime example of how research related to creating an environmentally and economically sustainable market for producing PHAs as biodegradable plastics continues to expand and develop.

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Chapter 3. The metabolism of (*R*)-3-hydroxybutyrate is regulated by the enhancer-binding protein PA2005 (HbrC) and the alternative sigma factor RpoN in *Pseudomonas aeruginosa* PAO1

Abstract

A variety of soil-dwelling bacteria produce polyhydroxybutyrate (PHB), which serves as a source of energy and carbon under nutrient deprivation and has also been developed as a mass-produced biodegradable plastic. Bacteria belonging to the genus *Pseudomonas* do not generally produce PHB but are capable of using the PHB degradation product (*R*)-3-hydroxybutyrate [(*R*)-3-HB] as a growth substrate. Essential to this utilization is the NAD^+- dependent dehydrogenase BdhA that converts (*R*)-3-HB into acetoacetate, a molecule that readily enters central metabolism. Apart from the numerous studies that had focused on the biochemical characterization of BdhA, there was nothing known about the assimilation of (*R*)-3-HB in *Pseudomonas*, including the genetic regulation of *bdhA* expression. This study aimed to define the regulatory factors that govern or dictate the expression of the *bdhA* gene and (*R*)-3-HB assimilation in *Pseudomonas aeruginosa* PAO1.

Importantly, expression of the *bdhA* gene was found to be specifically induced by (*R*)-3-HB in a manner dependent on the alternative sigma factor RpoN and the enhancer-binding protein PA2005 (HbrC). This mode of regulation was essential for the utilization of (*R*)-3-HB as a sole source of energy and carbon. However, non-induced levels of *bdhA* expression were sufficient for *P. aeruginosa* PAO1 to grow on (±)-1,3-butanediol, which is catabolized through an (*R*)-3-HB intermediate. Because this is, we believe, the first report of an enhancer-binding protein that responds to (*R*)-3-HB, PA2005 was named HbcR for (R)-3-<u>h</u>ydroxy<u>b</u>utyrate <u>c</u>atabolism <u>r</u>egulator. More broadly, this study serves as an example of the field of research necessary to determine the fate of biodegradable plastics in the environment and the genetic factors and strategies utilized by the ubiquitous bacteria responsible of this degradation.

The work presented in this chapter is a slight adaptation of a published article by B.R. Lundgren, J.R. Harris, et al. (2015) and can be found in *Microbiology*, Issue 161, pages 2232–2242.

***Note;** The author, JR Harris, was a primary contributor to this study and responsible for; some aspects of the molecular cloning, the majority of complementation growth studies, and the purification of* PA2005 *and subsequent EMSA experiments.*

3.1 Introduction

3.1.1 Natural Occurrence of (*R*)-3-Hydroxybutyrate

(*R*)-3-Hydroxybutyrate [(*R*)-3-HB] is commonly recognized for its role as a ketone body produced by mammalian cells when carbohydrate availability is limiting (Akram, 2013). However, there are a number of bacteria that biosynthesize CoA derivatives of (*R*)-3-HB and other (*R*)-3-hydroxy acids, which are polymerized into macromolecular structures called polyhydroxyalkanoates (PHAs) (Anderson & Dawes, 1990; Lu et al., 2009). Under starvation conditions, the PHA granule is degraded into its 3-hydroxy acid components, which can be used as sources of carbon and energy (Jendrossek & Handrick, 2002; Jendrossek et al., 1996). Additionally, there are a variety of microorganisms capable of degrading extracellular PHAs that have been naturally released into the environment via the death of PHA-producing bacteria (Müller et al., 1993; Schrimer et al., 1993; Miyazaki et al., 2000).

One of the most common and best understood PHAs is polyhydroxybutyrate (PHB), of which (*R*)-3-HB is the sole monomeric component. PHB was the first of this class of polymers to be characterized and over the course of decades has been at the center of intense research to develop these naturally-occurring, biodegradable polymers to serve as a potential substitute for petroleum-based plastics (Macrae and Wilkinson, 1958; Philip et al., 2007). The major impetus for the development of PHAs as bulk commodity plastics is to counterbalance the negative environmental effects caused by traditionally made plastics (Leberton et al., 2018). As the presence of PHB and other PHAs in environment increase, understanding how these materials naturally degrade is important to 1) validly claim that these materials are biodegradable and environmentally innocuous, and 2) help guide the future development of other biodegradable plastics with similarly sustainable properties. In addition to studying the role of catabolic

enzymes present in microorganisms capable of metabolizing PHAs, it is also of relevance to determine the regulatory elements and overall biosynthetic pathways involved in the environmental degradation of PHAs. As more diverse and robust PHAs are developed and implemented, it is important to understand how long these materials will persist in the environment once discarded. By studying the genetic regulatory elements required for the expression of enzymes involved in PHA metabolism, it becomes possible to predict how long certain types of PHAs will take to degrade once exposed to naturally occurring microbes. This will help to design PHAs based on their 'shelf-life' in order to prevent the environmental buildup of yet to be degraded PHA materials while still creating a plastic that can resist deterioration during its intended period of use.

3.1.2 Metabolism of (*R*)-3-HB in *Pseudomonas aeruginosa*

Bacteria of the genus *Pseudomonas* are renowned for their versatile metabolism in that they can assimilate and breakdown a wide assortment of compounds to meet their nutritional demands. One compound that is not often affiliated with *Pseudomonas* metabolism is (*R*)-3-hydroxybutyrate [(*R*)-3-HB or D-3-hydroxybutyrate]. *Pseudomonas* species do not biosynthesize nor incorporate (*R*)-3-HB into their own endogenous PHA reserves (Huisman et al., 1989; Timm & Steinbüchel, 1990). Nonetheless, these bacteria possess an NAD^+-dependent dehydrogenase (BdhA) that converts (*R*)-3-HB into acetoacetate, thereby allowing these bacteria to use (*R*)-3-HB as a growth substrate (Feller et al., 2006; Ito et al., 2006; Mountassif et al., 2010). After conversion to acetoacetate, the oligomeric enzyme dehydrocarnitine-CoA transferase converts acetoacetate to acetoacetyl-CoA followed by the generation of two molecules of acetyl-CoA through the catalytic action of acetoacetyl-CoA thiolase. Figure 3.1 describes the biosynthetic pathway in *P. aeruginosa* for the catabolism of (*R*)-3-HB to Acetyl-CoA where it can then serve

as a precursor in both fatty acid biosynthesis and the TCA cycle (Figure 3.1a). The enzymes responsible for the catabolism (*R*)-3-HB are transcribed and translated from genes that are all located adjacent to one another within the *P. aeruginosa* genome (Figure 3.1b).

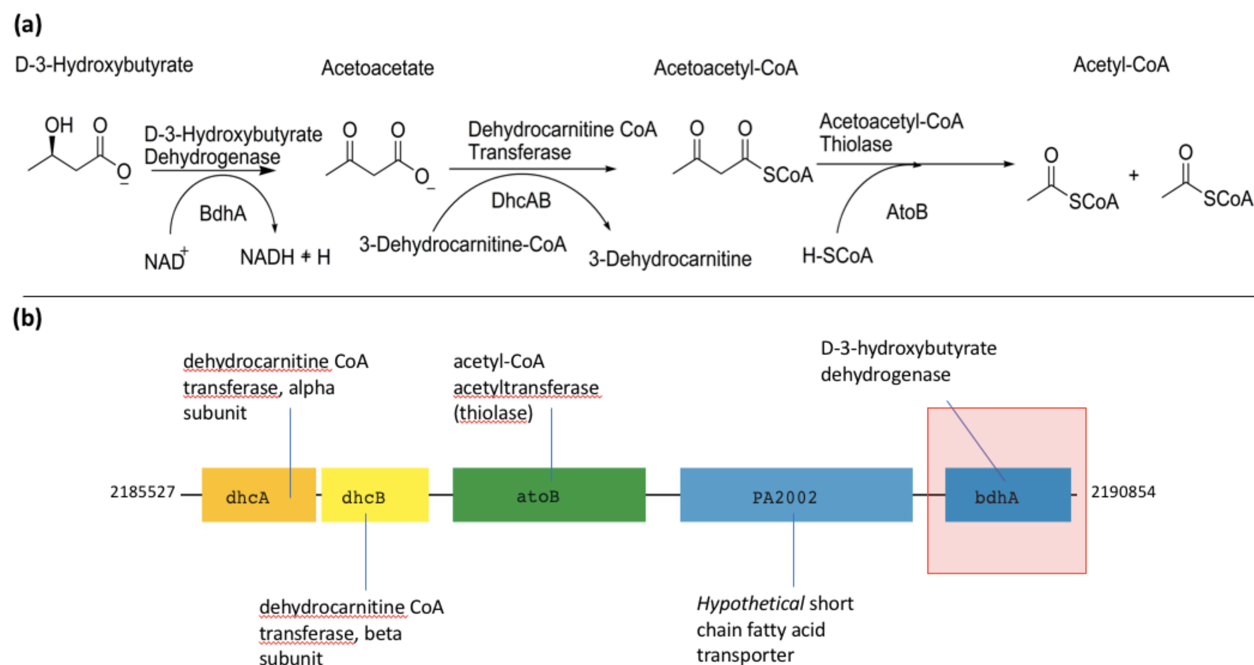

Figure 3.1 Catabolism of D-3-hydroxybutyrate in *Pseudomonas aeruginosa* PAO1. (a) Conversion of D-3-hydroxybutyrate [also known as (*R*)-3-hydroxybutyrate or (*R*)-3-HB] to two molecules of acetyl-CoA is accomplished through the concerted actions of D-3-hydroxybutyrate dehydrogenase (BdhA), dehydrocarnitine CoA transferase (DhcAB), and acetoacetyl-CoA thiolase (AtoB). (b) Within the PAO1 genome, D-3-hydroxybutyrate catabolic genes are appear adjacent to one another. Location within the genome is annotated and gene names can be found within the genes themselves with translated protein names labeled. PA2002 gene function has not yet been defined but may be involved in transport of D-3-hydroxybutyrate within the cell. The bdhA gene which codes for D-3-hydroxybutyrate dehydrogenase is outlined in red.

BdhA dehydrogenases have been biochemically characterized for some species of *Pseudomonas*, including *P. putida* (Feller et al., 2006; Paithankar et al., 2007), *P. fragi* (Ito et al., 2006; Nakajima et al., 2005) and *P. aeruginosa* PAO1 (Mountassif et al., 2010). These studies have provided extensive information regarding catalytic properties, mechanisms and structural features of (*R*)-3-HB dehydrogenases. Despite the wealth of information regarding the biochemical properties of enzymes involved in (*R*)-3-HB metabolism, the current study is, we

believe, the first to characterize the genetic regulation of *bdhA* expression and (*R*)-3-HB utilization for any given species of *Pseudomonas*.

3.1.3 Objectives of this Study

The existence of BdhA dehydrogenases among *Pseudomonas* spp. indicates that these bacteria can assimilate (*R*)-3-HB from external sources. In an effort to understand this process, we focused on defining the genetic mechanisms surrounding (*R*)-3-HB utilization in *P. aeruginosa* PAO1. The *bdhA* (*PA2003*) gene is actually part of a bicistronic operon that starts with *PA2004*, encoding a putative transporter related to the H^+ gluconate symporter family (Winsor et al., 2011). The *PA2004-bdhA* operon is preceded by a putative -24/-12 promoter recognized by the alternative sigma factor σ^{54} or RpoN (Conway & Boddy, 2012). RpoN-RNA polymerase (RNAP) holoenzymes cannot spontaneously isomerize from a closed to open complex for transcription initiation (Buck & Cannon, 1992). Additional transcriptional regulators called enhancer-binding proteins (EBPs) interact with the RpoN-RNAP holoenzyme and use the energy of nucleotide hydrolysis to mediate formation of the open complex (Morett & Segovia, 1993; Studholme & Buck, 2000). Adjacent to the *PA2004-bdhA* operon is the *PA2005* gene, which encodes a previously uncharacterized, putative EBP (Figure 3.2).

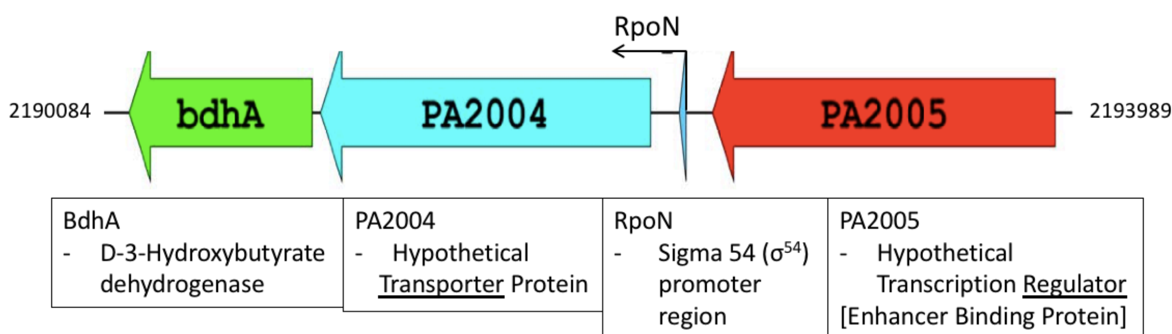

Figure 3.2 Organization of *bdha, PA2004,* and *PA2005* genes within the *Pseudomonas aeruginosa* PAO1 genome. The location of these genes within the PAO1 genome is annotated and the orientation of the arrows indicates the directionality of transcription. Both *PA2004* and *bdhA* are believed to be transcribed as a single operon. A description of the coded proteins is found below each gene with the addition of the RpoN promoter which is not a gene, but a regulator region believed to be involved in the expression of the *PA2004-bdhA* operon.

In addition to complementation studies designed to provide evidence for the necessity of the *PA2005* gene in (*R*)-3-HB catabolism, a series of experiments known as Electrophoretic Mobility Shift Assays (EMAs) were performed to further understand this relationship and definitively prove the role of the PA2005 protein (HbrC) as an EBP that interacts with the promoter region of DNA upstream of the *PA2004-bdhA* open reading frame (ORF). An EMSA, also referred to as a gel shift assay, is an electrophoresis technique used to determine the affinity of a specific protein for a sequence of DNA (probe) by comparing migration rates of the probe through an agarose gel (Kerr, 1995). If protein binds and interacts with the probe it retards the migration rate through the gel compared to unbound probe and is strongly indicative of a regulatory role of the protein for that specific sequence of DNA. The results of the current study show that the putative EBP PA2005 (HbrC) serves as a transcriptional regulator necessary for the induction of the *PA2004-bdhA* operon in response to (*R*)-3-HB.

3.2 Materials and Methods

3.2.1 Bacteria, Plasmids and Media

Bacteria and plasmids used in the study are given in Appendix A, Table 1. The *P. aeruginosa* strains obtained from the transposon mutant library (Jacobs et al., 2003) were verified using PCR as recommended by the library curators. Bacteria were grown in BD Difco Lennox broth (LB) or M63 minimal medium (Pardee et al., 1959). Unless otherwise stated, bacteria were grown in 50 ml medium (in 500 ml baffled shake flasks) in a rotary shaker at 37°C, 200 r.p.m. Medium used for growth of the *rpoN* mutant (PAO6359) of *P. aeruginosa* was supplemented with 5 mM *L*-Gln (Heurlier et al., 2003). Plasmid selection was achieved using the following concentrations of antibiotics: kanamycin (Km) (50 μg ml^{-1} for *Escherichia coli),*

carbenicillin (Cb) (100 μg ml^{-1} for *E. coli* or 200 μg ml^{-1} for *P. aeruginosa*) and gentamicin (Gm) (20 μg ml^{-1} for *E. coli* or 30 μg ml^{-1} for *P. aeruginosa*).

3.2.2 Standard DNA Procedures

DNA was purified using Promega nucleic acid purification kits. Restriction enzymes, ligases and polymerases were products of New England BioLabs. Oligonucleotides used for PCR applications were purchased from Integrated DNA Technologies and are listed in Appendix A, Table 1. Genomic DNA from *P. aeruginosa* PAO1 was used for all PCR applications. PCR products were gel-purified and cloned into pCR-Blunt (Invitrogen) according to the manufacturer's instructions. Cloned DNA was verified by sequencing (Genewiz).

3.2.3 Cloning of the *bdhA* (*PA2003*) and *PA2004* Genes

The *bdhA* and *PA2004* genes were PCR amplified using the primers BL444.f/BL444.r and BL443.f/BL443.r, respectively. The PA2004-bdhA operon was amplified with primers BL443.f/BL444.r. All three PCR products were individually cloned into pCR-Blunt. The *bdhA*, *PA2004* and *PA2004- bdhA* genes were then subcloned into the *Xba*I/*Sac*I sites of pBBR1MCS-5 to give pBRL496, pBRL501 and pBRL498, respectively.

3.2.4 Cloning of the *PA2005* ORF

The putative *PA2005* ORF was PCR amplified with the primers BL446.f/BL446.r, and the resulting PCR product was cloned into pCR-Blunt to give pBRL510. The PA2005 ORF from pBRL510 was subcloned into the *Nde*I/*Sac*I sites of pET28b (EMD Millipore) to give pBRL516. The pBRL516 plasmid was digested with *Xba*I/*Sac*I to liberate the *PA2005* ORF with a pET-derived RBS, which was cloned into the *Xba*I/*Sac*I sites of pBBR1MCS-5 (Kovach et al., 1995) to yield pJRH010. Lastly, the *PA2005* ORF from pBRL510 was cloned into the *Eco*RI site of

pTrc99a (Pharmacia) with either a forward orientation (pBRL595) or reverse orientation (pBRL596) relative to the *trc* promoter.

3.2.5 Construction of the *PA2004-lacZ* Reporter

The 1042 bp 5'-regulatory region positioned immediately upstream of the *PA2004* ORF was PCR amplified with primers BL442.f/BL442.r. This amplicon was then fused to the *lacZ* ORF of *E. coli* MG1655 with primers BL442.f/ BL342.r using PCR conditions as previously described (Lundgren et al., 2014). The *PA2004-lacZ* fusion PCR product was cloned into pCR-Blunt to give pBRL492. The *PA2004-lacZ* fusion in pBRL492 was cloned into the *Xba*I site of the promoterless ΔPlac-pBBR1MCS-5 plasmid (Lundgren et al., 2014) to yield pBRL499. The GG dinucleotide of the -24 element of the RpoN promoter located 148 bp upstream of the *lacZ* ORF in pBRL499 was changed to an AA dinucleotide using QuikChange (Agilent Technologies) with the primers BL445.f/BL445.r. The resulting plasmid pBRL505 was sequenced to verify the desired mutation.

3.2.6 Growth studies of *P. aeruginosa* on (*R,S*)-3-HB

BdhA has been shown to be specific for (*R*)-3-HB (Ito et al., 2006), and the BdhA of *P. aeruginosa* PAO1 was observed to synthesize acetoacetate when given (*R,S*)-3-HB as a substrate (Mountassif et al., 2010). Therefore, the sodium salt, racemic (*R,S*)-3-HB (Sigma Aldrich) was used as the growth substrate, because it was more cost effective than enantiopure (*R*)-3-HB. All *P. aeruginosa* strains were grown in quadruplicate. For each replicate, M63 minimal medium that was supplemented with 30 mM (*R,S*)-3-HB or (±)-1,3-butanediol was inoculated to an initial OD_{600} of ~0.1. Cultures were grown for 24 h and OD_{600} was periodically measured. For genetic complementation experiments, the medium was not supplemented with antibiotic for plasmid

selection. The use of 30 mM (*R,S*)-3-HB or (±)-1,3-butanediol as the sole carbon source was sufficient for selection of recombinant strains.

3.2.7 β-Galactosidase (LacZ) Assays

Each condition was tested in quadruplicate, and LacZ activity was determined using the Miller assay (Miller, 1992; Lundgren et al., 2013, 2014). The equation used to calculate Miller Units for all LacZ assays conducted in this study can be found in the description of Figure 3.4. To monitor the change in expression of *PA2004-lacZ* over time, *P. aeruginosa* strains harboring pBRL499 or pBRL505 were grown in LB or M63 minimal medium, which was supplemented with 30 mM sodium acetate or sodium succinate, to an OD_{600} of 0.3. Cells were then challenged with 30 mM (*R,S*)-3-HB, and LacZ activity was measured at 1, 2, 3 and 4 h post-induction.

The abilities of compounds to induce expression of *PA2004-lacZ* were examined by growing *P. aeruginosa* PAO1 harboring pBRL499 in M63 minimal medium supplemented with 30 mM sodium succinate to an OD_{600} of 0.3 and then adding (*R,S*)-3-HB, (*R*)-3-HB, (*R*)-2-hydroxybutyrate [(*R*)-2-HB], acetoacetate, (*R*)-lactate or (*S*)-carnitine to a final concentration of 1mM. LacZ activity was then measured 30 min post-addition of substrate. For LacZ assays involving (±)-1,3-butanediol, *P. aeruginosa* strains possessing pBRL499 were grown in LB, which was supplemented with 30 mM (±)-1,3-butanediol or 1,4-butanediol. After a 16 h incubation period (37°C, 200 r.p.m.), LacZ activity was measured for each sample.

LacZ assays involving *E. coli* were done using the lacZ-deficient *E. coli* strain Top10 (Invitrogen). *E. coli* Top10 was co-transformed with pBRL499 (or a *PA2004-lacZ* reporter derivative: ΔPlac-pBBR1MCS-5 or pBRL505) and pBRL595 (or a *PA2005* expression plasmid derivative: pTrc99a or pBRL596). Recombinant strains were grown in LB supplemented with gentamicin and carbenicillin to an OD_{600} of 0.3. Subsequently, (*R*)-3-HB, (*R,S*)-3-HB, 2-(*R*)-HB

or acetoacetate was added to a final concentration of 30 mM, and LacZ activity was measured at 2 h post-induction.

3.2.8 Purification and Electrophoretic Mobility Shift Assays (EMSAs) of His$_6$-PA2005

The *PA2005* ORF was cloned into pET28b, and the resulting plasmid pBRL516 encoded an N-terminal 6X histidine-tagged PA2005 fusion protein (His$_6$-PA2005). *E. coli* BL21(DE3) harboring pBRL516 was grown in LB to an OD_{600} of ~0.6 at 37°C with shaking (200 r.p.m.). Protein expression was induced by the addition of 0.1 mM IPTG, and the induced cultures were incubated for 12 h at 16°C (200 r.p.m.). Cells were harvested, suspended in buffer (100 mM sodium phosphate pH 8.0, 300 mM NaCl, 10 % v/v glycerol. 1 mg mL^{-1} lysozyme, 5 U mL^{-1} DNase I, 1 μg mL^{-1} pepstatin, 1 μg mL^{-1} leupeptin) and then lysed by sonication on ice. Unlysed cells and debris were removed via centrifugation. The His$_6$-PA2005 protein was then purified from the clarified lysate using Ni-NTA Superflow resin (Qiagen). The His$_6$-PA2005 protein was eluted off the resin using a step elution method with elution buffer (100 mM Tris, 300 mM NaCl, pH 8.0) containing 20, 100 and 250 mM imidazole. The purified His$_6$-PA2005 protein was concentrated using Amicon Ultra centrifugal filter units (EMD Millipore). Protein expression and purification were monitored visually using SDS-PAGE. The concentration of purified protein was determined using the Bradford assay (Pierce).

His$_6$-PA2005 was expected to bind to a region preceding the RpoN promoter positioned 148 bp upstream of the *PA2004* ORF. Therefore, a 116 bp probe (P_{PA2004}), which resembled the region 237–122 bp upstream of the *PA2004* ORF, was PCR amplified with the primers BL447.f/BL447.r. For the non-specific probe, the 200 bp promoter region of *gcvH2* (P_{gcvH2})

(Lundgren et al., 2013) was PCR amplified with the primers ZS406.f/ZS406.r. The P_{PA2004} and P_{gcvH2} PCR products were individually cloned into pJET1.2 (ThermoScientific) to yield the plasmids pBRL512 and pZS406, respectively. The 5'-labelled Cy5 primers JRH05.f/JRH05.r were used to PCR amplify P_{PA2004} and P_{gcvH2} from pBRL512 and pZS406, respectively. The resulting 5'-labelled Cy5 P_{PA2004} and P_{gcvH2} probes were gel-purified and used in subsequent EMSAs.

For the first set of EMSA reactions, 500 nM His_6-PA2005 was incubated with 1.0 nM 5'-labelled Cy5 P_{PA2004} probe (specific probe) or 1.0 nM 5'-labelled Cy5 P_{gcvH2} probe (non-specific probe) in EMSA buffer (25 mM Tris/acetate, 8.0 mM magnesium acetate, 10 mM KCl, 1.0 mM DTT, pH 8.0) for 30 min at 30°C. In the second set of EMSA reactions, 1.0 nM 5'-labelled Cy5 P_{PA2004} probe was incubated with 0, 6.25, 12.5, 25, 50, 100, 200 or 400 nM PA2005 in EMSA buffer for 30 min at 30°C. The samples were then analyzed using PAGE in non-denaturing conditions and imaged using a Typhoon imager.

3.3 Results

3.3.1 Transposon Insertions into the *bdhA* (*PA2003*), *PA2004* or *PA2005 (hbrC)* Gene Hindered the Growth of *P. aeruginosa* PAO1 on (*R,S*)-3-HB

The metabolism of (*R*)-3-HB was expected to be dependent on the *PA2004-bdhA* operon, because BdhA performs an essential function in that it oxidizes (*R*)-3-HB into acetoacetate. The role of PA2004 was less clear. Because PA2004 has homology to the H^+ gluconate symporters (Winsor et al., 2011), it was speculated that PA2004 might function as an (*R*)-3-HB transporter. The *PA2005* gene encodes a putative EBP, and because there is a -24/-12 or RpoN promoter 148

bp upstream of the *PA2004* ORF (Conway & Boddy, 2012), PA2005 (HbrC) was proposed to be the EBP that regulates expression of the *PA2004-bdhA* operon in response to (*R*)-3-HB. To test this hypothesis, a number of transposon mutants (*bdhA::Tn*, *PA2004::Tn*, and *PA2005::Tn*) were obtained from the University of Washington *Pseudomonas aeruginosa* transposon mutant library (Jacobs et al., 2003). A transposon mutation is generated by using a transposon, which is a mobile genetic sequence that is capable of transposition from one portion of the genome to another. It is possible to take advantage of these naturally occurring, mobile genetic elements and direct a transposon to locate itself within the sequence of a particular gene of interest to create a mutated strain that lacks the proper function of this gene. To verify the mutations of the transposon strains obtained, PCR of the mutated genes were performed and compared to PCR fragments of the wild-type genes (data not shown).

Consistent with this hypothesis, a *PA2005* transposon mutant (*PA2005*::Tn) could not use (*R,S*)-3-HB as a sole carbon source (Figure 3.3). Growth of the *PA2005* : : Tn mutant on (*R,S*)-3-HB was restored when the *bdhA* gene, the *PA2004-bdhA* operon or the *PA2005* gene was expressed from the *lac* promoter on the broad-host-range pBBR1MCS-5 plasmid (Figure 3.3a) [See Appendix A, Table 1 for full list of plasmids and primers used in this study]. This finding suggested that the diminished capacity of the *PA2005* : : Tn mutant to grow on (*R,S*)-3-HB might be a result of insufficient expression of the *bdhA* gene. Not surprisingly, a *bdhA* transposon mutant (*bdhA* : : Tn) (Figure 3.3b) and a *PA2004* : : Tn mutant (Figure 3.3c) also failed to utilize (*R,S*)-3-HB as a carbon source.

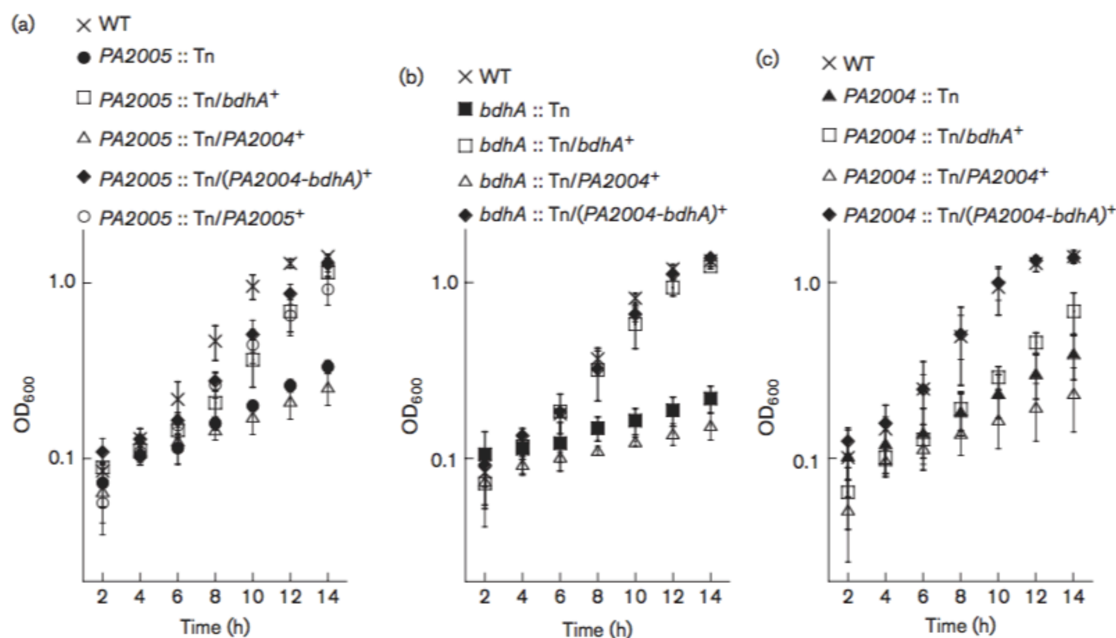

Figure 3.3 Transposon (Tn) insertions in the *bdhA*, *PA2004* and *PA2005* genes negatively affected the utilization of (*R,S*)-3-HB in *P. aeruginosa* PAO1.Growth deficiencies were observed for (a) *PA2005* : : Tn, (b) *bdhA* : : Tn and (c) *PA2004* : : Tn mutants of *P. aeruginosa* PAO1 on 30 mM (*R,S*)-3-HB. Expression of *bdhA* ($bdhA^{+}$), the *PA2004-bdhA* operon [$(PA2004\text{- } bdhA)^{+}$] or PA2005 ($PA2005^{+}$) from the *lac* promoter of the pBBR1MCS-5 plasmid restored growth of the *PA2005* : : Tn mutant on (*R,S*)-3-HB. The *bdhA* : : Tn and *PA2004* : : Tn mutants were also complemented with plasmid-derived expression of *bdhA* ($bdhA^{+}$) or the *PA2004-bdhA* operon [$(PA2004\text{-}bdhA)^{+}$]. Data points represent mean values±SD (n=4). ANOVA was performed using a Dunnett's post-hoc test (∝-value of 0.05) to identify significant changes (P< 0.0001).

3.3.2 Expression of a *PA2004-lacZ* Construct was Induced by (*R*)-3-HB

The expression of the *PA2004-bdhA* operon was expected to be regulated by PA2005 (HbrC) in response to (*R*)-3-HB availability. Therefore, it was first determined if the *PA2004-bdhA* operon was in fact inducible by (*R*)-3-HB. To achieve this goal, the 5'-regulatory region (1042 bp) located immediately upstream of the *PA2004* start codon was fused to the *lacZ* ORF of *E. coli* MG1665, and the resulting *PA2004-lacZ* fusion was cloned into the promoterless plasmid ΔPlac-pBBR1MCS-5 (Lundgren et al., 2014). *P. aeruginosa* PAO1 harboring the *PA2004-lacZ* reporter was grown in M63 minimal medium supplemented with 30 mM succinate. When the cells reached an OD_{600} of 0.3, they were challenged with various substrates added at a final

concentration of 1 mM. Within 30 min of the addition of either (*R*)-3-HB or (*R,S*)-3-HB, *PA2004-lacZ* expression increased twofold (Figure 3.4a). Other tested substrates, including (*R*)-2-HB, butyrate, acetoacetate, (*R*)-lactate and (*S*)-carnitine, did not induce expression of *PA2004-lacZ* (Figure 3.4b). Increased expression of *PA2004-lacZ* was specific to (*R*)-3-HB, indicating that this molecule is an inducer of the *PA2004-bdhA* operon in *P. aeruginosa* PAO1. It should be noted that in Figure 3.4b, a similar level induction was found for both (*R*)-3-HB and (*R,S*)-3-HB. Although it is believed that (*R*)-3-HB is the sole substrate capable of inducing the *PA2004-bdhA* operon, similar induction levels were seen for both the enantiopure and racemic mixtures because a minimum threshold for full induction of the operon was achieved with the amount of (*R*)-3-HB present in both reactions (minimum 500 mM).

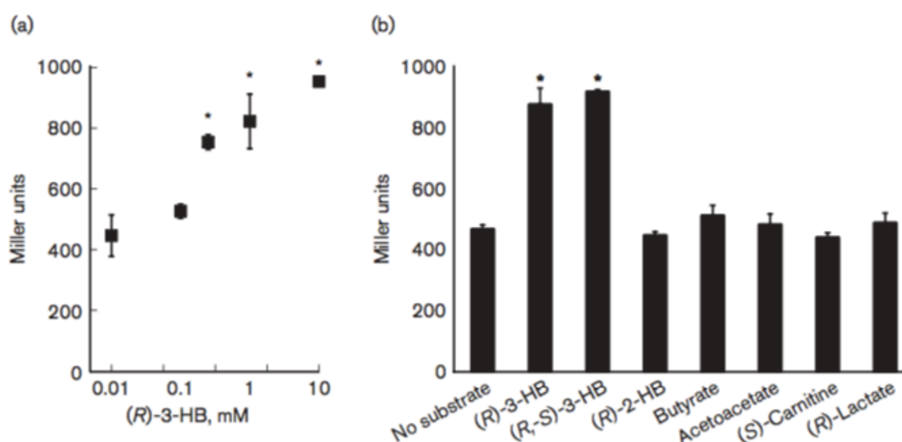

Figure 3.4 (*R*)-3-HB induced expression of a *PA2004-lacZ* reporter in *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 harboring a *PA2004-lacZ* reporter was grown to an OD_{600} of 0.3 in M63 minimal medium supplemented with 30 mM succinate and subsequently challenged with (a) various concentrations of (*R*)-3-HB or (b) various substrates provided at final concentrations of 1.0 mM. LacZ activity was measured 30 min post-addition of substrate. As shown, racemic and enantiopure (*R*)-3-HB were the only compounds that induced expression of the *PA2004-lacZ* reporter (twofold increase in LacZ activity). Notably, a concentration of 500 mM (*R*)-3-HB was found to be sufficient to induce expression of *PA2004-lacZ*. Data points represent mean values±SD (n=4). ANOVA was performed using a Dunnett's post-hoc test (∝-value of 0.05) to identify significant changes ($P < 0.0001$), which are marked with an asterisk. The equation used to determine Miller Units (MU) in this figure and all subsequent figures displaying *lacZ* assay data appearing in this chapter is as follows;

$$1\ \text{MU} = \frac{Abs420 - 1.75(Abs550)}{(t * v * Abs600)}$$

Where: Abs_{420} is the absorbance of the yellow *o*-nitrophenol, Abs_{550} is the scatter of cell debris, *t* is the reaction time in minutes, *v* is assay volume in milliliters, and Abs_{600} is the absorbance for the cell density (Miller 1992).

3.3.3 RpoN was Required for the Induction of *PA2004- lacZ* by (*R*)-3-HB

There is a putative RpoN promoter located 148bp upstream of the *PA2004* ORF (Conway & Boddy, 2012). This RpoN promoter, TGGCACGGTTATCGCA, has the highly conserved 'GG' and 'GC' (underlined) nucleotides positioned at the -24 and -12 elements, respectively (Barrios et al., 1999). Since one of the hallmark attributes of RpoN is its role in the assimilation of various organic compounds, RpoN was considered to regulate expression of the *PA2004-bdhA* operon in response to (*R*)-3-HB. In support of this hypothesis, expression levels of the *PA2004-lacZ* reporter were fourfold lower in an *rpoN* mutant compared with WT *P. aeruginosa* PAO1 challenged with 30 mM (*R,S*)-3-HB (Figure 3.5). In parallel, the conserved 'GG' nucleotides of the -24 element were changed to 'AA' in the RpoN promoter of the *PA2004-lacZ* reporter. This 'AA' substitution at the -24 element reduced the expression of *PA2004-lacZ* by more than fourfold (Figure 3.5). Collectively, RpoN and its cognate -24/-12 promoter were necessary for (*R*)-3-HB induction of *PA2004-lacZ*, increasing it from a background (non-induced) level of 200 MU to >800 MU.

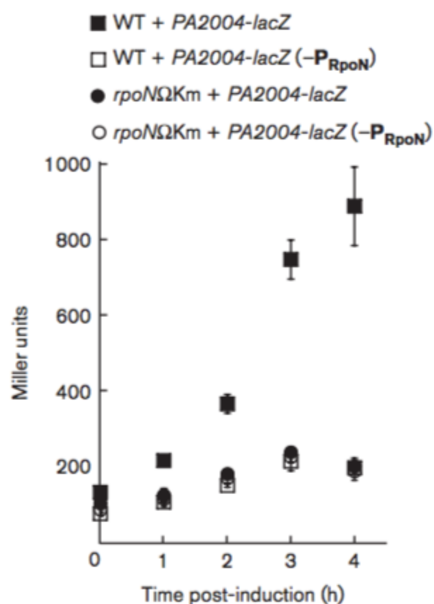

Figure 3.5 (*R*)-3-HB induction of *PA2004-lacZ* was RpoN dependent. There is a putative -24/-12 or RpoN promoter located 148 bp upstream of the *PA2004* ORF, suggesting that RpoN might be involved in the transcription of the *PA2004-bdhA* operon in response to (*R*)-3-HB. Addition of 30 mM (*R,S*)-3-HB did not induce expression of the *PA2004-lacZ* reporter in an *rpoN* mutant (*rpoN*ΩKm) of *P. aeruginosa* PAO1. Furthermore, substitution of the conserved 'GG' nucleotides of the -24 element with 'AA' in the RpoN promoter ($–P_{RpoN}$) of the *PA2004-lacZ* reporter made it unresponsive to 30 mM (*R,S*)-3- HB. Cells were grown in LB supplemented with 5 mM L-Gln to an OD_{600} of 0.3 and then challenged with 30 mM (*R,S*)-3-HB. LacZ activity was determined 1, 2, 3 and 4 h post-induction. L-Gln was provided to support the growth of the *rpoN* mutant (Heurlier et al., 2003). Data points represent mean values±SD (n=4). ANOVA was performed via a Dunnett's post-hoc test ($\propto$-value of 0.05) to identify significant changes ($P< 0.0001$).

3.3.4 Induction of the *PA2004-lacZ* Reporter was Dependent on *PA2005*

(*R*)-3-HB and RpoN were observed to be crucial determinants for the induction of the *PA2004-lacZ* reporter in *P. aeruginosa* PAO1. These results in combination with the previous finding that a *PA2005* : : Tn mutant had reduced growth on (*R*)-3-HB made PA2005 (HbrC) a prime candidate for being the EBP that participates with RpoN to activate transcription of the *PA2004-bdhA* operon in response to (*R*)-3-HB. Indeed, the addition of 30 mM (*R,S*)-3-HB did not induce expression of *PA2004-lacZ* in a *PA2005* : : Tn mutant (Figure 3.6). For *bdhA* : : Tn, *PA2004* : : Tn and WT *P. aeruginosa* PAO1, *PA2004-lacZ* expression increased more than threefold with the addition of 30 mM (*R,S*)-3-HB (Figure 3.6).

The LacZ findings were validated by assaying (*R*)-3-HB dehydrogenase activity for the *bdhA* : : Tn, *PA2004* : : Tn and *PA2005* : : Tn mutants. Cells were grown in LB to an OD_{600} of 0.3 and subsequently induced with either 0 or 30 mM (*R,S*)-3-HB. At 4 h post-induction, cells were harvested, washed, and lysed by sonication, and the resulting lysates were assayed for BdhA activity. For WT cells, BdhA activity increased from 93 (±5.9) to 395 (±17) U mg^{-1} with the addition of (*R,S*)-3-HB. There was no detectable BdhA activity present in the *bdhA* : : Tn mutant under either condition. The *PA2004* : : Tn mutants had a non-induced BdhA activity of 16 (±0.7) U mg^{-1}, which marginally increased to 23 (±2.4) U mg^{-1} with the addition of (*R,S*)-3-

HB. For the *PA2005* : : Tn mutant, non-induced and induced cells had BdhA activities of 23 (±0.9) and 19 (±2.3) U mg^{-1}, respectively. These values were ~20-fold lower than that of WT *P. aeruginosa* PAO1 challenged with (*R,S*)-3-HB. The measured BdhA activities are consistent with the LacZ data, indicating that PA2005 is necessary for the induction of BdhA expression in response to (*R*)-3-HB.

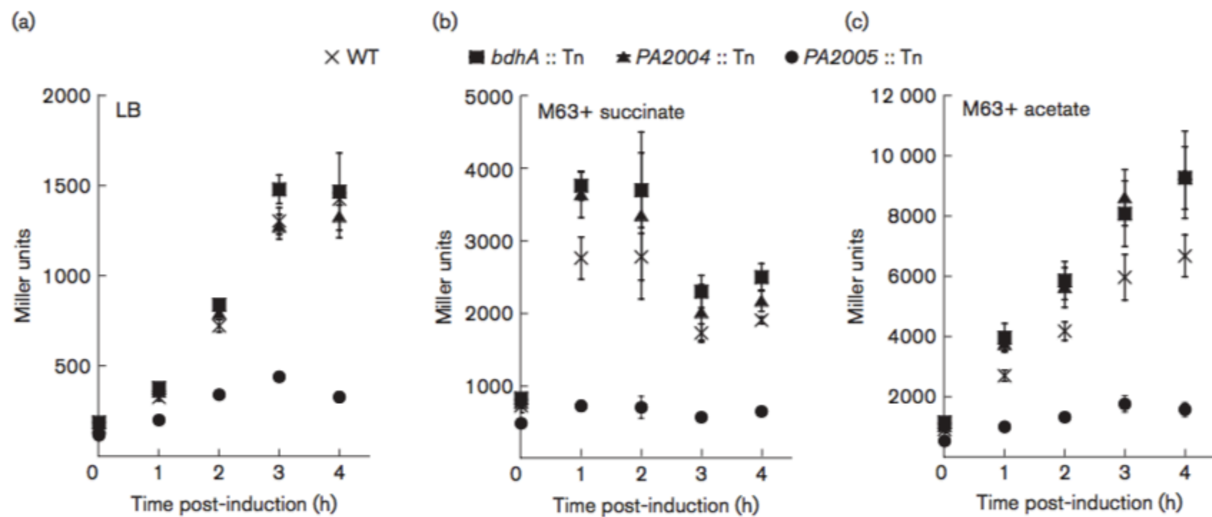

Figure 3.6 (*R*)-3-HB induction of *PA2004-lacZ* required the *PA2005* gene. *bdhA* : : Tn, *PA2004* : : Tn, *PA2005* : : Tn and WT *P. aeruginosa* PAO1 harboring the *PA2004-lacZ* reporter were grown in (a) LB or M63 minimal medium that was supplemented with either (b) 30 mM succinate or (c) 30 mM acetate. At an OD_{600} of 0.3, (*R,S*)-3-HB was added to a final concentration of 30 mM, and LacZ activity was determined 1, 2, 3 and 4 h post-induction. LacZ activity increased for *bdhA* : : Tn, *PA2004* : : Tn and WT cells when challenged with (*R,S*)-3-HB. In contrast, (*R,S*)-3-HB did not induce LacZ activity in the *PA2005* : : Tn mutant under any growth condition. Data points represent mean values±SD (n=4). ANOVA was performed using a Dunnett's post-hoc test (∝-value of 0.05) to identify significant changes (P< 0.0001).

3.3.5 (*R*)-3-HB Induced Expression of *PA2004-lacZ* in *E. coli* Top10 that Heterologously Expressed *PA2005 (hbrC)*

Expression of the *PA2004-lacZ* reporter was assayed in non-native *E. coli* Top10, which simultaneously expressed the *PA2005 (hbrC)* gene from pTrc99a. As shown in Figure 3.7, the basal expression of PA2005 (HbrC) from the *trc* promoter of pTrc99a caused a >60-fold induction of *PA2004-lacZ* in *E. coli* Top10 when challenged with either (*R*)-3-HB or (*R,S*)-3-HB.

LacZ activity increased from 50MU to >3000 MU at 2 h post-addition of 30 mM (*R*)-3-HB or (*R,S*)-3-HB. (*R*)-3-HB induction was not observed in the absence of *PA2005* or when the RpoN promoter in the *PA2004-lacZ* reporter was altered (Figure 3.7a). As observed earlier, the addition of (*R*)-2-HB or acetoacetate did not induce expression of *PA2004-lacZ* (Figure 3.7b). These findings demonstrate that, even in a non-native host, RpoN and PA2005 (HbrC) are sufficient and essential for the (R)-3-HB induction of the PA2004-lacZ reporter, and thus are key regulators of the *PA2004-bdhA* operon in *P. aeruginosa* PAO1.

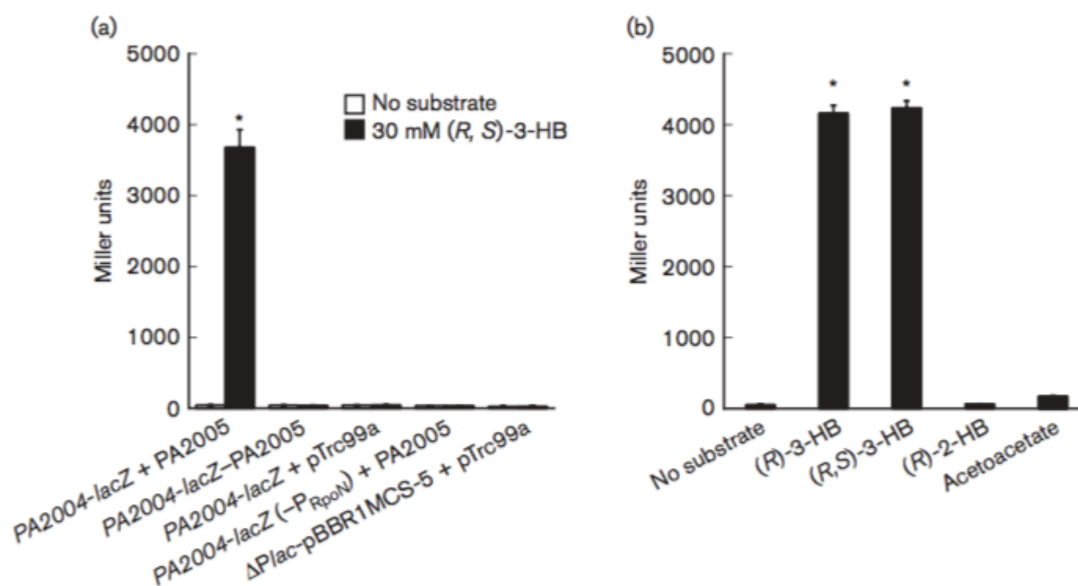

Figure 3.7 PA2005 was essential for (*R*)-3-HB induction of *PA2004-lacZ* in *E. coli.* The *lacZ*-deficient *E. coli* Top10 strain was co-transformed with the *PA2004-lacZ* reporter and the *PA2005* gene, which was carried on the pTrc99a expression plasmid. (a) Basal expression of the *PA2005* gene from the trc promoter of pTrc99a was sufficient for (*R*)-3-HB induction of *PA2004-lacZ* (*PA2004-lacZ* + *PA2005*). Induction was not observed for the *PA2004-lacZ* reporter when (i) the *PA2005* gene was cloned in a backwards orientation relative to the *trc* promoter in pTrc99a (*PA2004-lacZ – PA2005*), (ii) no *PA2005* gene was present, only pTrc99a plasmid (*PA2004-lacZ* + pTrc99a), or (iii) the 'GG' nucleotides of the -24 element of the RpoN promoter were substituted with 'AA' [*PA2004-lacZ* (–P_{RpoN}) + *PA2005*]. (b) Induction of *PA2004-lacZ* was specific for (*R*)-3-HB. In total, these results argue that *PA2005* is the EBP responsible for activating transcription from the RpoN promoter upstream of *PA2004-bdhA* in response to (*R*)-3-HB. Cells were grown in LB to an OD_{600} of 0.3 and then challenged with 30 mM of substrate. LacZ activity was measured 2 h post-induction. Data points represent mean values±SD (n=4). ANOVA was performed using a Dunnett's post-hoc test (∝-value of 0.05) to identify significant changes ($P < 0.0001$), which are marked with an asterisk.

3.3.6 Promoter Region of *PA2004* was Bound by Purified His_6-PA2005

In order to purify PA2005 protein, the gene was first introduced to an expression vector that yielded a fusion protein with six histidine residues added to the N-terminus of the translated PA2005 gene product. (pBRL516, Appendix A, Table 1). The His_6-tag allowed for affinity chromatography to be performed using an agarose resin with embedded nickel (Ni^{2+}) ions. Crude His_6-PA2005 chelated the nickel ions and when the mixture was applied to a column, non-chelated proteins were washed off. Bound His_6-PA2005 was then eluted using increasing concentrations of imidazole resulting in purified fractions of His_6-PA2005 containing little to no other contaminating proteins.

EMSA was used to determine if PA2005 actually binds to the *PA2004* promoter region. To this end, a 5'-labelled Cy5 probe, which spanned the region 237–122 bp upstream of the *PA2004* ORF, was used in an EMSA with purified His_6-PA2005. As shown in Figure 3.8a, purified His_6-PA2005 retarded the mobility of the *PA2004* promoter probe but did not change the mobility of a non-specific target promoter, i.e. the promoter region of *gcvH2*, which encodes a glycine cleavage protein (Lundgren et al., 2013). When increasing concentrations of His_6-PA2005 from 0 to 400 nM were incubated with the *PA2004* promoter probe, a mobility shift was observed for His_6-PA2005 concentrations as low as 12.5 nM and the shift intensity increased with the concentration of His_6-PA2005 (Figure 3.8b). These results indicate that PA2005 (HbrC) binds with high specificity and affinity to the *PA2004* promoter region.

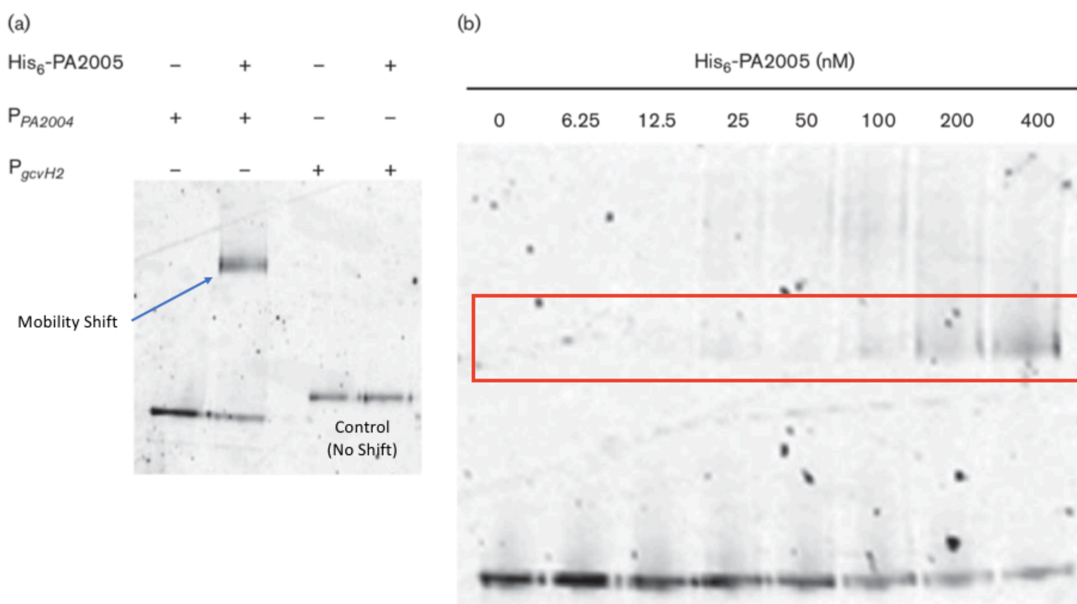

Figure 3.8 The promoter region of *PA2004* was bound by His_6-PA2005. EMSAs were performed with His_6-PA2005 and 1.0 nM 5'-labelled Cy5 probe DNA unless specified otherwise. (a) P_{PA2004} (specific) or P_{gcvH2} (non-specific) were incubated in the absence (lanes 1 and 3, respectively) or presence (lanes 2 and 4, respectively) of 500 nM PA2005. The shift in the position of P_{PA2004} in lane 2 (denoted by blue arrow) confirms that PA2005 binds to P_{PA2004}. No shift corresponding to binding of the non-specific probe P_{gcvH2} by PA2005 was observed in lane 4. (b) His_6-PA2005 (0 to 400 nM) was incubated with P_{PA2004}. A shift in P_{PA2004} was observed for His_6-PA2005 concentrations as low as 12.5 nM, indicating that His_6-PA2005 binds with high affinity to P_{PA2004}. The mobility shift bands (red box) indicate bound His_6-PA2005 protein to the P_{PA2004} DNA probe. The intensity of the shift increased with increasing His_6-PA2005 concentration.

3.3.7 Assimilation of (±)-1,3-butanediol Requires the *bdhA* Gene

(*R*)-3-HB utilization was dependent on the *bdhA* gene and its regulator PA2005 (HbrC). However, it was not known whether BdhA and/or PA2005 were necessarily essential for the assimilation of other compounds that are metabolized through (*R*)-3-HB intermediates. The compound (±)-1,3-butanediol is a common industrial chemical that is converted into (*R*)-3-HB by both eukaryotic and bacterial cells (Kersters & De Ley, 1963; Tate et al., 1971; Ugwu et al., 2011). Nothing is known about the metabolism of (±)-1,3-butanediol in *Pseudomonas* except for a study that identified this molecule as a substrate for a quinoprotein alcohol dehydrogenase of *P. putida* (Toyama et al., 1995). We found that *P. aeruginosa* PAO1 could grow on (±)-1,3-

butanediol when provided as a carbon source (Figure 3.9). This metabolism was dependent on *bdhA*, as the *bdhA* : : Tn mutant was unable to grow on (±)-1,3-butanediol (Figure 3.9a). Notably, this result reaffirms that (*R*)-3- HB is an intermediate of (±)-1,3-butanediol metabolism in *P. aeruginosa* PAO1. Disruptions of the *PA2004* and *PA2005* genes did not abolish growth of *P. aeruginosa* PAO1 on (±)-1,3-butanediol. The *PA2004* : : Tn (Figure 3.9b) and *PA2005* : : Tn (Figure 3.9c) mutants exhibited only reduced growth on this diol.

Expression of the *PA2004-lacZ* reporter remained unchanged (background levels) for *PA2004* : : Tn, *PA2005* : : Tn and WT *P. aeruginosa* PAO1 cells when grown on (±)-1,3-butanediol (Appendix A, Figure 1). In contrast, expression of *PA2004-lacZ* increased twofold for the *bdhA* : : Tn mutant in the presence of (±)-1,3-butanediol. Only in the absence of *bdhA* does intermediate (*R*)-3-HB reach concentrations that induce expression of the *PA2004-lacZ* reporter. It would appear that the catabolism of (±)-1,3- butanediol does not generate sufficient levels of (*R*)-3-HB to trigger the induction of the *PA2004-bdhA* operon via RpoN-PA2005.

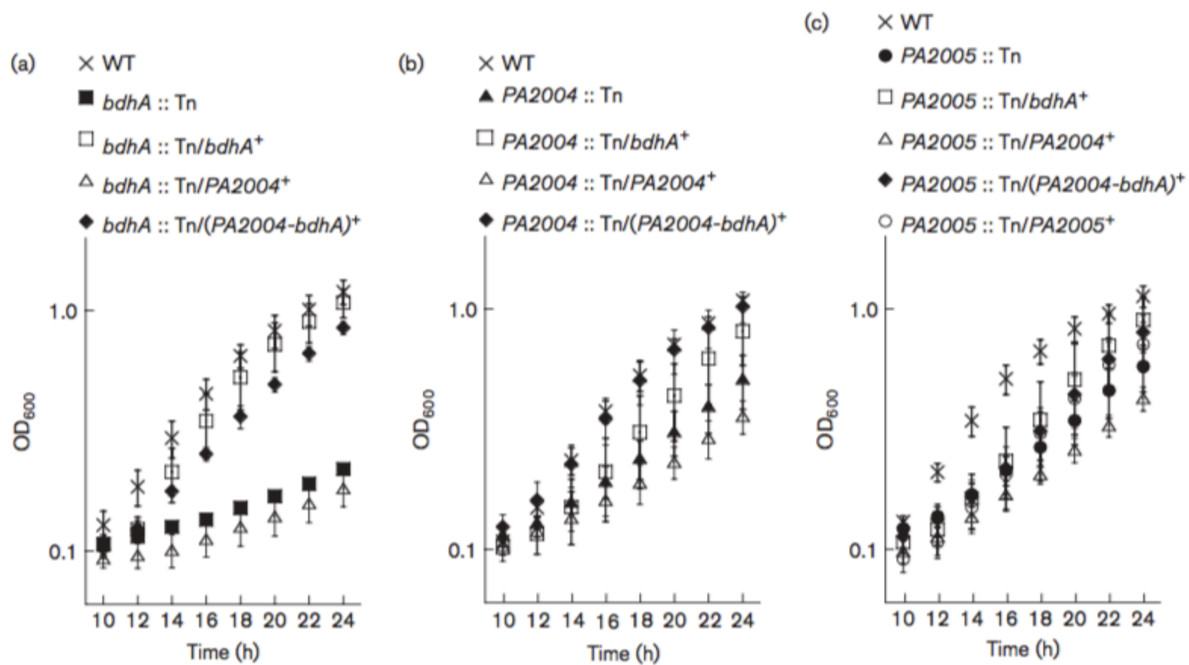

Figure 3.9 The *bdhA* gene was necessary for the growth of *P. aeruginosa* PAO1 on (±)-1,3-butanediol. (*R*)-3-HB is an intermediate of (±)-1,3-butanediol catabolism. (a) The *bdhA* : : Tn mutant failed to grow on (±)-1,3-butanediol as the sole carbon source. (b, c) The *PA2004* : : Tn (b) and *PA2005* : : Tn (c) mutants displayed reduced growth on (±)-1,3-butanediol, which suggested that the expression levels of the *bdhA* gene in these mutants were high enough to produce adequate amounts of (*R*)-3-HB dehydrogenase for converting intermediate (*R*)-3-HB into acetoacetate. Data points represent mean values±SD (n=4). ANOVA was performed using a Dunnett's post-hoc test (∝-value of 0.05) to identify significant changes (P< 0.0001).

3.4 Discussion

The *PA2004-bdhA* operon is conserved among many *Pseudomonas* spp., suggesting that it is a core unit and regulatory site in (*R*)-3-HB catabolism. Analysis of a *PA2004-lacZ* reporter and BdhA enzymatic activity identified several key factors involved in the expression of the *PA2004-bdhA* operon in *P. aeruginosa* PAO1. First, the expression of the *PA2004-bdhA* operon is inducible by (*R*)-3-HB (Figure 3.4). This induction or transcriptional activation was completely dependent on the alternative sigma factor RpoN and its cognate -24/-12 promoter positioned 148 bp upstream from the start codon of *PA2004*. (*R*)-3- HB did not induce expression of the *PA2004-lacZ* reporter in an *rpoN* mutant, while replacement of the conserved 'GG' motif of the -24 element with 'AA' in the RpoN promoter of the *PA2004-lacZ* reporter rendered it unresponsive to exogenous (*R*)-3-HB (Figure 3.5). Interestingly, the operons encoding the BdhA-PA2004 homologues in *P. putida* KT2440 (PP_3073-PP_3074) and *Pseudomonas fluorescens* SBW25 (PFLU2628-PFLU2629) are preceded by RpoN promoters at 65 and 72 bp upstream of PP_3074 and PFLU2629, respectively (Conway & Boddy, 2012). Additionally, adjacent to each of these operons is a gene (PP_3075, PFLU2630) encoding an EBP that is homologous to HbrC. EBPs are essential participants in RpoN-mediated transcription (Studholme & Buck, 2000), and therefore regulation of (*R*)-3-HB assimilation for some *Pseudomonas* spp. might be dependent on RpoN and an EBP homologous to HbrC.

The results of this study strongly suggest that the *hbrC* gene encodes the EBP that interacts with RpoN to activate transcription of the *PA2004-bdhA* operon in response to (*R*)-3-HB. It was observed that the addition of exogenous (*R*)-3-HB did not induce expression of the *PA2004-lacZ* reporter nor did it lead to an increase in BdhA activity in a *PA2005* : : Tn mutant compared with WT *P. aeruginosa* PAO1 (Figure 3.6). We also found that (*R*)-3-HB induced the expression of the *PA2004-lacZ* reporter in *E. coli*, but only if the *E. coli* cells were expressing the *hbrC* gene from the *trc* promoter of the plasmid pTrc99a (Figure 3.7). The presence of HbrC was essential for the (*R*)-3-HB induction of *PA2004-lacZ* in non-native *E. coli*. Lastly, purified HbrC protein did bind to a probe resembling the promoter region of *PA2004* (Figure 3.8), which supports HbrC being a direct regulator of the *PA2004-bdhA* operon.

The EBP HbrC is not a homologue of any previously characterized EBPs. Pfam analysis of HbrC identified an N-terminal PAS-4 domain (residues 25–116), the central RpoN-interaction domain (residues 158–326) and a C-terminal FIS-type helix–turn–helix (HTH) (residues 425–466). The PAS-4 domain most likely has a role in sensing intracellular (*R*)-3-HB, whereas the FIS-type HTH is expected to bind to a DNA motif upstream of the RpoN promoter of *PA2004-bdhA*. More in-depth EMSAs with HbrC are expected to identify the DNA-binding sites for this transcriptional regulator. Based on the results in the current study, HbrC was given the name HbcR for (*R*)-3- hydroxybutyrate catabolism regulator.

The assimilation of (*R*)-3-HB as a main carbon source was dependent on the induction of the *PA2004-bdhA* operon via RpoN-HbrC. In contrast, induction of the *PA2004- bdhA* operon was not observed when *P. aeruginosa* PAO1 was grown on (±)-1,3-butanediol even though (*R*)-3-HB is an intermediate in the breakdown of this diol (Appendix A, Figure 1). This finding indicates that non-induced levels of BdhA are sufficient to convert any available (*R*)-3-HB into

acetoacetate during the metabolism of (±)-1,3-butanediol. Importantly, non-induced levels of BdhA might support the growth of *P. aeruginosa* PAO1 on other molecules in which the formation of (*R*)-3-HB is a rate-limiting step in the catabolic process. For example, *Pseudomonas* spp. have been reported to hydrolyse PHB polyhydroxybutyrate (PHB) granules produced by other bacteria (Jendrossek et al., 1996), and subsequently use the liberated or free (*R*)-3-HB as a growth substrate. If the hydrolysis of the PHB granule is relatively slow then (*R*)-3-HB is not expected to accumulate to concentrations that lead to the induction of the *PA2004-bdhA* operon, and thus a similar situation to that described for (±)-1,3-butanediol metabolism will be observed. Whether *P. aeruginosa* PAO1 can degrade PHB granules remains to be determined, and what implications (*R*)-3-HB induction of *PA2004-bdhA* has for the assimilation of PHB granules in the environment is a subject worth exploring.

It is of interest to mention that (*R*)-3-HB is naturally produced in humans during starvation conditions as ketone bodies (Akram, 2013). Considering *P. aeruginosa* is a known pathogen of humans and can cause chronic, lethal respiratory infections, it is possible that activation of the *PA2004-bdhA* operon by HbrC occurs when an infected host has been unable to eat and high amounts of (*R*)-3-HB are being produced to meet energy demands. However, high sequence homology of both *bdhA* and *hbrC* was found in a variety of other *Pseudomonas* species including *P. chlororaphis* and *P. jinjuensis*, both of which naturally occur in soil. Overall, it is possible that activation of HbrC and catabolism of serum (*R)*-3-HB occurs in humans infected with *P. aeruginosa* but it is unlikely that this system evolved for this purpose and more likely has its origins in environmentally (soil) induced conditions.

3.5 Conclusion

Overall, this study was able to accurately determine the regulatory mechanisms required for the uptake and catabolism of (*R*)-3-hydroxybutyrate by *Pseudomonas aeruginosa* PAO1. The proposed regulatory mechanism begins with activation of the HbrC enhancer binding protein in the presence of (*R*)-3-HB. Formation of an HbrC hexamer occurs and then binds to the intergenic region between the *hbrC* and *PA2004* genes where ATP hydrolysis is coupled with transcriptional activation of an σ^{54}-RNA polymerase holoenzyme present at the -24/-12 promoter region of the *PA2004-bdhA* operon. Transcription and translation of the *PA2004* and *bdhA* genes occur, thus initiating the first step of this catabolic pathway in which translated BdhA converts (*R*)-3-HB to acetoacetate (Figure 3.10).

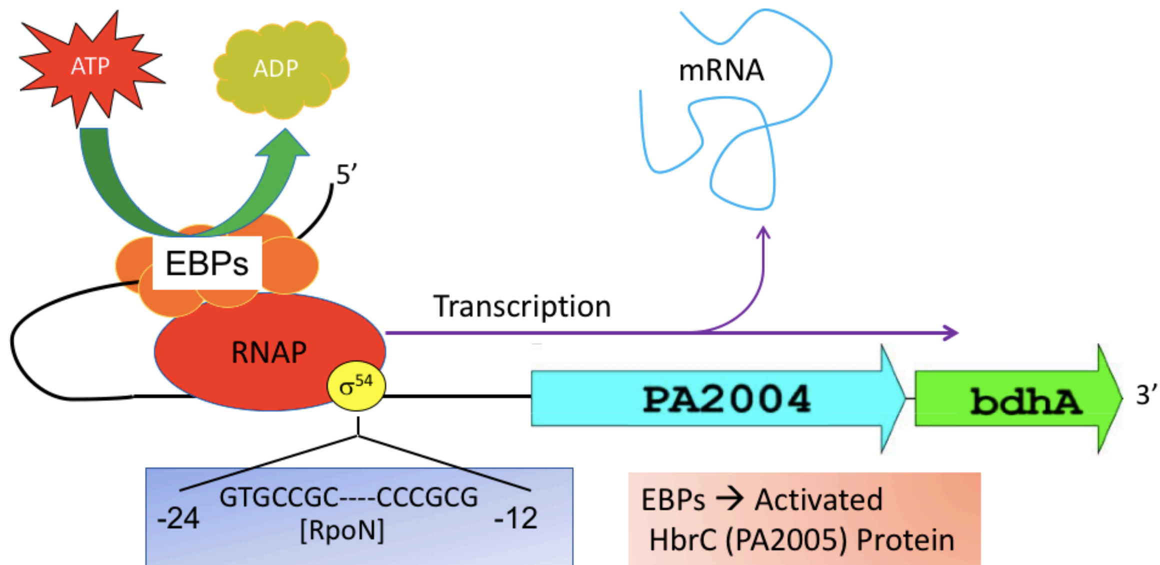

Figure 3.10 HbrC regulates the expression of the *PA2004-bdhA* gene operon. In the presence of (*R*)-3-HB, activated HbrC forms a hexamer and hydrolyses ATP to activate the σ^{54}-RNA polymerase holoenzyme and subsequent transcription of the *PA2004-bdhA* gene operon. The conserved RpoN binding sequence is denoted within the promoter region of *PA2004*. mRNA containing the transcribed *PA2004* and *bdhA* genes is then translated allowing for the subsequent catabolism of (*R*)-3-HB.

The overall implications of these findings are a greater understanding of how bacteria naturally present in the environment are capable of degrading and metabolizing the components of PHB. Interestingly, the potentially slow breakdown of PHB granules and resulting low-levels of available (*R*)-3-HB for uptake brings into question the necessity of a transcriptional regulator for high levels of BdhA expression. Although PHA depolymerase homologs have been identified in other *Pseudomonas* species, there has yet to be a specific study related to the ability of *P. aeruginosa* to catabolize PHB and other extracellularly present PHAs (de Eugenio et al., 2007). Future studies to determine the kinetic rates of PHA depolymerization by endogenously produced depolymerase enzymes may provide some insight into the evolutionary origins of the regulatory mechanisms described in this study.

Broadly, studies investigating the breakdown of PHA plastics is a vital aspect of the increased implementation of these materials into society and the environment. Although it has been observed that many PHA materials naturally degrade in the environment, more studies are needed to determine the rate at which various PHA polymers would be naturally metabolized as well as a kinetic analysis of the enzymes involved, especially those involved in initial depolymerization. Lastly, the genetic regulation of the expression of these catabolic genes is a pertinent area of study in order to fully understand the mechanisms of degradation.As the PHA industry continues to grow, this specific study can serve as one example of how to further explore the regulatory mechanisms used by bacteria to naturally assimilate biodegradable plastics.

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Chapter 4. Conclusions and Outlook

The primary objective of the work presented here was to exemplify and add to the body of research being conducted to further progress the PHA industry as a whole. Currently, a major issue preventing the further advancement of PHA technologies and implementation as petrochemical plastic substitutes is the cost of production (Yin et al., 2014). A number of the naturally PHA-producing, halophilic *Halomonas* species have been the focus of intense research and represent extremely viable candidates for the development of industrial level production strains for these polymers (Ying et al., 2015). A major asset of these bacteria in regard to industrial processing is their capacity to produce PHAs in batch fermenters using an open process in which non-sterile seawater is continuously pumped through the reaction (Tan et al., 2014). The need for fresh water, especially in water shortage areas, and the energy toll involved in water sterilization has been cited as a barrier in the further advancement of the PHA industry (Chen, 2012). Use of *Halomonas* species for generating PHAs circumvents this issue and has created a demand for recombinant 'super production' strains (Chen, 2009)

One major finding of this work was the establishment of the first described method for the transformation of a member of the *Halomonas* genus via electroporation using plasmid DNA. The method described in Chapter 2 is relatively quick to perform, reproducible, and efficient in generating positive transformants. Although the method of conjugation for creating recombinant strains of *Halomonas* has already been well-established, it can be time-consuming and tedious; characteristics that negate the general incentive to streamline the PHA production process (Li et al., 2016; Wang et al., 2015). Through the use of the described method in Chapter 2, it is now possible to rapidly introduce plasmids into *Halomonas* sp. O-1, and potentially other members of this genus, which can lead to the development of quicker and more reliable methods for creating

recombinant strains capable of producing high amounts of PHAs with a variety of carbon feedstocks.

As previously discussed, a major impetus for the development of industrial-level PHA-producing *Halomonas* strains is the potential ability to use these bacteria in an open and continuous batch fermentation process that utilizes non-sterile sea-water instead of sterilized freshwater. While this method has the potential to greatly reduce energy costs related to sterilizing freshwater as well as the barriers related to the increasing scarcity of freshwater, it should be noted that there are also drawbacks to industrial processes that involve salt-water. A major issue is related to the rapid corrosion of stainless steel by ocean water (Beccaria et al, 1995). Given that many pieces of equipment used in industrial processes for culturing bacteria are made of stainless steel, such as fermenters, it is possible that the use of seawater for media preparation my lead to increased costs in equipment maintenance and replacement. Overall, in-depth cost-benefit analyses of the economic benefits and drawbacks of using non-sterile ocean water in these fermentation processes will be the ultimate determinant of further developmental steps. Nonetheless, regardless of the use of unsterile saltwater or not, *Halomonas* species remain to be of great interest to the growing PHA industry and the study presented in Chapter 2 of this work represents one example of the steps necessary in order to make PHAs economically viable alternatives to bulk-commodity petroleum-based plastics.

Another goal of this work was to explore the fate of PHA plastics once they are no longer needed and are disposed of in the environment. One of the most desirable aspects of these materials is their ability to naturally degrade in the environment. This is due largely in part to the fact that PHAs are created by bacteria and over the course of evolution, many microorganisms

have adapted methods for capitalizing on these macromolecules as a source of energy (Numata et al., 2009). However, with the continued industrialization of PHA production, copolymers not present in nature that contain non-PHA components have begun to be developed (Salamanca-Cardona et al. 2017). This trend will likely continue in the direction of incorporating various additives into these materials to broaden their properties and applications A review of the sustainability of the bio-based plastics industry in 2012 found that many marketed biodegradable plastics still pose some environmental hazards, including potential toxicity by degradation products due to the chemical modifications performed during processing (Álvarez-Chávez et al., 2012). Therefore, as this industry grows, in order to maintain the benchmark of environmental sustainability it is vital that research is conducted to determine the fate and impact of each newly developed biodegradable plastic material.

For example, the marketed biodegradable polymer polylactic acid (PLA) has been shown to natural degrade and be totally assimilated by fungi and bacteria when placed in soil or compost (Madhavan Nampoothiri et al., 2010). This has made development of PLA plastic products an area of intense research and, in 2015, in terms of volume, PLA represented 45.1% of the entire biodegradable market with continued growth projected to increase (Rohan 2015). Although PLA represents a more environmentally-friendly alternative to petroleum-based plastics that do not naturally decompose, further studies of PLA materials has shown that this polymer does not experience significant biodegradation when placed in marine conditions (Greene, 2012). This finding implies that PLA materials do not satisfy many definitions of biodegradable when considering marine conditions. Furthermore, due to the slow rate of decomposition of PLA in the ocean, this material is actually believed to be contributing to the buildup of plastics in the oceans and the formation trash gyres as described in the introductory

chapter of this work. Considering this information, is has become increasing important to the study all the mechanisms involved in PHA biodegradation in order to prevent the creation of a material that is marketed as 'environmentally-friendly' but actually causes a detriment to natural systems.

To help address this issue, in Chapter 3 we investigated the regulatory mechanisms required for the catabolism of (*R*)-3-hydroxybutyrate [(*R*)-3HB] by *Pseudomonas aeruginosa* PAO1. (*R*)-3HB is the sole monomer of poly(hydroxybutyrate) [PHB], one of the most widely used PHA materials and a highly common component of PHA co-polymers (Zinn et al, 2001). Furthermore, *P. aeruginosa* is a ubiquitous soil bacterium and served as an adequate representative of the capabilities of naturally present microorganisms (Teitzel & Parsek, 2003). The result of this study proved that the *PA2005* gene product of PAO1 codes for a putative enhancer binding protein (named HbrC) involved in the transcriptional regulation of the genes involved in (*R*)-3HB catabolism. Additionally, we showed that high levels of expression of the enzyme (BdhA) responsible for the degradation of (*R*)-3HB to acetoacetate, a natural metabolite, is dependent on activation by HbrC. These results further support the claim that examinations of PHA biodegradation should focus on regulatory elements in addition to metabolic enzymes.

Understanding how PHA polymers biodegrade in terms of enzymology and regulatory processes is important for a number of additional reasons. PHA co-polymer composition and crystallinity can greatly influence the rate of environmental degradation (Numata et al., 2009). Studying the precise mechanisms of the degradation of various polymers will help to design and engineer materials that are perfectly suited for their intended use and desired shelf life and will further prevent negative environmental impacts by decreasing the time that PHA plastic litter persists in the environment, especially the oceans. Given that there have been isolates of

Pseudomonas aeruginosa found in marine environments, it is believed that degradation of PHB by this organism will not only occur in terrestrial conditions, but marine environments as well (Khan et al., 2007). Additionally, standards of biodegradation of PHA materials such as PHB and poly(3HB-*co*-3HV) were achieved when placed in marine conditions, thus mitigating and circumventing the issues posed by PLA waste in the oceans.

Another area of interest in terms of PHA degradation is related to the *in vivo* breakdown of PHAs designed for use in medical implants and other medical applications. Studies to determine the biocompatibility and rates of catabolism of PHA implants have enhanced relevance due to the increased use of these materials in food packaging (Siracusa et al., 2008). The *in vivo* compatibility of PHAs is a characteristic that also has environmental implications. A potential issue of concern regarding PHA build up in the environment is related to the potential consumption of PHA materials by marine and avian species prior to its biodegradation, as has been seen for many other plastic materials present in the ocean. However, given the chemical configuration of PHA materials, especially the ester bonds present in the backbone structure, it is believed that animals that consumed PHA waste would be able to digest this material. This belief is based on the presence of many serine hydrolase enzymes present in the gut and digestive tracts of animal species. There are many studies that show the same enzymes that are capable of breakdown fats and proteins during digestion are also highly capable of the catalyzing the scission of ester bonds such as those found in PHAs (Patricelli & Cravatt, 1999; Miled, 2000). Therefore, the possibility of PHA bioaccumulation causing detrimental effects to marine fauna is much less likely than other plastic-like materials, and the consumption and digestion of PHAs may even yield carbon and energy sources rather than cause toxic buildup. In summation, as the PHA industry continues to develop in its technological capacity it is of importance to mirror this

growth with in-depth studies to accurately determine how these materials will impact the environment and human health.

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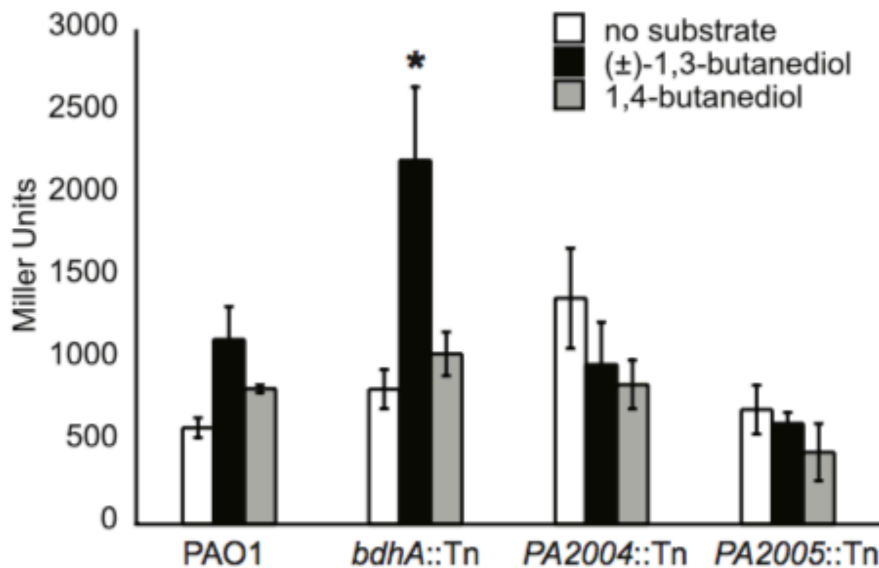
Appendix A; Chapter 3 Supplementary Tables and Figures

Table 1 - Bacteria, plasmids and oligonucleotides

Bacterium, plasmid or oligonucleotide	Relevant characteristics	Source
P. aeruginosa		
PAO1 M	WT	Jacobs *et al.* (2003)
PW4481	*PA2003*-H08 : : IS*phoA*/*hah* derivative of PAO1	Jacobs *et al.* (2003)
PW4483	*PA2004*-E10 : : IS*phoA*/*hah* derivative of PAO1	Jacobs *et a.* (2003)
PW4484	*PA2005*-D07 : : IS*phoA*/*hah* derivative of PAO1	Jacobs *et al.* (2003)
PAO6359	*rpoN* : : Ω-Km derivative of PAO1	Heurlier *et al.* (2003)
E. coli		
BL21(DE3)	*fhuA2* [*lon*] *ompT gal* (λ DE3) [*dcm*] Δ*hsdS*	EMD Millipore
Top10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL* (Str^R) *endA1* λ⁻	Invitrogen
Plasmid		
pCR-Blunt	Cloning plasmid; Km^r	Invitrogen
pJET1.2	Cloning plasmid; Cb^r	ThermoScientific
pTrc99a	Expression plasmid; Cb^r	Pharmacia
pET28b	Expression plasmid; Km^r	EMD Millipore
pBBR1MCS-5	Broad-host strain plasmid; Gm^r	Kovach *et al.* (1995)
Δ*Plac*-pBBR1MCS-5	pBBR1MCS-5 minus *lac* promoter; Gm^r	Lundgren *et al.* (2014)
pBRL491	*bdhA* gene in pCR-Blunt; Km^r	This study
pBRL492	*PA2004-lacZ* in pCR-Blunt; Km^r	This study
pBRL494	*PA2004-bdhA* genes in pCR-Blunt; Km^r	This study
pBRL496	*bdhA* gene in pBBR1MCS-5; Gm^r	This study
pBRL498	*PA2004-bdhA* genes in pBBR1MCS-5; Gm^r	This study
pBRL499	*PA2004-lacZ* in Δ*Plac*-pBBR1MCS-5; Gm^r	This study
pBRL500	*PA2004* gene in pCR-Blunt; Km^r	This study
pBRL501	*PA2004* gene in pBBR1MCS-5; Gm^r	This study
pBRL505	*PA2004-lacZ* with mutated RpoN promoter; Gm^r	This study
pBRL510	*PA2005* ORF in pCR-Blunt; Km^r	This study
pBRL512	P_{PA2004} probe in pJET1.2; Cb^r	This study
pBRL516	*PA2005* ORF in pET28b; Km^r	This study
pBRL595	*PA2005* in pTrc99a with forwards orientation; Cb^r	This study
pBRL596	*PA2005* in pTrc99a with backwards orientation; Cb^r	This study
pJRH10	*PA2005* in pBBR1MCS-5	This study
pZS406	P_{gcvH2} probe in pJET1.2; Cb^r	This study
Oligonucleotide		
BL342.f	atgaccatgattacggattcactg	*E. coli lacZ*
BL342.r	gcagttatttttgacaccagaccaactggta	*E. coli lacZ*
BL442.f	gctggaggccgaattcttc	5′ Regulatory region of *PA2004*
BL442.r	gaatccgtaatcatggtcatcgggcatatctccacg	5′ Regulatory region of *PA2004*
BL443.f	gcatctagagccttccccatgcgaagc	*PA2004* gene
BL443.r	gcagagctctcagaccaggccggtggc	*PA2004* gene
BL444.f	gcatctagacatccagcgggagaaacagatg	*bdhA* gene
BL444.r	gcagagctcctactgcgccacccagcc	*bdhA* gene
BL445.f	cgcgattctggggcctaacacggttatcgcaacg	Mutation of RpoN promoter in 5′ regulatory region of PA2004
BL445.r	cgttgcgataaccgtgttaggccccagaatcgcg	Mutation of RpoN promoter in 5′ regulatory region of *PA2004*
BL446.f	gccatatgaacgacgccgacagcc	*PA2005* ORF
BL446.r	gcgagctctcagttcgtctctatttcgagac	*PA2005* ORF
BL447.f	gctcgattacgtctccattttg	P_{PA2004} probe
BL447.r	gaaaggcacgttgcgataacc	P_{PA2004} probe
JRH05.f	ggctcgagtttttcagcaagat	5′-Labelled Cy5 pJET1.2 primer
JRH05.r	gaatattgtaggagatcttctagaaag	5′-Labelled Cy5 pJET1.2 primer
ZS406.f	ggggctgtgccatcggctgtaacg	P_{gcvH2} probe
ZS406.r	gcggccccggccctgtcgccacgg	P_{gcvH2} probe



Appendix A, Figure 1. Growth on (±)-1,3-butanediol did not induce expression of *PA2004-lacZ***.** Expression of the *PA2004-lacZ* reporter remained at non-induced levels when *PA2004*::Tn, *PA2005*::Tn and wild-type cells were grown in LB supplemented with 30 mM (±)-1,3- butanediol. For *bdhA*::Tn, a 2-fold increase in LacZ activity was observed in the presence of 30 mM (±)-1,3-butanediol. Only in the absence of *bdhA* does intermediate (*R*)-3-HB reach concentrations that induce expression of the *PA2004-lacZ* reporter. [Cells were grown in LB supplemented with 30 mM (±)-1,3-butanediol or 1,4-butanediol, and LacZ activity was measured at 16 h post-inoculation. Data points represent mean values ± SD (n = 4). ANOVA was performed using a Dunnett's post-hoc test (α-value of 0.05) to identify significant changes ($p < 0.0001$), which are marked with an asterisk.]

Appendix B; Peer-review Publication Covers

Journal of Microbiological Methods 129 (2016) 127–132

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A rapid and efficient electroporation method for transformation of *Halomonas* sp. O-1

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ARTICLE INFO

Article history:
Received 14 July 2016
Received in revised form 15 August 2016
Accepted 15 August 2016
Available online 16 August 2016

Keywords:
Halomonas
Competent cells
Transformation
Electroporation

ABSTRACT

Halomonas sp. O-1 is a halophilic bacterium with a high potential for industrial application due to its natural ability to produce polyhydroxyalkanoates (PHAs) using seawater-based media. However, a major barrier preventing industrial scale implementation of this organism is a lack of molecular methodologies capable of readily transforming members of the *Halomonas* genus. Currently, the only reliable method used for introducing DNA into *Halomonas* spp. is bacterial conjugation, a somewhat tedious and time-consuming technique compared to electroporation-based methodologies. Here we describe a rapid and reproducible method for the electroporation of *Halomonas* sp. O-1 with plasmid DNA. Electrocompetent cells were generated by growing *Halomonas* sp. O-1 in a yeast extract-tryptone medium with a final salinity of 3.5%, pH of 7.5, followed by several washes using 300 mM sucrose. Results show that plasmids containing chloramphenicol (Cm^R) and gentamicin (Gm^R) resistance cassettes are suitable antibiotic selection markers for transformation and yields of 10^4 transformants per μg of DNA were obtained. This method is simple to perform and the materials used are readily available in most research labs. Additionally, this plasmid-based transformation procedure has the potential to be adapted for a number of applications including the creation of recombinant stains and the generation of deletion mutants of *Halomonas* spp.

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1. Introduction

Halophiles are a class of extremophiles characterized by their ability to thrive in high salt environments that are typically toxic to most other forms of life (Oren, 2008). There has been increasing interest in the use of these microorganisms for various biotechnology and industrial applications due to the overall enzymatic stability required for survival in such harsh environments and a decreased risk of contamination during processing as a result of these conditions (Aguilar, 1996). The genus of Gram-negative halophilic bacteria known as *Halomonas* was first established in 1980 with the discovery and morphological characterization of *Halomonas elongata* (Vreeland et al., 1980). Since then, isolates of this genus have been identified in a myriad of habitats including coastal sea waters (Ilham et al., 2014), salt-mines (Cardinali-Rezende et al., 2016), saline lakes (Zhang et al., 2016), salt-secreting leaves (Finkel et al., 2011), industrial brine (Carlson et al., 2016), heavy metal polluted shores (Jain et al., 2016), rust found on the sunken RMS *Titanic* (Sánchez-Porro et al., 2010), and even as part of the human skin microbiome (Cosseau et al., 2016). New members of the *Halomonas* genus are being identified at an extraordinary rate and the habitats in which they thrive are equally astounding.

Polyhydroxyalkanoates (PHAs) are a class of diverse polyesters that are naturally produced by a variety of microorganisms to serve as intracellular carbon storage molecules (Anderson and Dawes, 1990). PHAs have attracted great interest as biobased and biodegradable replacements for petroleum-based plastics (Lu et al., 2009) and as biomaterials for biomedical applications (Levine et al., 2015). In addition to the inherent environmentally friendly properties of PHA-based polymers, studies have also shown that bacteria can be engineered to make these materials from a variety of renewable resources and waste products such as woody biomass and biodiesel glycerol, thus enhancing overall sustainability (Pan et al., 2012; Salamanca-Cardona et al., 2014; Zhu et al., 2010). However, major barriers that exist for wide-scale industrial application and integration of these processes include the large amount of freshwater needed to sustain PHA production and the associated energy costs to sterilize medium for bacterial growth (Chen, 2012).

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http://dx.doi.org/10.1016/j.mimet.2016.08.009
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Microbiology (2015), **161**, 2232–2242 **DOI** 10.1099/mic.0.000163

The metabolism of (*R*)-3-hydroxybutyrate is regulated by the enhancer-binding protein PA2005 and the alternative sigma factor RpoN in *Pseudomonas aeruginosa* PAO1

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A variety of soil-dwelling bacteria produce polyhydroxybutyrate (PHB), which serves as a source of energy and carbon under nutrient deprivation. Bacteria belonging to the genus *Pseudomonas* do not generally produce PHB but are capable of using the PHB degradation product (*R*)-3-hydroxybutyrate [(*R*)-3-HB] as a growth substrate. Essential to this utilization is the NAD^+-dependent dehydrogenase BdhA that converts (*R*)-3-HB into acetoacetate, a molecule that readily enters central metabolism. Apart from the numerous studies that had focused on the biochemical characterization of BdhA, there was nothing known about the assimilation of (*R*)-3-HB in *Pseudomonas*, including the genetic regulation of *bdhA* expression. This study aimed to define the regulatory factors that govern or dictate the expression of the *bdhA* gene and (*R*)-3-HB assimilation in *Pseudomonas aeruginosa* PAO1. Importantly, expression of the *bdhA* gene was found to be specifically induced by (*R*)-3-HB in a manner dependent on the alternative sigma factor RpoN and the enhancer-binding protein PA2005.This mode of regulation was essential for the utilization of (*R*)-3-HB as a sole source of energy and carbon. However, non-induced levels of *bdhA* expression were sufficient for *P. aeruginosa* PAO1 to grow on (±)-1,3-butanediol, which is catabolized through an (*R*)-3-HB intermediate. Because this is, we believe, the first report of an enhancer-binding protein that responds to (*R*)-3-HB, PA2005 was named HbcR for (*R*)-3-*h*ydroxy*b*utyrate *c*atabolism *r*egulator.

Received 28 May 2015
Accepted 21 August 2015

INTRODUCTION

Bacteria of the genus *Pseudomonas* are renowned for their versatile metabolism in that they can assimilate and break down a wide assortment of compounds to meet their nutritional demands. One compound that is not often affiliated with *Pseudomonas* metabolism is (*R*)-3-hydroxybutyrate [(*R*)-3-HB]. (*R*)-3-HB is commonly recognized for its role as a ketone body produced by mammalian cells when carbohydrate availability is limiting (Akram, 2013). However, there are a number of bacteria that biosynthesize CoA derivatives of (*R*)-3-HB and other (*R*)-3-hydroxy acids, which are polymerized into macromolecular structures called polyhydroxyalkanoates (PHAs) (Anderson & Dawes, 1990; Lu *et al.*, 2009). Under starvation conditions, the PHA granule is degraded into its 3-hydroxy acid components, which can be used as sources of carbon and energy (Jendrossek & Handrick, 2002; Jendrossek *et al.*, 1996).

Pseudomonas species do not biosynthesize nor incorporate (*R*)-3-HB into their PHA reserves (Huisman *et al.*, 1989; Timm & Steinbüchel, 1990). Nonetheless, these bacteria possess an NAD^+-dependent dehydrogenase (BdhA) that converts (*R*)-3-HB into acetoacetate, thereby allowing these bacteria to use (*R*)-3-HB as a growth substrate (Feller *et al.*, 2006; Ito *et al.*, 2006; Mountassif *et al.*, 2010). BdhA dehydrogenases have been biochemically characterized for some species of *Pseudomonas*, including *P. putida* (Feller *et al.*, 2006; Paithankar *et al.*, 2007), *P. fragi* (Ito *et al.*, 2006; Nakajima *et al.*, 2005) and

Abbreviations: EMSA, electrophoretic mobility shift assay; EBP, enhancer-binding protein; PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate; (*R*)-2-HB, (*R*)-2-hydroxybutyrate; (*R*)-3-HB, (*R*)-3-hydroxybutyrate.

A supplementary figure is available with the online Supplementary Material.

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Appendix C; Additional Publication Contributed to by this Author

Genetic Analysis of the Assimilation of C_5-Dicarboxylic Acids in *Pseudomonas aeruginosa* PAO1

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There is a wealth of information on the genetic regulation and biochemical properties of bacterial C_4-dicarboxylate transport systems. In sharp contrast, there are far fewer studies describing the transport and assimilation of C_5-dicarboxylates among bacteria. In an effort to better our understanding on this subject, we identified the structural and regulatory genes necessary for the utilization of α-ketoglutarate (α-KG) in *Pseudomonas aeruginosa* PAO1. The PA5530 gene, encoding a putative dicarboxylate transporter, was found to be essential for the growth of *P. aeruginosa* PAO1 on both α-KG and glutarate (another C_5-dicarboxylate). Metabolite analysis confirmed that the PA5530 gene was necessary for the uptake of extracellular α-KG. Like other substrate-inducible transporter genes, expression of the PA5530 gene was induced by extracellular C_5-dicarboxylates. It was later found that the expression of the PA5530 gene was driven solely by a −24/−12 promoter recognized by the alternative sigma factor RpoN. Surprisingly, the enhancer binding protein MifR, which is known to have an essential role in biofilm development, was required for the expression of the PA5530 gene. The MifR protein is homologous to other transcriptional regulators involved in dicarboxylate assimilation, suggesting that MifR might interact with RpoN to activate the expression of the PA5530 gene in response to extracellular C_5-dicarboxylates, especially α-KG. The results of this study provide a framework for exploring the assimilation of α-KG in other pseudomonads.

The C_5-dicarboxylate α-ketoglutarate (α-KG) is widely recognized for being an intermediate of both carbon and nitrogen metabolism. The other C_5-dicarboxylate commonly encountered is glutarate, which is an intermediate of lysine and tryptophan metabolism. It has become increasingly apparent that α-KG plays a larger and more profound role in bacterial physiology than originally believed. For example, it is now known that α-KG directly regulates nitrogen metabolism via binding to and controlling the activity of the nitrogen regulatory protein PII (1–3). There is also compelling evidence that endogenous α-KG is a natural protectant against both oxidative damage (4, 5) and cyanide poisoning (6). α-KG-dependent hydroxylases are ubiquitous among bacteria and participate in various cellular functions (7), including the assimilation of key nutrients, e.g., sulfur and phosphate, and the biosynthesis of complex molecules such as antibiotics and lipid A. Interestingly, there have been a few recently published papers reporting that α-KG might be a preferred nutrient for some bacteria during the course of an infectious state (8–10). α-KG is a suitable growth substrate for most bacteria, but the genes involved in the transport and assimilation of α-KG have been investigated for only a small number of bacterial species, including *Escherichia coli* (10–12), *Staphylococcus aureus* (13), *Bacillus licheniformis* (14), *Lactococcus lactis* (15), *Xanthomonas oryzae* (9), and *Rhizobium tropici* (8). Missing from the literature are studies describing the genetic regulation of α-KG assimilation in *Pseudomonas*, which is surprising, because these bacteria are well known for their versatile metabolism, enabling them to consume a wide range of substrates.

The assimilation of α-KG has been documented in previous studies involving *Pseudomonas* (16–20). Campbell and Stokes observed that acetate-grown *P. aeruginosa* displayed a significant lag time when transferred into α-KG medium (19), hinting at the probable existence of an inducible α-KG transporter. *Pseudomonas* cultures grown under conditions of nitrogen limitation are well known to accumulate extracellular α-KG, which is readily depleted with the addition of exogenous ammonium. Probably the most complete study on α-KG transport in *Pseudomonas* was that by Edwards et al., which biochemically characterized C_5-dicarboxylate transport in *P. putida* (21). Those authors observed that transport of α-KG in *P. putida* was induced by extracellular C_5-dicarboxylates such as α-KG and glutarate (21). Despite these observations, the genes encoding the transport and regulatory proteins involved in α-KG uptake had not been identified for any given *Pseudomonas* species.

Because of the limited information available on α-KG assimilation in *Pseudomonas*, we decided to identify the genetic network surrounding α-KG transport in *Pseudomonas aeruginosa* PAO1. An initial survey of the sequenced genome of *P. aeruginosa* PAO1 revealed that the predicted PA5530 protein is homologous to the well-characterized α-KG transporter, KgtP, of *E. coli* K-12 (11). Subsequent analysis confirmed that the PA5530 gene was required for the uptake of extracellular α-KG for *P. aeruginosa* PAO1. Expression of the PA5530 gene was induced by extracellular C_5-dicarboxylates, and this expression was dependent on the alternative sigma factor σ^{54} or RpoN. Unexpectedly, the expression of the PA5530 gene also required the transcriptional regulator MifR, which was found previously to be involved in biofilm formation (22). MifR shares significant homology (>60%) with other regu-

Received 24 February 2014 **Accepted** 25 April 2014
Published ahead of print 2 May 2014
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doi:10.1128/JB.01615-14

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TABLE 1 Strains, plasmids, and oligonucleotides used in the current study

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence	Reference or source
Strains		
Pseudomonas aeruginosa		
PAO1	Wild type	23
PW9687	PA5166-F04::ISphoA/hah derivative of PAO1	24
PW10371	PA5530-C01::ISphoA/hah derivative of PAO1	24
PAO6359	*rpoN*::Ω-Km derivative of PAO1	25
Δ*mifR* PAO1	Δ*mifR* derivative of PAO1	This study
Δ*mifR* PW9687	Δ*mifR* derivative of PW9687	This study
PA14	Wild type	26
ΔPA5530 PA14	ΔPA5530 derivative of PA14	This study
PAK	Wild type	27
ΔPA5530 PAK	ΔPA5530 derivative of PAK	This study
Escherichia coli Top10	F⁻ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)*7697 galE15 galK16 rpsL* (Str^r^) *endA1* λ⁻	Invitrogen
Plasmids		
pCR-Blunt	Cloning plasmid; Km^r^	Invitrogen
pTrc99a	Expression plasmid; Cb^r^	Pharmacia
pBBR1MCS-5	Broad-host-range plasmid; Gm^r^	28
pEX18ApGW	Plasmid for gene deletions in *P. aeruingosa*; Cb^r^ Gm^r^	29
Δ*Plac*-pBBR1MCS-5	pBBR1MCS-5 minus *lac* promoter; Gm^r^	This study
pBRL474	PA5530 gene in pCR-Blunt; Km^r^	This study
pBRL479	PA5530 gene in pBBR1MCS-5; Gm^r^	This study
pBRL476	PA5530-*lacZ* in pCR-Blunt; Km^r^	This study
pBRL485	PA5530-*lacZ* in Δ*Plac*-pBBR1MCS-5; Gm^r^	This study
pBRL487	PA5530-*lacZ* with mutated RpoN promoter; Gm^r^	This study
pBRL511	*mifR*::Gm^r^ in pEX18ApGW; Cb^r^ Gm^r^	This study
pBRL538	PA5530::Gm^r^ in pEXT18ApGW; Cb^r^ Gm^r^	This study
pBRL549	*mifSR* operon in pCR-Blunt; Km^r^	This study
pBRL561	*mifS*-Δ*mifR* operon in pCR-Blunt; Km^r^	This study
pBRL566	*mifSR* operon (forward orientation) in pTrc99a; Cb^r^	This study
pBRL567	*mifSR* operon (backward orientation) in pTrc99a; Cb^r^	This study
pBRL570	*mifS*-Δ*mifR* operon (forward orientation) in pTrc99a;Cb^r^	This study
pJRH07	*mifR* gene in pCR-Blunt; Km^r^	This study
pJRH08	*mifR* gene in pBBR1MCS-5; Gm^r^	This study
Oligonucleotides		
BL342.f	ATGACCATGATTACGGATTCACTG	
BL342.r	GCAGTTATTTTTGACACCAGACCAACTGGTA	
BL434.f	GCATCTAGACTGATCAGCACATCCAAGACAAC	
BL434.r	GCAGAGCTCTCAATCGGTCGTGATCTTCGA	
BL435.f	GCAGATCGGCGAGTTCTCC	
BL435.r	GAATCCGTAATCATGGTCATCGTGTTTCCTCTTTTCGTTGTTG	
BL438.f	GCTTTCCCGCGTTAACACGGCACCTGCTATC	
BL438.r	GATAGCAGGTGCCGTGTTAACGCGGGAAAGC	
BL466.f	TACAAAAAAGCAGGCTGACCAGGTGATCTTCGTCGAC	
BL466.r	TCAGAGCGCTTTTGAAGCTAATTCGGAAGGGTTTCTCGATGAAGTCG	
BL467.f	AGGAACTTCAAGATCCCCAATTCGCGAACATTTCGCCAGCGTTG	
BL467.r	TACAAGAAAGCTGGGTCTCTTCGCCGACGAAATCGC	
BL552.f	TACAAAAAAGCAGGCTGAAAGCGCCAACGCCATTTC	
BL552.r	TCAGAGCGCTTTTGAAGCTAATTCGGATGATCAGCGAGCCAAAGC	
BL553.f	AGGAACTTCAAGATCCCCAATTCGCTTCTACACCTACACCACCTAC	
BL553.r	TACAAGAAAGCTGGGTCAATCGGTCGTGATCTTCGAG	
BL554.f	GCATCTAGACGTTTCGATCCGCCGATGTC	
BL554.r	GCAGAGCTCAGTTGGCGAAGGATCTCTGAC	
JRH04.f	GACTCTAGATAAGAAGGAGATATACCATGAGCGACCAGGTGATCTTC	
JRH04.r	GACCTCGAGAGTTGGCGAAGGATCTCTGAC	

lators involved in α-KG utilization, including KgtR of *R. tropici* CIAT899 (8) and KguR of *E. coli* CTF073 (10). The results presented here indicate that MifR is a homolog of the KgtR/KguR proteins and participates with RpoN to activate the expression of the putative α-KG transporter (PA5530) in response to extracellular α-KG.

MATERIALS AND METHODS

Bacteria, plasmids, and media. Bacterial strains and plasmids used in the current study are given in Table 1. Bacteria were grown in Lennox broth (BD Difco), nutrient broth no. 2 (Oxoid), or modified M9 minimal medium (30), containing 5 μM $FeSO_4$. Solid bacteriological medium was prepared with the addition of BD Bacto agar at 15 g liter^{-1}. The following

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antibiotics were used for plasmid selection: kanamycin (Km) (50 μg ml^{-1} for *E. coli*), carbenicillin (Cb) (100 μg ml^{-1} for *E. coli* or 200 μg ml^{-1} for *P. aeruginosa*), and gentamicin (Gm) (10 μg ml^{-1} for *E. coli* or 30 μg ml^{-1} for *P. aeruginosa*).

Molecular biology methods. DNA was purified by using Promega nucleic acid purification kits. Restriction enzymes and ligases were products of New England BioLabs. PrimeStar polymerase (TaKaRa Biosciences) was used for all PCRs, which were done according to the recommended protocols for PrimeStar polymerase. Oligonucleotides used for PCR are provided in Table 1. Genomic DNA from *P. aeruginosa* PAO1 was used for all PCR applications. PCR products were cloned into pCR-Blunt by using the Zero Blunt PCR cloning kit (Invitrogen). Cloned DNA was verified by sequencing (Genewiz).

Construction of Δ*Plac*-pBBR1MCS-5. A promoterless plasmid suitable for the housing and analysis of reporter genes in *P. aeruginosa* was constructed. The *lac* promoter positioned upstream of the multiple-cloning site of plasmid pBBR1MCS-5 was removed via SphI-KpnI double digestion. The SphI-KpnI-digested pBBR1MCS-5 fragment was blunted by using *Pfu* DNA polymerase (Agilent Technologies) and subsequently religated to yield Δ*Plac*-pBBR1MCS-5.

Cloning of the PA5530, *mifR* (PA5511), and *mifSR* (PA5512-PA5511) genes. The PA5530, *mifR*, and *mifSR* genes were PCR amplified with primer pairs BL434.f/BL434.r, JRH04.f/JRH04.r, and BL554.f/BL554.r, respectively. The PA5530, *mifR*, and *mifSR* PCR products were cloned into pCR-Blunt to generate plasmids pBRL474, pJRH07, and pBRL549, respectively. The PA5530 and *mifR* genes were individually subcloned into the XbaI/SacI sites of pBBR1MCS-5 to yield pBRL479 and pJRH08, respectively.

The XbaI/SpeI *mifSR* fragment from pBRL549 was cloned into the XbaI site of pTrc99a with either a forward or backward orientation to give plasmid pBRL566 or pBRL567, respectively. Plasmid pBRL549 was digested with SalI to excise an internal portion (nucleotides 27 to 830) of the *mifR* gene, thereby yielding plasmid pBRL561. The resulting XbaI/SpeI *mifS*-Δ*mifR* fragment was cloned from pBRL561 into the XbaI site of pTrc99a with a forward orientation, generating pBRL570.

Cloning of the PA5530-*lacZ* fusion. The 1,069-bp 5′ region upstream of the predicted PA5530 open reading frame (ORF) was PCR amplified with primer pair BL435.f/BL435.r, and the β-galactosidase (*lacZ*) ORF of *E. coli* K-12 MG1655 was PCR amplified with primer pair BL342.f/BL342.r. The PA5530 promoter and *lacZ* PCR products were assembled into a single fragment via fusion PCR (31), using primer pair BL435.f/BL342.r. The PA5530-*lacZ* fusion was cloned into pCR-Blunt to give pBRL476. The XbaI/SpeI PA5530-*lacZ* fragment of pBRL482 was subcloned into the XbaI site of Δ*Plac*-pBBR1MCS-5 to yield pBRL485. The "GG" dinucleotide positioned 105 bp upstream of the *lacZ* ORF in the PA5530-*lacZ* reporter (in pBRL485) was changed to "AA" by using the QuikChange kit (Agilent Technologies) and primer pair BL438.f/BL438.r. The resulting plasmid, pBRL488, was sequenced to verify that the desired substitution was present.

Deletion of the PA5530 and *mifR* (PA5511) genes in *P. aeruginosa*. The PA5530 and *mifR* genes were deleted in *P. aeruginosa* according to the method of Choi and Schweizer (29). Procedures for plasmid construction, electroporation, screening, selection, and marker removal were conducted exactly as described previously (29). Briefly, the PA5530::Gm and *mifR*::Gm cassettes were cloned into plasmid pEX18ApGW to give pBRL511 and pBRL538, respectively. The PA5530 gene was deleted in *P. aeruginosa* PA14 and PAK by using pBRL511, whereas the *mifR* gene was deleted in *P. aeruginosa* PAO1 and PW9687 by using pBRL538. The Gm markers were removed by using plasmid pFLP2. The ΔPA5530 and Δ*mifR* mutations were verified by PCR.

Growth experiments on C_5-dicarboxylates. All *P. aeruginosa* strains were grown in triplicate. For each replicate, 50 ml of M9 minimal medium supplemented with 5 μM $FeSO_4$ and 20 mM C_5-dicarboxylate (α-KG or glutarate) (in a 500-ml baffle shake flask) was inoculated to an initial optical density at 600 nm (OD_{600}) of ~0.1. Cultures were grown at 37°C at 200 rpm, and the absorbance at 600 nm (OD_{600}) was measured at 0, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, and 24 h postinoculation. Note that M9 minimal medium did not have to be supplemented with gentamicin to select for recombinant *P. aeruginosa* strains harboring plasmid pBRL479 or pJRH08; the presence of C_5-dicarboxylate as a sole carbon source was sufficient for selection.

Measurement of extracellular α-KG from *P. aeruginosa* cultures. Experiments were done in triplicate. *P. aeruginosa* PAO1 and the PA5530 transposon mutant (strain PW10317) were grown in 2.0 ml of nutrient broth no. 2 (Oxoid) at 37°C at 200 rpm for 18 h. Afterwards, 50 ml of nutrient broth no. 2 (Oxoid) (in a 500-ml baffle shake flask) was inoculated with 0.5% of a seed culture, and the culture was grown at 37°C at 200 rpm. At OD_{600} values of 0.2, 0.4, 0.8, and 1.6, an aliquot of culture (1.0 ml) was passed through a 0.22-μm membrane (Millipore) via vacuum filtration. The filtrate (0.5 ml) was added to an equal volume (0.5 ml) of 0.8 M perchloric acid, and the solution was vortexed vigorously for 1 min.

Each prepared sample (20 μl) was combined with an equal volume (20 μl) of 1,2-diamino-4,5-methylenedioxybenzene (DMB) derivatization reagent (32), and the mixture was incubated in the dark at 50°C for 2.5 h. If needed, samples were diluted prior to the DMB derivatization so that the measured concentration of the DMB–α-KG derivative was between 20 and 200 μM (dynamic range of the method). The DMB-derivatized α-KG was analyzed in each sample by electrospray ionization-liquid chromatography mass spectrometry (ESI-LC-MS), which consisted of an API2000 LC tandem mass spectrometry (LC/MS/MS) system equipped with a turbo-ion spray source interfaced with a Prominence UFLC. The column used for separation was a C_{18} reversed-phase Hypersil (100-mm- by 2.1-mm internal diameter [ID], 3-μm particle size, and 120-Å pore size). Mobile phase A (H_2O with 0.05% formic acid) and mobile phase B (95% acetonitrile–5% H_2O with 0.05% formic acid) were used for all separations. The elution procedure consisted of an isocratic profile of 15 min of 20% mobile phase B in mobile phase A and a linear gradient from 20 to 100% mobile phase B in mobile phase A over 1 min, followed by an isocratic profile of 100% mobile phase B for 4 min with a flow rate of 0.30 ml min^{-1}. Electrospray ionization in the positive mode was performed by using the turbo-ion spray source with an ion spray voltage of 4,500 V, a desolvation temperature of 300°C, and gas 1 and gas 2 set at 20 and 30, respectively. Mass spectra were collected over the range 200 to 300 *m/z* with a declustering potential of 22 V, a focusing potential of 400 V, and an entrance potential of 5 V. The DMB–α-KG derivative was detected by ion extraction of the $[M + H]^+$ *m/z* of 262.9 to 263.5. All traces were smoothed by using a Savitzky-Golay filter, and the area under the peak was determined by using Analyst classic parameters from the Applied Biosystems software (version 1.5). Concentrations reported for α-KG represent mean values (± standard deviations [SD]).

PA5530-*lacZ* expression experiments. Each treatment was tested in triplicate, and galactosidase (LacZ) activity was measured by using the Miller assay as described previously (23). Recombinant *P. aeruginosa* and *E. coli* strains harboring pBRL485 were grown in nutrient broth no. 2 (Oxoid) at 37°C at 200 rpm. When the cells reached an OD_{600} of ~0.3, exogenous substrates were added to a final concentration of 0.02 to 20 mM, and LacZ activity was assayed at either 30 or 90 min postinduction.

Microarray analysis. Microarray experiments were done in triplicate. *P. aeruginosa* PAO1 was grown in nutrient broth no. 2 (Oxoid) at 37°C at 200 rpm to an OD_{600} of 0.3. α-KG was then added to the cultures at a final concentration of either 0 or 20 mM. At 30 min postinduction, an aliquot of culture (1.0 ml) was mixed with 0.5 ml RNAprotect Bacteria reagent (Qiagen). The bacteria in the treated samples were lysed by using an enzymatic proteinase K digestion approach, as detailed in the RNAprotect Bacteria reagent handbook. The RNA was purified by using the RNeasy minikit (Qiagen) with an on-column DNase digestion step. PCR and a Bioanalyzer (Agilent Technologies) were used to check the purified RNA samples for DNA contamination and overall quality. Microarray studies were performed by the Microarray Core Facility (Upstate Medical University, Syracuse, NY) by using *P. aeruginosa* PAO1 Affymetrix GeneChip

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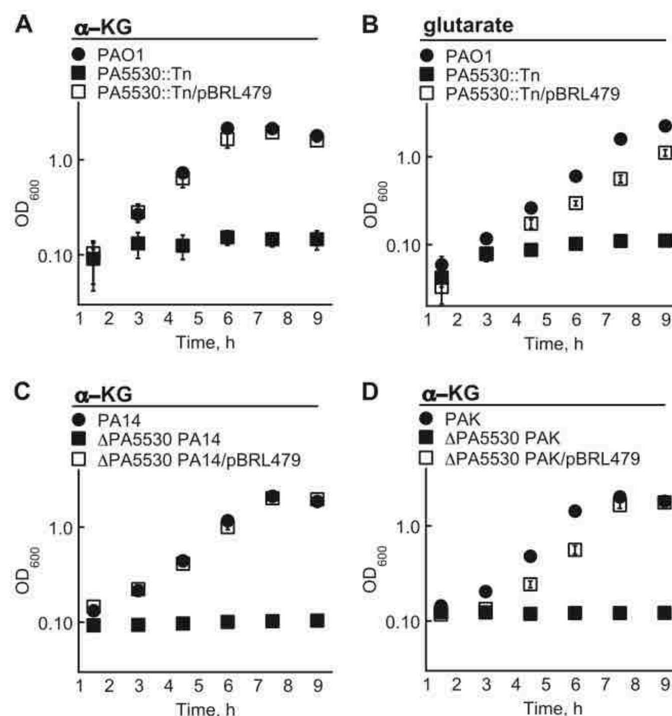

FIG 1 The PA5530 gene is required for the growth of *P. aeruginosa* on C_5-dicarboxylates. (A and B) A PA5530 transposon mutant (PA5530::Tn) of *P. aeruginosa* PAO1 did not grow in M9 minimal medium when the sole carbon source was 20 mM α-KG (A) or glutarate (B). (C and D) α-KG was also not a viable carbon source for ΔPA5530 derivatives of *P. aeruginosa* PA14 (C) and PAK (D). Growth on C_5-dicarboxylates was recovered for all strains when the PA5530 gene was expressed from the *lac* promoter of pBBR1MCS-5 (pBRL479). Data points represent mean values ± SD ($n = 3$).

arrays. Methods for conducting the microarray experiments, including data processing and statistical analysis, were done exactly as previously described (23).

Microarray data accession number. Data from the microarray experiment were deposited in the Gene Expression Omnibus (GEO) database (33) under accession no. GSE54032.

RESULTS

The PA5530 gene is required for growth of *P. aeruginosa* on C_5-dicarboxylates. The protein responsible for α-KG transport in *P. aeruginosa* PAO1 was expected to possess significant homology to the α-KG:H^+ symporter (KgtP) of *E. coli* K-12 MG1655. After performing a BLASTP search against the protein database of *P. aeruginosa* PAO1, the putative PA5530 protein was found to have 70% homology (55% identity) to KgtP of *E. coli* K-12. Consistent with the idea that the PA5530 protein functions as an α-KG transporter, a *P. aeruginosa* PAO1 strain harboring a transposon insertion within the PA5530 gene could not utilize α-KG as a carbon source (Fig. 1A). Additionally, the PA5530 mutant did not grow on glutarate-minimal medium (Fig. 1B), showing that the assimilation of C_5-dicarboxylates converges on the PA5530 gene. Growth on C_5-dicarboxylates was restored in the PA5530 mutant when the PA5530 gene was expressed from the *lac* promoter on the broad-host-range plasmid pBBR1MCS-5.

The PA5530 gene was also required for the growth of *P. aeruginosa* strains PA14 and PAK on α-KG. The PA5530 gene was deleted in *P. aeruginosa* strains PA14 and PAK, and the resulting ΔPA5530 *P. aeruginosa* mutants were assayed for growth on α-KG. As expected, neither ΔPA5530 mutant grew on α-KG unless the PA5530 gene was heterologously expressed from pBBR1MCS-5 (Fig. 1). The necessity of the PA5530 gene for α-KG assimilation appears to be a shared trait of *Pseudomonas aeruginosa* strains PAO1, PA14, and PAK.

We next confirmed that the PA5530 gene is required for α-KG uptake in *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 and the PA5530 mutant were grown in nutrient broth no. 2 (Oxoid), and the extracellular α-KG concentrations were measured at cell densities (OD_{600}) of 0.2, 0.4, 0.8, and 1.6. At an OD_{600} of 0.2, *P. aeruginosa* PAO1 and the PA5530 mutant had comparable extracellular α-KG concentrations of 240 (±38) μM and 384 (±32) μM, respectively. By the next doubling (OD_{600} of 0.4), extracellular α-KG concentrations decreased to <20 μM (lower detection limit) for *P. aeruginosa* PAO1 but increased to 898 (±122) μM for the PA5530 mutant. Interestingly, *P. aeruginosa* PAO1 at an OD_{600} of 0.8 accumulated extracellular α-KG to a concentration of 88 (±24) μM, which decreased to <20 μM at an OD_{600} of 1.6. In contrast, the PA5530 mutant exhibited extracellular α-KG concentrations of 868 (±72) μM and 1,134 (±48) μM at OD_{600} values of 0.8 and 1.6, respectively. The elevated extracellular α-KG

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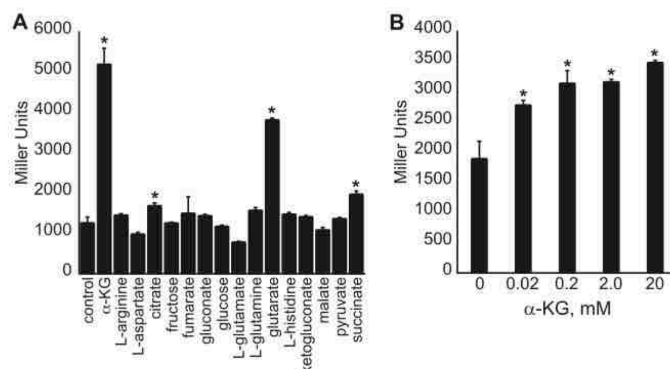

FIG 2 Expression of a PA5530-*lacZ* reporter is induced by extracellular C_5-dicarboxylates. The 5′ 1,069-bp regulatory region upstream of the PA5530 ORF was fused to *lacZ*, and the resulting PA5530-*lacZ* reporter was introduced into *P. aeruginosa* PAO1. Recombinant cells were grown in nutrient broth no. 2 (Oxoid) to an OD_{600} of 0.3 and subsequently induced with various organic compounds provided at a final concentration of 20 mM. (A) At 90 min postinduction, LacZ activity was highest for cells treated with α-KG or glutarate. (B) The expression level of PA5530-*lacZ* increased within 30 min after the addition of α-KG to a final concentration of 0.02 to 20 mM. Data points represent mean values ± SD ($n = 3$). Analysis of variance was performed by using Dunnett's *post hoc* test (α-value of 0.05) to identify significant changes ($P < 0.0001$), which are marked (asterisks).

concentration observed for the PA5530 mutant is a characteristic also found in a Δ*kgtP E. coli* strain (12) and shows that the disruption of the PA5530 gene hinders the uptake of α-KG in *P. aeruginosa* PAO1. It should be noted that the α-KG content of nutrient broth no. 2 (Oxoid) was found to be <20 μM. Therefore, the measured extracellular α-KG concentration was a product of *Pseudomonas* metabolism and not an artifact of nutrient broth no. 2 (Oxoid).

Expression of the PA5530 gene is regulated by extracellular α-KG. Previous biochemical studies have shown the existence of an inducible α-KG transporter in *Pseudomonas* (21). To determine if the expression of the PA5530 gene is induced by the presence of extracellular C_5-dicarboxylates, the 1,069-bp 5′-regulatory region immediately upstream of the PA5530 start codon was fused to a DNA segment encoding the β-galactosidase (*lacZ*) ORF of *E. coli* K-12 MG1655. The resulting PA5530-*lacZ* fusion was cloned into a promoterless plasmid, Δ*Plac*-pBBR1MCS-5, and subsequently electroporated into *P. aeruginosa* PAO1. *P. aeruginosa* PAO1 harboring the PA5530-*lacZ* reporter was grown in nutrient broth no. 2 (Oxoid) to an OD_{600} of 0.3, at which time various organic acids were added to a final concentration of 20 mM. As shown in Fig. 2A, expression of PA5530-*lacZ* increased by 330% (±28%) within 90 min of the addition of α-KG. Glutarate also induced the expression of PA5530-*lacZ* by 224% (±8.0%). Of the remaining carbon sources tested, only succinate and citrate were found to have an inducing effect, increasing PA5530-*lacZ* expression levels by ~50%. These data confirm that expression of the PA5530 gene is strongly induced in the presence of extracellular C_5-dicarboxylates, with preference toward α-KG. For example, the PA5530-*lacZ* expression level increased by 48% (±22%) within 30 min after the addition of α-KG to a final concentration of 20 μM (Fig. 2B).

The data from the PA5530-*lacZ* reporter experiments showed that the expression of the PA5530 gene is induced by extracellular α-KG; however, the number and range of genes whose expression is regulated by α-KG were not known. We therefore determined the transcriptional response of *P. aeruginosa* PAO1 to extracellular α-KG using Affymetrix GeneChips. In triplicate, *P. aeruginosa* PAO1 cells were grown in nutrient broth no. 2 (Oxoid) to an OD_{600} of 0.3 and then treated with either 0 or 20 mM α-KG. At 30 min postinduction, cells were harvested, and total RNA was isolated. Following microarray and statistical analyses, a total of four genes displayed changes in transcript levels by >2-fold with the addition of exogenous α-KG.

The changes in transcript levels for three of the four genes were near the 2-fold cutoff: PA0865 (−2.8 ± 0.7), PA4131 (−2.5 ± 0.3), and PA5170 (3.7 ± 1.6). The PA4131 gene encodes a putative iron-sulfur protein and has no known function. The physiological roles of the PA0865 (*hpd*) and PA5170 (*arcD*) genes were previously analyzed (34, 35), and their known functions cannot be readily extrapolated to α-KG metabolism. Because of this uncertainty and the slight changes observed in their transcript levels, these genes are not considered to directly participate in α-KG assimilation. Expectedly, the transcript level of the PA5530 gene increased by 17-fold (±5.0-fold) with the addition of exogenous α-KG. The microarray results reinforce the findings of the PA5530-*lacZ* experiments, and collectively, they show that α-KG induces the expression of the PA5530 gene. The inducible expression of the PA5530 gene is a behavior reminiscent of other bacterial transporter genes and argues that the PA5530 gene most likely encodes the α-KG transporter in *P. aeruginosa* PAO1.

Expression of the PA5530 gene is dependent on RpoN. The sigma factor RpoN recognizes a −24/−12 promoter with the consensus sequence 5′-TGGCACG-N4-TTGCW-3′ (W is A/T) (36). Located 92 bp upstream of the predicted start codon of the PA5530 ORF is a putative RpoN promoter, 5′-TGGCACG-N4-CTGCT-3′. To determine if this predicted RpoN promoter is functional, the highly conserved "GG" motif of the −24 element was changed to "AA" in the PA5530-*lacZ* reporter. This mutation in the RpoN promoter of the PA5530-*lacZ* reporter reduced its expression by >95% in *P. aeruginosa* PAO1 (Fig. 3); this finding is consistent with the "GG" motif being a key determinant of RpoN-mediated transcription. Furthermore, there was a >95% decrease in the expression level of the PA5530-*lacZ* reporter in an *rpoN*

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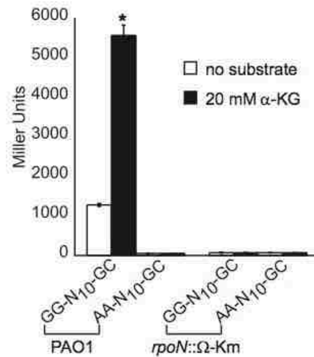

FIG 3 RpoN is essential for the expression of the PA5530-*lacZ* reporter. Positioned 93 bp upstream of the PA5530 ORF is a sequence resembling a −24/−12 promoter (GG-N_{10}-GC). Replacement of the conserved "GG" dinucleotide of the −24 element with "AA" in the PA5530-*lacZ* reporter (denoted AA-N_{10}-GC) reduced its expression by >95% in *P. aeruginosa* PAO1. Additionally, expression of the PA5530-*lacZ* reporter was null in an *rpoN*::Ω-Km derivative of *P. aeruginosa* PAO1. Note that all strains were grown in nutrient broth no. 2 (Oxoid) to an OD_{600} of 0.3 and then induced with 20 mM α-KG. LacZ activity was measured at 90 min postinduction. Data points represent mean values ± SD (n = 3). Analysis of variance was performed by using Dunnett's *post hoc* test (α-value of 0.05) to identify significant changes ($P <$ 0.0001), which are marked (asterisk).

mutant compared to wild-type *P. aeruginosa* PAO1 (Fig. 3). The addition of exogenous α-KG did not change the expression of PA5530-*lacZ* in the *rpoN* mutant. These results support a model in which RpoN and its cognate −24/−12 promoter direct the transcription of the PA5530 gene in *P. aeruginosa* PAO1.

The *mifR* gene is required for expression of the PA5530 gene in *P. aeruginosa* PAO1. RpoN requires additional regulators, known as enhancer binding proteins (EBPs), to activate the transcription of targeted genes (37). Having established that RpoN is completely necessary for the expression of the PA5530 gene, we next sought to determine the EBP that participates with RpoN in this process. To this end, we scanned the chromosomal region surrounding the PA5530 locus to identify genes encoding known or hypothetical EBPs. In the vicinity of the PA5530 locus is the *mifR* (PA5511) gene. The EBP MifR has been observed to be required for microcolony formation (22, 38), but it also possesses significant homology (>60%) to the DctD family of response regulators. DctD response regulators are EBPs that activate the transcription of genes whose products participate in the transport of dicarboxylates from the environment (39, 40). For *P. aeruginosa* PAO1, DctD (PA5166) regulates the transport of C_4-dicarboxylates such as fumarate, malate, and succinate (41). We therefore wondered if MifR might be a DctD homolog that regulates the transport of C_5-dicarboxylates in *P. aeruginosa* PAO1.

In support of MifR having a role in the utilization of C_5-dicarboxylates, a Δ*mifR* mutant of *P. aeruginosa* PAO1 could not use α-KG or glutarate as a growth substrate (Fig. 4). Expression of the PA5530 gene from plasmid pBBR1MCS-5 rescued the growth of the Δ*mifR* mutant on both of these C_5-dicarboxylates. This finding suggested that the diminished capacity of the Δ*mifR* mutant to grow on C_5-dicarboxylates might be a result of insufficient expression of the PA5530 gene. This suspicion was confirmed when it was observed that the expression of the PA5530-*lacZ* reporter was reduced by >95% in the Δ*mifR* mutant compared to wild-type *P. aeruginosa* PAO1 (Fig. 5). We also found that the *dctD* gene had no impact on the expression of PA5530-*lacZ*; α-KG induced the expression of PA5530-*lacZ* in a *dctD* mutant to levels observed in wild-type cells (Fig. 5). As expected, deletion of the *mifR* gene in the *dctD* mutant abolished the expression of PA5530-*lacZ*. The inability of the Δ*mifR* mutant to grow on C_5-dicarboxylates or express the PA5530-*lacZ* reporter gives credibility to the idea that the primary function of MifR is the regulation of C_5-dicarboxylate transport in *P. aeruginosa* PAO1.

The response regulator gene *mifR* is in a putative operon with its predicted sensor kinase partner gene *mifS*. Sensing and responding to extracellular α-KG are most likely mediated by the MifSR two-component signal transduction system (TCS) in *P. aeruginosa* PAO1. To test this hypothesis, the *mifSR* genes were

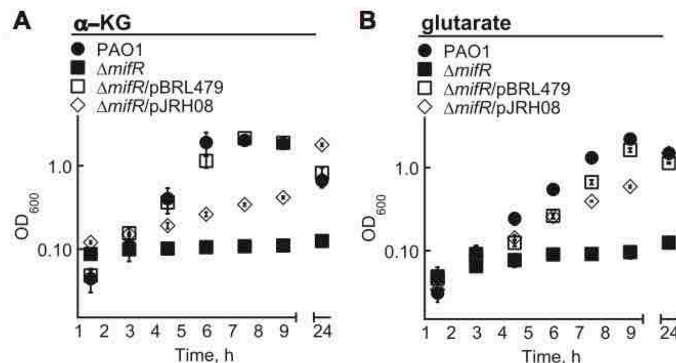

FIG 4 Heterologous expression of the PA5530 gene rescues the growth of a Δ*mifR* mutant of *P. aeruginosa* PAO1 on C_5-dicarboxylates. A Δ*mifR* mutant of *P. aeruginosa* PAO1 could not grow in M9 minimal medium with either 20 mM α-KG (A) or glutarate (B) as the sole carbon source. Expression of the PA5530 gene from the *lac* promoter of pBBR1MCS-5 (pBRL479) restored the growth of the Δ*mifR* mutant on these C_5-dicarboxylates. There was partial complementation of the Δ*mifR* mutant with pJRH08 (*mifR* gene in pBBR1MCS-5). Full complementation most likely requires optimizing the expression of the *mifR* gene in plasmid pJRH08 (for an example, see reference 34). Data points represent mean values ± SD (n = 3).

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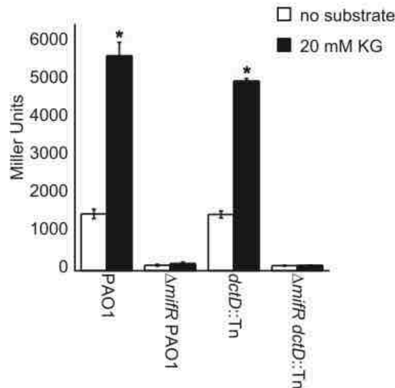

FIG 5 Expression of the PA5530-*lacZ* reporter is dependent on the *mifR* gene. Expression of the PA5530-*lacZ* reporter was reduced by >95% in a Δ*mifR* mutant of *P. aeruginosa* PAO1 compared to that observed in wild-type cells. Cells harboring a *dctD* transposon insertion (*dctD*::Tn) displayed wild-type levels of LacZ activity. However, deletion of the *mifR* gene in *dctD*::Tn cells eliminated the expression of PA5530-*lacZ*. These findings suggest that MifR regulates C_5-dicarboxylate transport, whereas DctD is involved in the transport of C_4-dicarboxylates. Note that all strains were grown in nutrient broth no. 2 (Oxoid) to an OD_{600} of 0.3 and then induced with 20 mM α-KG. LacZ activity was measured at 90 min postinduction. Data points represent mean values ± SD ($n = 3$). Analysis of variance was performed by using Dunnett's *post hoc* test (α-value of 0.05) to identify significant changes ($P < 0.0001$), which are marked (asterisks).

heterologously expressed in *E. coli* Top10 cells that also harbored the PA5530-*lacZ* reporter. As shown in Fig. 6, the basal expression of the *mifSR* operon from the *trc* promoter of plasmid pTrc99a allowed *E. coli* Top10 to express the PA5530-*lacZ* reporter when challenged with external α-KG. Specifically, LacZ activity increased by >1,000% within 120 min of the addition of 20 mM α-KG. This induction was dependent on the *mifR* gene, because *E. coli* Top10 harboring (i) a *mifS*-Δ*mifR* operon or (ii) a *mifSR* operon in the backward orientation of the *trc* promoter of pTrc99a failed to express the PA5530-*lacZ* reporter in the presence of extracellular α-KG. Glutarate induced the expression of the PA5530-*lacZ* reporter by 125% (±10%), whereas C_4-dicarboxylates had no effect on PA5530-*lacZ* expression. These findings show that the *mifSR* genes are sufficient for activating the expression of the PA5530-*lacZ* reporter in nonnative *E. coli* and thus suggest that MifR is a direct regulator of the PA5530 gene.

DISCUSSION

The main objective of this study was to determine the gene encoding the α-KG transporter in *P. aeruginosa* PAO1. Based on homology to the prototypic α-KG transporter KgtP, we identified two genes, PA0229 (*pcaT*) and PA5530, which might encode the α-KG transporter. PA0229 or PcaT is annotated as a putative α-KG transporter in the Pseudomonas Genome Database (42), but PcaT proteins function in the transport of β-ketoadipate (43), which is a C_6-dicarboxylate. In *P. aeruginosa* PAO1, the *pcaT* gene is part of the predicted *pcaTBCD* operon, whose products participate in the breakdown of aromatic compounds, i.e., the protocatechuate

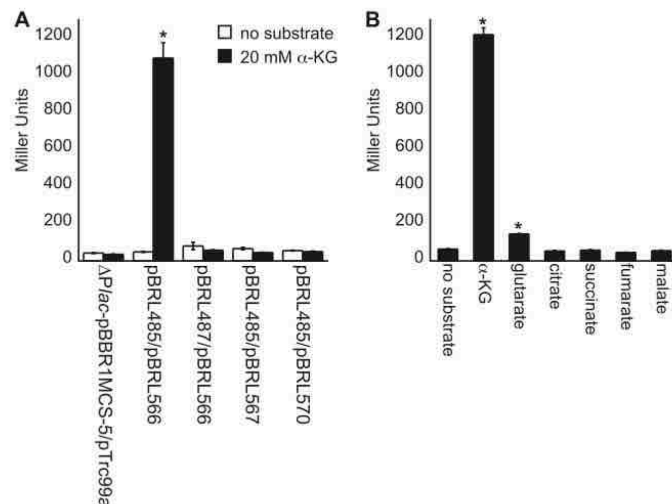

FIG 6 α-KG induces expression of the PA5530-*lacZ* reporter in nonnative *E. coli* only when the *mifSR* genes are heterologously expressed. (A) *E. coli* Top10 cells that basally expressed the *mifSR* operon from the *trc* promoter of pTrc99a (pBRL566) responded to extracellular α-KG via increasing the expression level of the PA5530-*lacZ* reporter by >1,000% compared to the level in noninduced cells. *E. coli* Top10 cells expressing nonfunctional *mifSR* alternatives, including a *mifSR* operon oriented in the opposite direction of the *trc* promoter (pBRL567) or a *mifS*-Δ*mifR* operon (pBRL570), did not express the PA5530-*lacZ* reporter. Furthermore, the *mifSR* operon did not cause a change in the expression of the PA5530-*lacZ* reporter in which the "GG" dinucleotide of the −24 element of the putative RpoN promoter was replaced with "AA" (pBRL487). (B) Lastly, expression of PA5530-*lacZ* in *E. coli*/pBRL566 was induced by extracellular C_5-dicarboxylates. These results show that the *mifSR* genes can directly regulate the expression of the PA5530 gene in response to extracellular α-KG. For all experiments, cells were grown in nutrient broth no. 2 (Oxoid) to an OD_{600} of 0.3 and then induced with 20 mM substrate. LacZ activity was measured at 120 min postinduction. Data points represent mean values ± SD ($n = 3$). Analysis of variance was performed by using Dunnett's *post hoc* test (α-value of 0.05) to identify significant changes ($P < 0.0001$), which are marked (asterisks).

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branch of the β-ketoadipate pathway (44). Consequently, PcaT was considered to be a transporter for β-ketoadipate and not α-KG. This made the PA5530 gene a favorable candidate for encoding the α-KG transporter in *P. aeruginosa* PAO1.

A transposon insertion within the PA5530 gene in *P. aeruginosa* PAO1 prevented this bacterium from using C_5-dicarboxylates as growth substrates. Follow-up experiments showed that the disruption of the PA5530 gene caused a significant accumulation of extracellular α-KG. For example, both wild-type *P. aeruginosa* PAO1 and a PA5530 mutant produced ~200 μM extracellular α-KG within 90 min of being inoculated into a rich medium. Whereas wild-type cells were able to assimilate this extracellular α-KG, lowering its levels below 20 μM, the PA5530 mutant continued to accumulate extracellular α-KG, which eventually reached concentrations of >1.0 mM. This result is consistent with the PA5530 gene functioning in α-KG transport, but we did not anticipate that the levels of extracellular α-KG would be in the millimolar range. The generation and subsequent uptake of extracellular α-KG are well-known characteristics of *P. aeruginosa* cultures. However, the magnitude or significance of this activity when *P. aeruginosa* is grown in a rich medium has not been reported previously. The metabolic process responsible for the continuous production of extracellular α-KG is a topic worth exploring.

α-KG did induce the expression of the PA5530 gene, as determined by both microarray and *lacZ* reporter experiments. In fact, the exogenous addition of 20 μM α-KG was sufficient to stimulate the expression of the PA5530 gene. Expression of the PA5530 gene was also induced by extracellular glutarate, reaffirming that the PA5530 gene has all the elements associated with an inducible C_5-dicarboxylate transporter. Namely, the PA5530 gene (i) encodes a putative KgtP homolog, (ii) was essential for the utilization of C_5-dicarboxylates, (iii) was required for the uptake of extracellular α-KG, and (iv) was genetically regulated by extracellular C_5-dicarboxylates.

Upstream of the PA5530 ORF is a −24/−12 promoter recognized by the sigma factor RpoN. We did not find any sequence resembling a σ^{70}-type promoter within the vicinity of the predicted start codon of the PA5530 ORF. The changing of the highly conserved "GG" dinucleotide of the −24 element to "AA" completely eliminated the expression of the PA5530 gene. Additionally, the PA5530 gene was not expressed in an *rpoN*-deficient derivative of *P. aeruginosa* PAO1. Both of these results suggest that RpoN is the sigma factor responsible for the expression of the PA5530 gene. The genetic regulation of C_5-dicarboxylate transport by RpoN might be a common mechanism employed by *Pseudomonas*, because the *rpoN* gene was also required for the growth of *P. putida* on α-KG (45).

One of the most interesting findings of this study was the requirement of the *mifR* gene for the assimilation of C_5-dicarboxylates in *P. aeruginosa* PAO1. The response regulator MifR (PA5511) and its partner sensor kinase MifS (PA5512) comprise a TCS that was initially found to be essential for the development of mature biofilms (22). Later studies showed that in the absence of the *mifR* gene, pyruvate fermentation was suboptimal and thus unable to support the formation of microcolonies (38). In our study, a Δ*mifR* mutant could not utilize C_5-dicarboxylates due to a significant reduction (>95%) in the expression of the PA5530 gene.

A reasonable explanation as to why the *mifR* gene was required for the expression of the PA5530 gene was found when we closely examined the amino acid sequence of the MifR protein. Like all other enhancer binding proteins, MifR has the conserved RpoN interaction domain, but more importantly, MifR is 70% homologous (54% identical) to the response regulator DctD (PA5166) of *P. aeruginosa* PAO1. The presence of dual DctD regulators (commonly annotated DctD1 and DctD2) has been reported for the sequenced genomes of several *Pseudomonas*-related bacteria, including *Ralstonia solanacearum*, *Azotobacter vinelandii*, *Pseudomonas protegens*, and *Burkholderia mallei* (NCBI Genome Database). The DctBD TCS of *P. aeruginosa* PAO1 is involved in the assimilation of C_4-dicarboxylates (41), and we found that the *dctD* gene had no effect on the expression of the PA5530 gene. It is plausible that MifS and MifR function as a DctBD-type TCS that responds to extracellular C_5-dicarboxylates via activating the expression of the PA5530 gene. Some bacteria are known to use a TCS, e.g., KguSR (10) and KgtSR (8), to regulate α-KG assimilation, and we found that the heterologous expression of the *mifSR* genes in *E. coli* was sufficient to activate the expression of the PA5530-*lacZ* reporter in response to extracellular α-KG. Biochemical evidence is needed to verify that MifR binds to and regulates the expression of the PA5530 gene.

Lastly, it will be valuable to determine if the PA5530 gene or α-KG has any role in biofilm development in *P. aeruginosa* PAO1. Cells within the interior of matured biofilms are considered to be in an anaerobic environment (46, 47), and recently, pathogenic *E. coli* strain CTF073 was reported to preferentially assimilate α-KG as a carbon source under anaerobic growth conditions (10). It is possible that MifR is required for the anaerobic metabolism of α-KG in *P. aeruginosa* PAO1. A recent transcriptomic study found that the transcript levels of several genes whose products are involved in anaerobic metabolism were deregulated in a Δ*mifR* mutant of *P. aeruginosa* PAO1 (38). We have now just begun to analyze α-KG utilization for anaerobically grown *P. aeruginosa* PAO1, including its ΔPA5530 and Δ*mifR* derivatives.

ACKNOWLEDGMENTS

We acknowledge NIH grant P30 DK089507 for funding the *P. aeruginosa* PAO1 transposon mutant used for our study. This study was made possible by NIH R15 GM104880-01A1 and NSF CBET 1263905 awards to C.T.N.

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Research Experience

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- **Upstate Bone and Joint Center – Over 100 hours** — *March 2016- May 2016*
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Teaching Experience

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  Focus on protein purification and related analytical techniques
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Volunteer/ Service Work

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Second Place: NY Biotechnology Symposium Poster Competition, **2014**

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Dean's List, SUNY-ESF, Spring **2012**, Spring **2013**

Outstanding Senior, SUNY-ESF Department of Chemistry, **2014**

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