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Exploring the Petrochemical Bioremediation Capacities of Aerobic Thermophilic Bacteria

by

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Dissertation

Submitted in fulfilment of the requirements for the degree

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Co-supervisor: Prof. D. Cowan

January 2020

Declaration

I declare that this dissertation is my own work, aided by Dr P. De Maayer (supervisor). It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

_____

(Signature of candidate)

29th day of **January 2020** at **Johannesburg**

ABSTRACT

Crude oil is a common environmental contaminant that has detrimental effects on marine, freshwater and soil environments and the biota that inhabit these ecological niches. Numerous oil remediation strategies have been developed, with different efficacies and downstream effectiveness. One approach that has garnered a lot of attention is the use of bacteria for hydrocarbon bioremediation. This study explored the hydrocarbon bioremediation capabilities of aerobic, thermophilic bacteria on alkane hydrocarbons. A substantial collection of aerobic thermophilic bacteria was screened through liquid and plate-based assays for their capacity to degrade oil hydrocarbons and the potential to produce biosurfactants. Three strains of *Bacillus licheniformis*, namely But5A1A, But5A1B and Wam9A3, showed promise in terms of their oil degradation and biosurfactant production capabilities. Complete genome sequencing of these strains was undertaken with the purpose of identifying the molecular basis underlying the observed hydrocarbon degradation. Although no clear pathway was established, a number of enzymes with known roles in hydrocarbon degradation, for example alkane monooxygenases, were identified. Moreover, genes that encode the biosurfactant lichenysin, which may be responsible for the oil displacement activities observed during the screening assays, were also identified. This biosurfactant is likely responsible for the enhanced degradation capacity observed in *B. licheniformis* But5A1A. Finally, GC-MS analysis was used to analyse the degradation capacities of these strains. These results support the observations made during the liquid assays and further suggests the production of alkanes by these bacterial strains of an unconfirmed function. Overall, these strains demonstrate great potential for future bioremediation ventures.

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Finally, my deepest thanks go towards my family, friends, lab mates and lab partner, Michael Mol. Their unfailing support and encouragement have helped me through this MSc - this accomplishment would not have been possible without them.

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Chapter 1

A study background and literature review on the bacterial bioremediation of oil

1.1. Introduction

Crude oil has become a primary energy commodity in the past century - more so than any other fuel source (Jobling and Jamasb, 2017). This is owing to its extensive usage in both daily lives, the generation of heat and electricity, for transportation and in industry (Atlas and Hazen, 2011). As a result, there have been increased efforts in the acquisition and transport (~35 million barrels are ferried across oceans every year) of crude oils over the past century (Macaulay, 2014). Although the trade of this invaluable resource has created powerful relations amongst countries (Overland, 2016), constant shipment and usage of these oils has made the environment vulnerable to crude oil pollution (Holliger *et al.,* 1997).

Crude oil spills and leakages are a major contributing factor in overall environmental pollution (Das and Chandran, 2011; Xu *et al*., 2018). The hydrocarbons in these oils have both mutagenic and fatal effects on the organisms inhabiting these environments (Das and Chandran, 2011) and furthermore, hinders their ability to execute basic ecosystem functions vital for the maintenance of environmental homeostasis (Jonsson and Haller, 2014). Therefore, effective methods of environmental oil decontamination are imperative.

Presently, techniques used in environmental hydrocarbon decontamination include physicochemical and biological processes such as washing, incineration and bioventing (Das and Chandran, 2011; Yuniati, 2018). Physicochemical methods, however, are often unfeasible as they are expensive (when used on large contaminant volumes), do not fully remove contaminants, and are not environmentally friendly (Das and Chandran, 2011; Koshlaf and Ball, 2017; Vidali, 2001). A solution to this issue is the use of microorganisms, such as those used in bioventing, to remove pollutants from the environment: a process termed bioremediation (Das and Chandran, 2011; Koshlaf and Ball, 2017; Xu *et al*., 2018). This alternative has shown great potential as it is non-invasive and, presents less of an economic challenge, owing to the natural abundance of microorganisms that are capable of metabolising hydrocarbons (Das and Chandran, 2011; Koshlaf and Ball, 2017; Xu *et al*., 2018).

When hydrocarbons enter an environment, their primary degradation is carried out by bacteria and fungi; this happens because petroleum hydrocarbons exist naturally and has led to the

evolution of diverse mechanisms in microorganisms to utilise these hydrocarbons as both an energy and carbon source for growth (Atlas and Hazen, 2011). The susceptibility of hydrocarbons to microbial attack varies depending on which class the hydrocarbons belong to (Das and Chandran, 2011). Excessive hydrocarbon pollution, especially those hydrocarbons that are less inherently biodegradable, remain in the environment for extensive periods of time and is not only toxic to the animals occupying these niches but also disrupt the microorganisms inhabiting these environments (Hakima and Ian, 2017). Thus, there has been a developing interest in the exploration and exploitation of microorganisms which are capable of hydrocarbon degradation, particularly the more recalcitrant hydrocarbons.

Because the use of aerobic mesophilic bacteria in hydrocarbon biodegradation has been more extensively explored and elucidated (Das and Chandran, 2011; Nzila, 2018; Rojo, 2009), in comparison to their thermophilic counterparts, this study will focus on the hydrocarbon-degrading capabilities amongst aerobic thermophilic bacteria, where information is still largely lacking (Nzila, 2018). Thermophiles operate at higher temperatures of >50˚C – a temperature range at which oil viscosity decreases (Leahy and Colwell, 1990; Mohammad *et al*., 2017). This increases the bioavailability of oil which helps enhance the microbial hydrocarbon degrading process (Leahy and Colwell, 1990). Therefore, it would be beneficial to gauge the potential of thermophilic bacteria, who naturally thrive at higher temperatures, in bioremediation strategies (Vieille and Zeikus, 2001).

The assessment of these degradation capabilities will pertain specifically to the biodegradation of long-chain linear alkanes, which provide the bacteria with a source of carbon and energy (Atlas and Hazen, 2011). Furthermore, this experiment will also consider the molecular aspects of these hydrocarbon-degrading bacteria and the enzymes and pathways they utilise in the aerobic catabolism of these recalcitrant hydrocarbons. This is important because the discovery of these molecular determinants may provide a variety of solutions to improve current petroleum hydrocarbon bioremediation techniques (Peixoto, 2011).

1.2. Petroleum hydrocarbon pollution of the environment and its consequences

'Energy globalization' is a term that can be defined as the increasing interconnectedness of the earth's energy supplies through the transportation of increasing volumes of energy over larger distances and across international borders (Overland, 2016). The advent of this term came with the development of modern civilizations, as emphasis was placed on a source of energy for

both industrial and daily human activities (Atlas and Hazen, 2011; Xu *et al*., 2018). Although energy can be sourced from natural gases, coal and nuclear fuel, more than one third of the energy used globally, is sourced from crude oil (Jobling and Jamasb, 2017).

Because crude oil/petroleum resources are unevenly distributed amongst countries (Jobling and Jamasb, 2017), there have been increased efforts in the exploration, production, refining, storage and transport of crude oil internationally (Das and Chandran, 2011; Joshi *et al.*, 2013; Rahman *et al*., 2002). During the acquisition, transport and usage of these oils and its derivatives, accidental spills and leakages are an unfortunate, but common occurrence (Das and Chandran, 2011; Rahman *et al*., 2002). When crude oil enters the aquatic or terrestrial environment, it is considered a major contaminant in both prevalence and quantity (Rahman *et al*., 2002). This is owing to the presence of hydrocarbons - organic, mostly water-insoluble compounds containing carbon and hydrogen - in these oils (Peixoto *et al*., 2011; Yuniati, 2018).

Petroleum hydrocarbons are considered harmful towards fauna, flora, microorganisms and even humans and are therefore classified as priority pollutants (Xia *et al*., 2015; Yuniati, 2018). Hydrocarbon contamination has both mutagenic and fatal effects on the organisms inhabiting these environments (Das and Chandran, 2011). This then hinders their ability to fulfil their ecological roles and execute basic ecosystem functions, all of which are vital to the maintenance of environmental homeostasis, thus, the result is a deterioration in both soil and water quality (Hakima and Ian, 2017; Jonsson and Haller, 2014).

Prime examples of how oil spills can cause a wide range of detrimental effects on the environment include the sinking of the Exxon Valdez in 1989 and the Deepwater Horizon oil spill of 2010 (Atlas and Hazen, 2011; Beyer *et al*., 2016). The Exxon Valdez incident occurred in Prince William Sound, Alaska where 41.6 million litres of oil was released into the environment when the oil tanker sank (Atlas and Hazen, 2011). Although it is not the largest ever recorded oil spill, it was deemed the most detrimental in terms of short-term effects to the environment (Atlas and Hazen, 2011). This spill caused approximately 36,000 bird mortalities and the deaths of more than 1000 sea otters and billions of Pacific herring and pink salmon eggs (Maki, 1991). As it was impossible to collect all animal carcasses, the mortality rate amongst wildlife was likely much higher (Maki, 1991).

The largest recorded oil spill was the Deepwater Horizon oil spill that occurred in the northern Gulf of Mexico. Here, 3.19 barrels or 779 million litres worth of oil was spilt into the ocean from the oil rig after an explosion (Atlas and Hazen, 2011; Beyer *et al.*, 2016). Although this

incident did not yield as many animal mortalities as the Exxon Valdez spill, it did still result in negative biological impacts, such as hampering the flight of some migratory bird species which eventually led to increased death rates (Beyer *et al*., 2016). Furthermore, this event has raised many concerns for the long-term impacts on the deep-sea corals, large fish species and sea turtles of the Gulf (Beyer *et al*., 2016). In order to limit such negative effects in the future, effective remediation strategies are crucial.

1.3. Remediation methods to counteract petroleum hydrocarbon contaminants

1.3.1. Existing remediation strategies to remedy oil spills

Because the potential dangers of chemical mixtures, such as petroleum hydrocarbons, are now widely recognized by the public, industries, and the government, greater effort has been placed on hydrocarbon remediation (Khan *et al*., 2004; Koshlaf and Ball, 2017). Remediation is the removal, degradation or transformation of harmful contaminants into less harmful products (Arora, 2018; Yuniati, 2018). Thus, a range of physical, chemical and biological remediation techniques have been designed and employed to decrease the amount of petroleum hydrocarbon pollution in the environment (Azubuike *et al*., 2016; Khan *et al*., 2004). The advantages and disadvantages of some of these techniques are briefly outlined in **Table 1** below.

Table 1: Advantages and disadvantages of some of the physical, chemical and biological petroleum hydrocarbon remediation techniques currently employed.

Technique name	Brief overview of the technique	Pros	Cons
Bioventing	Air is injected into the contaminated environment to maximize bio-degradation.	- Equipment is readily available and easy to install - Short treatment times are required	- Cannot be applied to all site conditions. - Only effective on unsaturated soils. - Not always able to reach low clean-up limits
Thermal desorption	Soil is heated (between 100-600˚C) to release petroleum.	- Desorption efficiency can reach up to 99% - Not sensitive to concentration of contaminants.	- Expensive: equipment is pricey to setup and transport
Soil washing	Uses water (sometimes combined with solvents) and mechanical action to physically scrub soil.	- Cost effective. - *In situ* – no excavation costs. - Permits recovery of metals. - Allows for removal of contaminants from course soils.	- Contaminants are not destroyed – resulting soil has to be destroyed. - Contaminant site is exposed to solvents used in the process. - Wash water has to be treated prior to disposal.
Solidification/ Stabilization	Solidification – waste is encapsulated in a solid material of great structural integrity. Stabilization – contaminant is converted to an immobile, less soluble, less toxic product. Both reduce the mobility of contaminant.	Relatively inexpensive	- Depth of contaminants are a limiting factor. - Needs to be monitored long-term - If not properly completed, contaminant volume could increase.
Soil flushing	Soil is flushed with a solution that relocates contaminants to an area where they can be removed	*In situ* (no excavation costs).	- Heterogenous soils are difficult to treat. - Lengthy remediation times. - Environment is exposed to the solvents required for removal of hydro-phobic contaminants

(Khan *et al*., 2004; Watson, 1996)

Although these techniques are applied to improve the state of hydrocarbon-contaminated sites, they can cause further harm to the environment e.g. in the case with soil flushing, where solvents are added or in soil washing where soil has to be disposed of because contaminants are not destroyed (Khan *et al*., 2004). Furthermore, some of these methods, e.g. bioventing and thermal desorption, require the implementation of specialized machinery/instrumentation which is expensive to employ - owing to set-up and transportation costs (Khan *et al*., 2004). Lastly, these methods are generally only partially effective when employed as can be seen with the solidification/solubilization methods (Das and Chandran, 2011; Khan *et al*., 2004; Vidali, 2001). Thus, the study and use of bioremediation methods - where microorganisms are used to remove pollutants from the environment - are a growing trend; this is because it is both cheaper and these microorganisms, who possess the ability to metabolise petroleum hydrocarbons, are ubiquitous (Atlas and Hazen, 2011).

1.3.2. Bioremediation of oil contaminants

Upon entry into the environment, initial hydrocarbon degradation is mediated by the indigenous bacteria (Atlas and Hazen, 2011; Leahy and Colwell, 1990; Xu *et al*., 2018); this is because petroleum hydrocarbons are naturally occurring products that are derived from aquatic algae laid down between 180 - 85 million years ago (Atlas and Hazen, 2011). These microorganisms function through the process of biomineralization of different hydrocarbons into innocuous products such as water, inorganic compounds, organic compounds and carbon dioxide (Das and Chandran, 2011; Seo *et al*., 2009). This prevents the potential stress-inducing build-up of hydrocarbons in the environment and allows bacteria to acquire both carbon and energy (obtained through the tricarboxylic acid cycle) – products that are vital to their reproduction and growth (Xu *et al*., 2018).

The concept of using bacteria in oil bioremediation was prompted by the successful application of hydrocarbon-degrading bacteria in the Exxon Valdez oil spill of 1989 and the BP Deepwater Horizon oil spill of 2010 (Atlas and Hazen, 2011; Das and Chandran, 2011). Here, the bacteria, along with a range of other factors such as photochemical degradation by natural sunlight (Beyer *et al*. 2016), functioned to remove great quantities of oil and in so doing were able to reduce the overall environmental impact of these spillages (Atlas and Hazen, 2011; Das and Chandran, 2011).

The problem with bioremediation arises when one takes into consideration the presence of recalcitrant hydrocarbons contained within petroleum and its derivatives. Different hydrocarbons have distinct degrees of susceptibility towards microbial degradation (Leahy and Colwell, 1990; Maletić *et al*., 2011). Monocyclic aromatic hydrocarbons (benzene, toluene, ethylbenzene and xylene – BTEX), polycyclic aromatic hydrocarbons (naphthalene, anthracene, pyrene and phenanthrene) and acyclic alkanes (paraffins), due to their chemical structures, or factors in the environment, are considered recalcitrant compounds and are less susceptible to degradation (Holliger *et al.*, 1997). These tend to accumulate in the environment (Holliger *et al.*, 1997), thus leading to long-term detrimental effects on the biota and the environment (Das and Chandran, 2011).

Monocyclic aromatic hydrocarbon (MAH) and polycyclic aromatic hydrocarbon (PAH) pollution has raised great concern over the past years. MAHs (i.e. BTEX) are common contaminants found in groundwater plumes and in other water resources intended for human consumption (Fayemiwo *et al*., 2017). Ingestion of these compounds causes liver lesions, drowsiness, cancer and irritation of organs, while long-term exposure can cause adverse effects on the respiratory and central nervous systems (Holliger *et al*., 1997; Fayemiwo *et al*., 2017). PAHs, although they are a minor component of crude oils, raise significant concerns due to their mutagenic and carcinogenic properties (Atlas and Hazen, 2011; Holliger *et al*., 1997; Ghosal *et al*., 2016). The PAHs, because of their recalcitrant nature to microbial degradation, tend to accumulate in the environment where they enter various food chains (Das and Chandran, 2011; Ghosal, 2016); thus, PAHs are viewed as major hazards to animal and plant health (Ghosal *et al.*, 2016).

MAHs and PAHs demonstrate low bioavailability owing mostly to their chemical structures. Benzene, toluene, ethylbenzene and xylenes (MAHs) are mono-aromatic, non-oxygenated, ring structures with a single six-carbon benzene ring as its basis (Figure 1; Chen and Taylor, 1995; Fayemiwo *et al*., 2017). Despite possessing a greater water solubility than PAHs, these compounds are non-reactive species with low bioavailability which is a result of their closed structures (Fayemiwo *et al*., 2017). PAHs consist of two or more fused benzene and/or pentacyclic rings in linear, angular, or cluster arrangements (Figure 2; Chauhan *et al*., 2008; Ghosal *et al*., 2016). PAHs can remain in the environment owing to their highly hydrophobic and chemically stable natures that increase with increasing molecular weight (Chauhan *et al*., 2008; Seo *et al*., 2009).

Generally, the inability of the bacteria to degrade these aromatic hydrocarbons is the result of external environmental factors and not solely due to the structural composition of these contaminants (Bamforth and Singleton, 2005; Chauhan *et al*., 2008). A range of physico-chemical factors, such as oxygen availability, temperature and pH determine the survival and activity of the degrading bacteria in the contaminated environments (Bamforth and Singleton, 2005; Chauhan *et al*., 2008; Holliger *et al*., 1997). As bacteria generally encounter these aromatic hydrocarbons in niches where little or no oxygen is present, PAHs and MAHs remain in the environment where they continue to act as a threat to the biota (Bamforth and Singleton, 2005; Chauhan *et al*., 2008; Ghosal *et al*., 2016; Holliger *et al*., 1997).

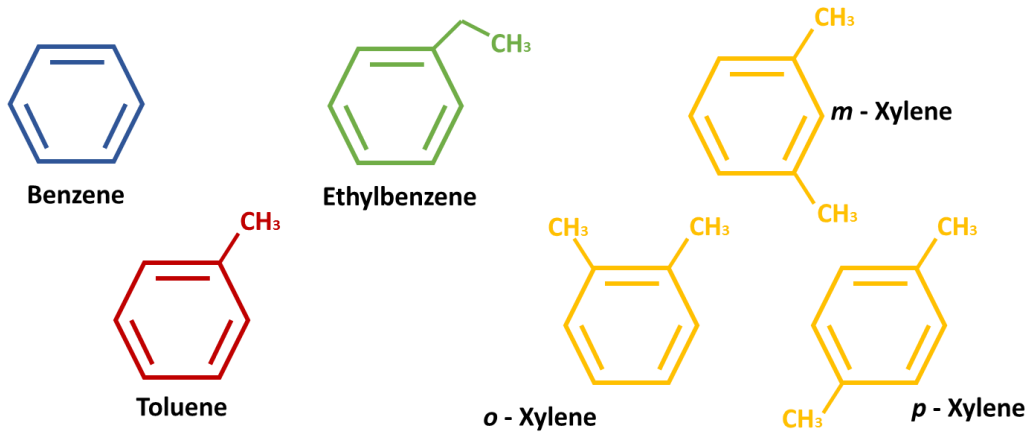


Figure 1: The monocyclic aromatic hydrocarbons (MAHs) benzene, toluene, ethylbenzene and the xylene (BTEX) compounds (Fayemiwo *et al*., 2017). As a result of their closed ring structures they are considered non-reactive species with low bioavailability. Because of this, they have limited bioavailability for bacteria, and they remain in the environment as recalcitrant hydrocarbons (Chen and Taylor, 1995; Fayemiwo *et al*., 2017).

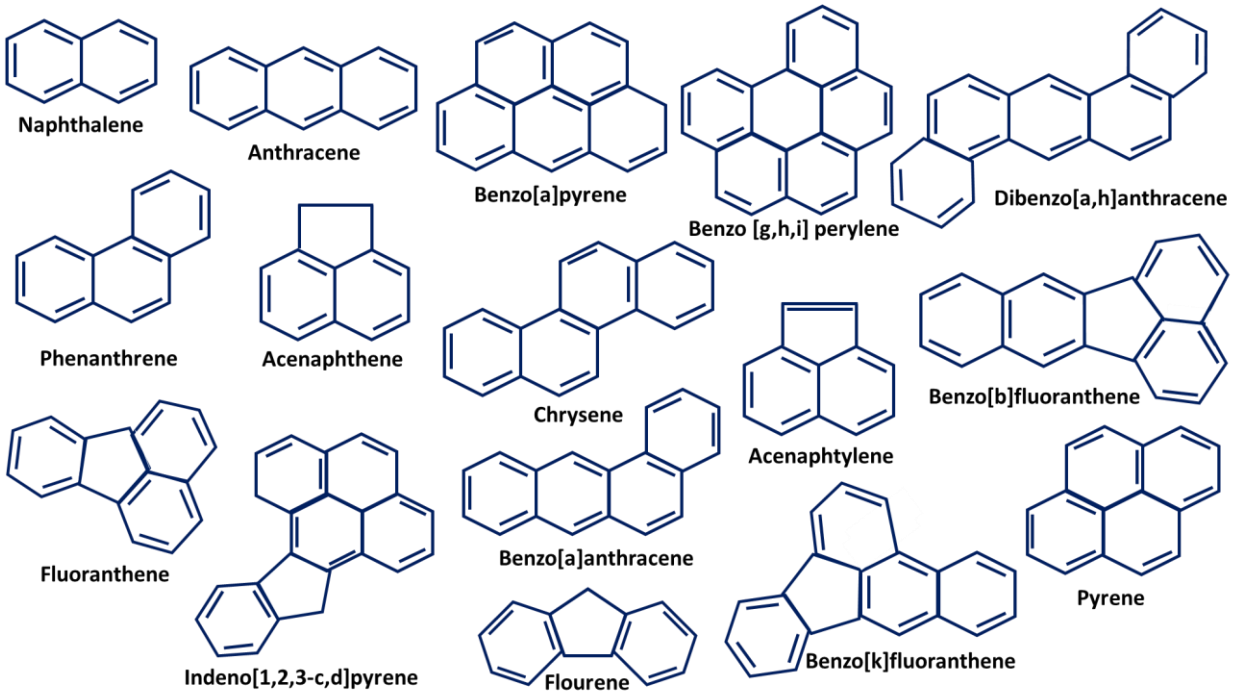


Figure 2: The 16 polycyclic aromatic hydrocarbons (PAHs) classified as priority pollutants by the United States Environmental Protection Agency (US EPA). Because of their highly hydrophobic and chemically stable natures, which increase with increasing molecular weight, they are difficult targets for bacterial degradation (Chauhan *et al*., 2008; Seo *et al*., 2009). As a result, they remain in the environment where they leach into water sources intended for human consumption. Ingestion of these compounds cause a range of detrimental defects on animal and plant health and are of great concern (Atlas and Hazen, 2011; Holliger *et al*., 1997; Ghosal *et al*., 2016).

Acyclic hydrocarbons (n-alkanes), formed exclusively by carbon and hydrogen atoms, are non-polar and very chemically inert (Labinger and Bercaw, 2002; Rojo, 2009). Because of their low solubility in water, their likelihood to accumulate in cell membranes, and the energy required to activate the molecule, some microorganisms may experience difficulty when utilizing them (Rojo, 2009; Singh, 2012). Nevertheless, n-alkanes, amongst the other aliphatic hydrocarbons, are the most readily degraded (Abbasian *et al*., 2015). Alkanes can be sorted into four different groups, namely the gaseous alkanes, and the aliphatic hydrocarbons with low molecular weight (C_8–C_{16}), medium molecular weight (C_{17}–C_{28}), and high molecular weight (>C_{28}) (Abbasian *et al*., 2015). n-Alkanes, C_{10}-C_{25} in length, are favoured as substrates and tend to be the most readily degraded (Maletić *et al*., 2011). By contrast, longer chained alkanes, C_{25}-C_{40} in length, are hydrophobic solids and are accordingly more difficult to metabolise owing to their poor water solubility and bioavailability (Abbasian *et al*., 2015;

Maletić *et al*., 2011, Nozari *et al*., 2018). These alkane types must first undergo enzymatic activation prior to degradation (Abbasian *et al*., 2015).

How bacteria respond to certain hydrocarbons, and in certain environments, can be easily understood upon comparison of the Exxon Valdez oil spill and the Deepwater Horizon oil spill and how naturally occurring bacteria allowed for bioremediation to occur. These oil spills were vastly different in terms of the nature of the oil, volume of the oil, and the environments impacted (Atlas and Hazen, 2011; Sylves and Comfort, 2012). The BP Deepwater Horizon occurred in a warm, subtropical climate that is prone to natural oil seeps; thus, bacteria native to this environment were already well-adapted to hydrocarbon degradation (Atlas and Hazen, 2011). Furthermore, the spill was a light crude which is inherently more biodegradable than the heavy crude oil spilt in the cold, sub-Artic climate of Alaska where the Exxon Valdez spill occurred (Atlas and Hazen, 2011; Sylves and Comfort, 2012). This reiterates the concept that the volume of oil leaked is not the sole factor in how easily or how well petroleum hydrocarbons are removed from the environment via bacterial biodegradation (Atlas and Hazen, 2011; Bamforth and Singleton, 2005; Chauhan *et al*., 2008; Holliger *et al*., 1997).

This project will focus on the bacterial degradation of n-alkane hydrocarbons. The focus on alkanes stems from the fact that they are major components (> 50%) of crude oil and are commonly found in oil-contaminated environments (Feng *et al*., 2007; Rojo, 2009; Singh, 2012). Because alkanes can demonstrate such chemical inertness, especially long-chain alkanes, their persistence in the environments poses a great threat (Feng *et al*., 2007; Singh, 2012). Therefore, biotechnological applications for bacterial degradation of these hydrocarbons, is of great interest (Feng *et al*., 2007).

1.3.3. Existing bacterial bioremediation strategies

Microbial bioremediation methods can be applied as either *ex situ* or *in situ*. *In situ* methods, are applied at the site of contamination (Gallego, 2001), for example, bioventing (**Table 1**) which is the controlled delivery of oxygen to unsaturated zones to increase bioremediation activities amongst naturally occurring microorganisms (Gallego, 2001). *Ex situ* methods, by contrast, require excavation of the contaminated environment and typically includes the use of bioreactors (Gallego, 2001).

Although *in situ* bioremediation strategies are thought to be less costly than *ex situ* strategies, they generally require the installation of equipment on-site and this is a costly process (Azubuike *et al.*, 2016). Additionally, *in situ* strategies require suitable environmental conditions such as appropriate temperature, pH levels and nutrient availability for a successful outcome to be achieved; this represents a hindrance to implementation of such a strategy as one cannot control the environmental conditions of the subsurface of polluted sites (Azubuike *et al*., 2016). In contrast, *ex situ* strategies which generally incorporate the use of bioreactors allow for: 1) operating parameters to be optimized; 2) the environment to be controlled more easily; 3) faster bioremediation times and 4) can be used to treat a wide range of pollutants (Azubuike *et al*., 2016). Regardless of the advantages and disadvantages associated with each method, methods are only selected and employed once the scenario, environment and contaminant type have been adequately assessed (Vidali, 2001).

1.4. Mechanisms of petroleum hydrocarbon degradation in bacteria

1.4.1. Hydrocarbon uptake

A major constraint on efficient hydrocarbon biodegradation is the limited bioavailability of hydrocarbons to bacteria, and limited direct contact between hydrocarbon substrates and the bacterial cell – a step necessary for oxygenases to function effectively (Xu *et al*., 2018). Most hydrocarbons are particularly hydrophobic and are insoluble in aqueous environments thus hindering their bioavailability to bacteria (Peixoto *et al.*, 2011; Xu *et al*., 2018; Yuniati, 2018). If bacterial cells were unable to take them up and use them, the environmental build-up of these hydrocarbons would cause toxicity to the microorganisms inhabiting this environment (Abbasian *et al*., 2015). Bacteria have developed a range of mechanisms such as the production and secretion of biosurfactants and bio-emulsifiers that increase their ability to target hydrocarbon substrates, and the expression of surface components that allows them to improve adhesion to petroleum hydrocarbons (Das and Chandran, 2011; Uzoigwe *et al*., 2015; Xu *et al*., 2018).

Biosurfactants are amphiphilic compounds composed of amino acids, sugars, fatty acids and functional groups, such as carboxylic acids, and usually have low molecular weights (Uzoigwe *et al*., 2015). They lower the surface and interfacial tension between hydrophobic and hydrophilic phases; causing more interaction and mixing between the phases (Uzoigwe *et al*.,

2015). Thus, in oil contaminated environments, biosurfactants work to increase effective dispersion and bioavailability of hydrophobic hydrocarbons for bacterial access and degradation (Das and Chandran, 2011; Uzoigwe *et al*., 2015).

Biosurfactants increase hydrocarbon bioavailability through a process termed micelle solubilization (Das and Chandran, 2011; Uzoigwe *et al*., 2015; Kaczorek *et al*., 2018). The surfactant surrounds the hydrophobic hydrocarbon (**Figure 3**) and owing to its amphiphilic nature, creates micelles with a hydrophobic core, thereby allowing for the accumulation of these hydrocarbons within the core (Das and Chandran, 2011; Uzoigwe *et al*., 2015; Kaczorek *et al*., 2018). Owing to this process, the solubility of hydrocarbons within an aqueous medium increases substantially and thereby enhances its bioavailability to bacterial cells (Das and Chandran, 2011; Uzoigwe *et al*., 2015; Kaczorek *et al*., 2018). It must be noted that not all biosurfactants secreted by bacteria are advantageous as not all of them assist in enhancing the degradation of the hydrocarbons (Xu *et al*., 2018). Furthermore, enhancement or inhibition of bioremediation, by biosurfactants, is dependent on a range of physico-chemical factors such as hydrocarbon type, properties of the biosurfactant and physiological characteristics of the hydrocarbon-degrading bacteria (Xu *et al*., 2018).

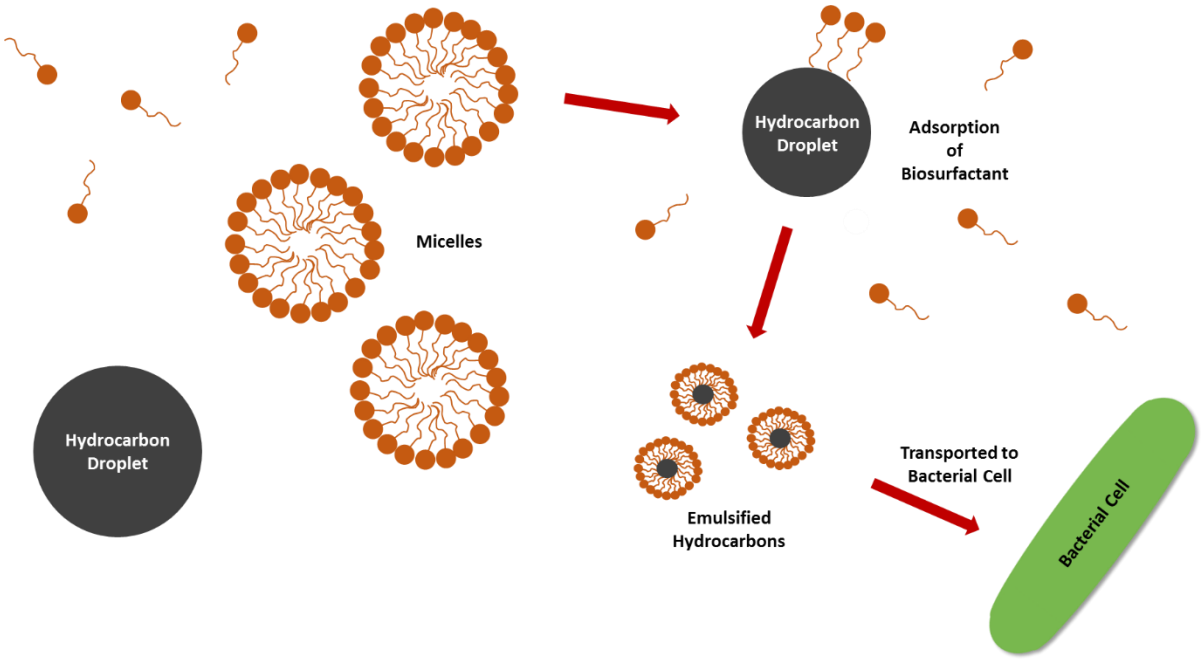


Figure 3: How biosurfactants act to form micelles around hydrophobic hydrocarbons. This action increases their bioavailability and makes it easier to transport across the bacterial

cell wall/membrane (Das and Chandran, 2011; Uzoigwe *et al*., 2015; Kaczorek *et al*., 2018). Here, the hydrophobic tails of the biosurfactant interacts with the hydrocarbon droplet creating a 'hydrophobic core' while their hydrophilic heads are free to interact with the aqueous medium (Das and Chandran, 2011; Uzoigwe *et al*., 2015; Kaczorek *et al*., 2018).

To improve uptake of specific hydrocarbons, bacteria also secrete substances called bio-emulsifiers (Uzoigwe *et al*., 2015; Xu *et al*., 2018). Bio-emulsifiers, in comparison to biosurfactants, have higher molecular weights and are composed of complex mixtures of lipopolysaccharides, heteropolysaccharides, proteins and lipoproteins (Uzoigwe *et al*., 2015). These molecules work to effectively emulsify immiscible liquids (Uzoigwe *et al*., 2015). Furthermore, they allow for the solubilization of substrates that possess poor solubility and in oil-polluted environments, they act by tightly binding to dispersed hydrocarbons to prevent them from aggregating - increasing their bioavailability to bacteria; this process is termed stabilization of emulsion (Uzoigwe *et al*., 2015).

Because hydrocarbons disturb bacterial cell membrane fluidity, membrane composition of the exposed bacterium must change for the phospholipid bilayer to achieve a stabilised state (Abbasian *et al*., 2015). Bacteria can alter their membrane composition to protect themselves and to attract insoluble hydrocarbons. Microbial adhesion to hydrocarbons (MATH) describes the affinity of microbial cells to adhere to a hydrophobic interface, a property which they change depending on the hydrocarbons in their environment (Kaczorek *et al*., 2018; Rosenburg *et al*., 1980). Bacteria do so by remodelling their outer cell layers to increase cell surface hydrophobicity; this allows biodegradable hydrocarbons, and other organic pollutants, to adhere to their surface more easily (Kaczorek *et al*., 2018; Van Hamme *et al*., 2003).

Remodelling may include changes to the saturated alcohols, lipopolysaccharides, intramolecular hydrogen, phosphoryl, amine, and carboxyl groups located on their cell surface (Kaczorek *et al*., 2018). Overall, this allows bacterial cells to utilise water-insoluble hydrocarbons through direct contact (Rosenburg *et al*., 1980). Regardless of the mechanism employed by the bacterium, biosurfactants, bio-emulsifiers and changes to cell surface play important roles in enhancing hydrocarbons bioavailability to the bacterial cells (Kaczorek *et al*., 2018).

1.5. Enzymatic pathways linked to hydrocarbon degradation

A range of bacteria are naturally capable of metabolising hydrocarbons as a source of carbon and energy (Atlas and Hazen, 2011; Rojo, 2009). Although biodegradation of both aliphatic and aromatic hydrocarbons may occur under aerobic or anaerobic conditions (Peixoto *et al*., 2011), bacteria mainly do so in aerobic conditions, as it allows for the most rapid and complete degradation of most organic pollutants (Das and Chandran, 2011). Some of these bacteria use hydrocarbons as a reserve and tend to utilise other sources such as amino acids, fatty acids, and sugars first (Rojo, 2009). Other bacteria use hydrocarbons as a primary source and are accordingly referred to as hydrocarbonoclastic bacteria (Rojo, 2009). Regardless of their trophic affinity for hydrocarbons, bacteria possess special enzyme-encoding genes that allow them to take up and metabolise these hydrocarbons (Das and Chandran, 2011; Nzila, 2018; Rojo, 2009).

Hydrocarbon-degrading bacteria have evolved over time and now contain a repertoire of genes encoding various enzymes, forming metabolic pathways, which allows them to deal with the versatility of molecular structures amongst hydrocarbons (Abbasian *et al*., 2015). Bacteria, through catabolism, convert hydrocarbons into simple organic molecules which they either process further or release into the environment to be further degraded, into non-hazardous compounds, by other microorganisms (Abbasian *et al*., 2015).

1.5.1. Aerobic bacterial degradation of monoaromatic and polyaromatic hydrocarbons

Aerobic bacteria capable of degrading monoaromatic BTEX compounds primarily belong to two phyla, the Actinobacteria (includes members of the genera *Rhodococcus* and *Mycobacterium*), and the *Proteobacteria* (including members of the genera *Pseudomonas* and *Azoarcus*) (Wang *et al*., 2010). These bacteria are more inclined to degrade the compound benzene – a widespread contaminant, and the most toxic amongst the BTEX compounds - a Group A human carcinogen according to the US EPA (Lovely *et al*., 1997).

Bacteria capable of degrading BTEX compounds, aerobically, synthesize the enzymes mono-oxygenase and/or dioxygenase (Abbasian *et al*., 2015; Vogt *et al*., 2011; Wang *et al*., 2010). Genes that encode these enzymes have been discovered in a variety of different bacterial strains (Vogt *et al*., 2011; Wang *et al*., 2010). These oxygenases catalyse the addition of oxygen

molecules and work to activate the stable aromatic nucleus of the monoaromatic hydrocarbon; the addition of oxygen yields various reaction intermediates (**Figure 4**), however, the final product of oxidation is generally catechol (Abbasian *et al*., 2015; Tao *et al*., 2004; Vogt *et al*., 2011; Wang *et al*., 2010; Van Hamme *et al*., 2003). Thereafter, a catechol dioxygenase is required to cleave the aromatic ring structure and create intermediates (Wang *et al*., 2010; Van Hamme *et al*., 2003). These intermediates are then oxidized further and degraded upon entry into the tricarboxylic acid (TCA) cycle to yield energy (Wang *et al*., 2010; Van Hamme *et al*., 2003).

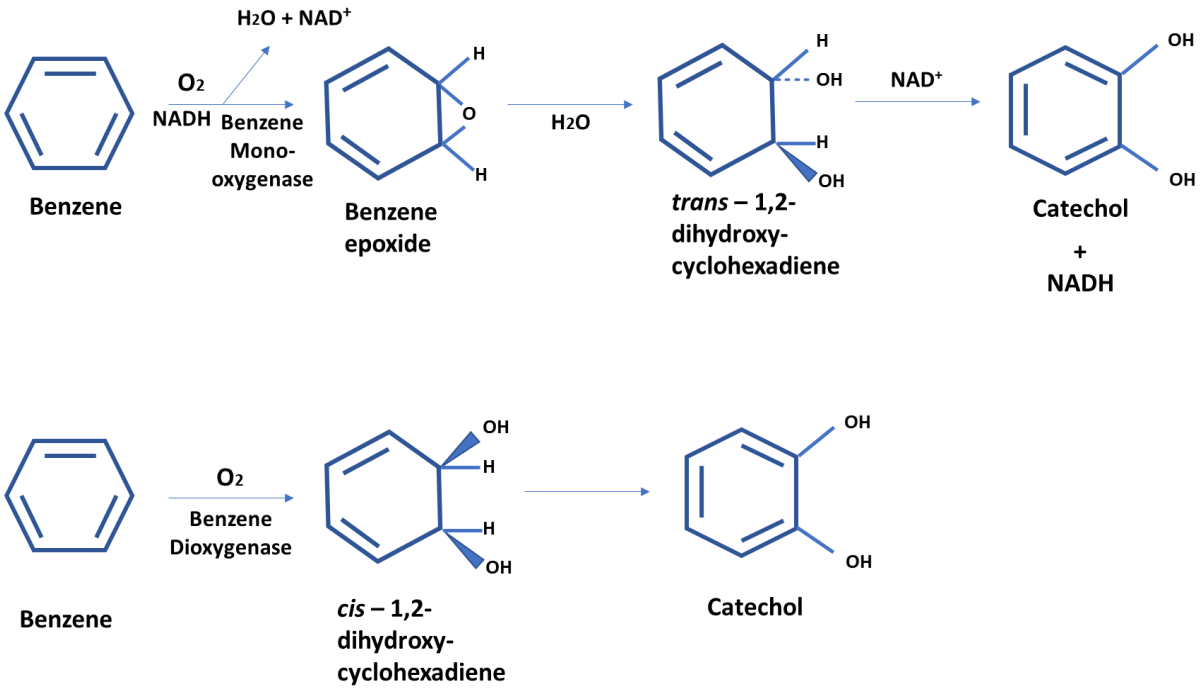


Figure 4: The possible ways, and intermediates, in which benzene can be aerobically degraded by bacteria (Mancini *et al.*, 2003). The final intermediate of this reaction is a catechol molecule.

With regards to polyaromatic hydrocarbons, naphthalene degradation is the most well-characterised system; this is owing to its high solubility and simple nature relative to other PAHs (Seo *et al*., 2009). As a result, this model is sometimes used to predict and understand pathways used in the degradation of other three-ringed PAHs (Seo *et al*., 2009). The model explores the degradation of the molecule naphthalene into salicylate, which is then further degraded and processed by the TCA cycle (Abbasian *et al*., 2015; Seo *et al*., 2009).

The first step of this pathway is catalysed by a dioxygenase enzyme which inserts two oxygen molecules into the aromatic structures of the PAHs; this enzyme works on a range of PAHs other than naphthalene, e.g. anthracene and phenanthrene (Abbasian *et al*., 2016; Seo *et al*., 2009). The resultant product undergoes a set of changes through interactions with different enzymes in the pathway (**Table 2**) before salicylate is formed (Abbasian *et al*., 2016; Seo *et al*., 2009). Salicylate is then processed into catechol which, as is the case in BTEX degradation, is further acted upon to create intermediates which are degraded upon entry into the TCA cycle (Abbasian *et al*., 2016; Seo *et al*., 2009; Van Hamme *et al*., 2003).

Table 2: A summary of the basic chemical reactions and enzymes responsible for the biodegradation of the polyaromatic hydrocarbon naphthalene.

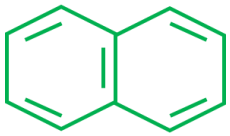
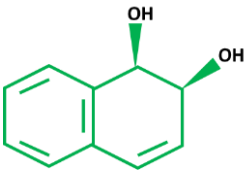
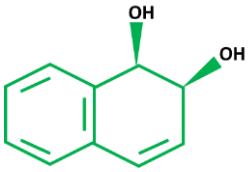
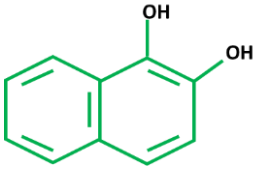
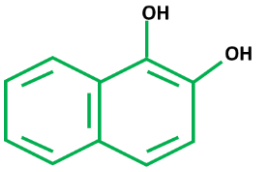
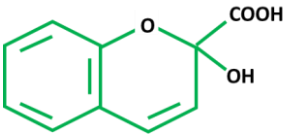
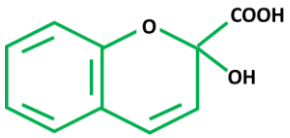
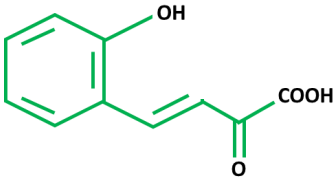
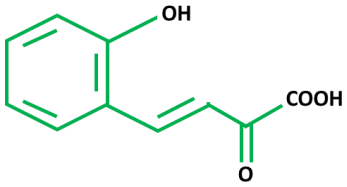
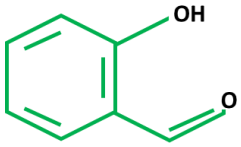
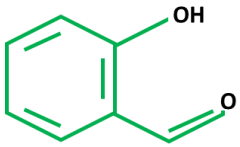
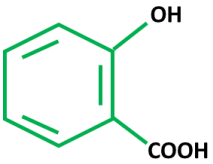
Molecule acted upon	Enzyme-type used	Enzyme	Main product
 Naphthalene	Dioxygenase	NahA	 *cis*-naphthalene dihydrodiol
 *cis*-naphthalene dihydrodiol	Dehydrogenase	NahB	 1,2-dihydroxynaphthalene
 1,2-dihydroxynaphthalene	Dioxygenase	NahC	 2-hydroxy-2*H*-chromene-2-carboxylic acid

Table 2 contd.: A summary of the basic chemical reactions and enzymes responsible for the biodegradation of the polyaromatic hydrocarbon Naphthalene.

Molecule acted upon	Enzyme-type used	Enzyme	Main product
 2-hydroxy-2*H*-chromene-2-carboxylic acid	Isomerase	NahD	 *trans*-o-hydroxybenzylidene pyruvate
 *trans*-o-hydroxybenzylidene Pyruvate	Hydratase-aldolase	NahE	 Salicylaldehyde
 Salicylaldehyde	Dehydrogenase	NahF	 Salicylate

(Abbasian *et al*., 2016; Seo *et al*., 2009; Van Hamme *et al*., 2003)

1.5.2. Aerobic bacterial degradation of alkane hydrocarbons

The best-characterized alkane degradation pathway was first discovered in *Pseudomonas putida* Gpo1 (Van Hamme, 2003). The genes which encode the enzymes of this pathway are carried on the OCT plasmid and are split into two operons, namely: *alkBFGHJKL* and *alkST (Dinamarca et al*., 2003; Van Hamme *et al*., 2003). The expression of the *alkBFGHJKL* genes is controlled by the action AlkS found in the *alkST* operon, which is located 9.7 kb away from the former operon (van Beilen *et al*., 2001; Van Hamme *et al*., 2003). The *alkBFGHJKL* operon encodes the proteins responsible for oxidation of the alkane substrate into to its fatty acid counterpart; this fatty acid then undergoes further catabolism through the action of β-oxidation before it can enter the TCA cycle (Dinamarca *et al*., 2003).

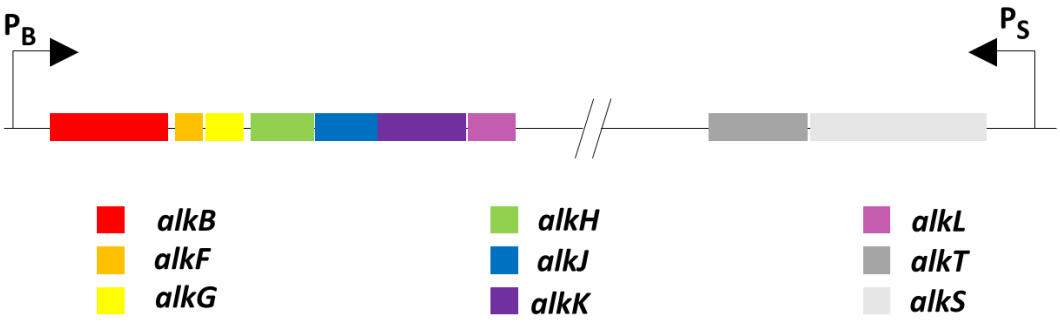


Figure 5: Diagrammatic representation of the *alkBFGHJKL* and *alkST* operons housed on the OCT plasmid of *Pseudomonas putida* Gpo1. These two operons, located 9.7 kb from each other, possess genes that encode for the most well-characterized aerobic alkane hydrocarbon degradation pathway in bacteria (van Beilen *et al*., 2001; Van Hamme *et al*., 2003). Here, gene names and their corresponding position on the operon are colour-coded.

When the bacterium detects the presence of alkanes, the transcriptional activator AlkS protein induces expression of the *alkBFGHJKL* genes from the promoter *PalkB* (P_B in **Figure 5**) and expression of the *alkST* genes from the promoter *PalkS2* (P_S in **Figure 5**) (Dinamarca *et al*., 2003; Wang and Shao, 2013). Because *alkS* activates the *PalkS2* promoter, thus inducing its own transcription, this pathway is said to be under the control of a positive feedback mechanism (Wang and Shao, 2013).

The second gene, *alkT*, yields a rubredoxin reductase enzyme that, when paired with the products of the *alkB* gene (a membrane-bound monooxygenase/hydroxylase enzyme) and *alkG* gene (a rubredoxin), forms an alkane hydroxylase system - the first enzyme of this alkane degradation pathway (Abbasian *et al*., 2016; Dinamarca *et al*., 2003; Van Hamme *et al*., 2003). The enzyme encoded by *alkB* is categorized as a rubredoxin-dependent monooxygenase (Abbasian *et al*., 2016; Dinamarca *et al*., 2003; Van Hamme *et al*., 2003) and therefore requires rubredoxin reductase and rubredoxin to act as an electron transporter subunit (Abbasian *et al*., 2016; Dinamarca *et al*., 2003; Van Hamme *et al*., 2003). These three proteins work together to shunt electrons to the monooxygenase (Van Hamme *et al*., 2003).

This shunt involves the transfer of electrons, by rubredoxin reductase (AlkT), from a NADH molecule to the rubredoxin protein (AlkG), followed by subsequent transfer to the membrane-bound monooxygenase (AlkB) (Rojo, 2005). This transfer prompts the monooxygenase to transfer a single oxygen atom, from an O_2, to one of the terminal methyl groups of the alkane

substrate (Rojo, 2005). The main product of this reaction is a primary alcohol while an H_2O molecule is produced as a by-product – a result of the reduction of the other oxygen atom by the electrons transferred by rubredoxin (Rojo, 2005; Van Hamme *et al*., 2003).

The primary alcohol is then further oxidized by the products of the *alkJ* gene (alcohol dehydrogenase) and the *alkH* gene (aldehyde dehydrogenase) to its corresponding aldehyde and fatty acid, respectively (Abbasian *et al*., 2016; Rojo, 2005; Van Hamme *et al*., 2003). The fatty acid is then conjugated to CoA – a reaction catalysed by the product of the *alkK* gene (acyl-CoA ligase/synthetase) (Abbasian *et al*., 2016; Rojo, 2005; Van Hamme *et al*., 2003). This product then undergoes β-oxidation to generate acetyl-CoA, the substrate that will finally enter the TCA cycle to generate energy (Rojo, 2005; Van Hamme *et al*., 2003). A diagrammatic representation of this process can be seen in **Figure 6** below.

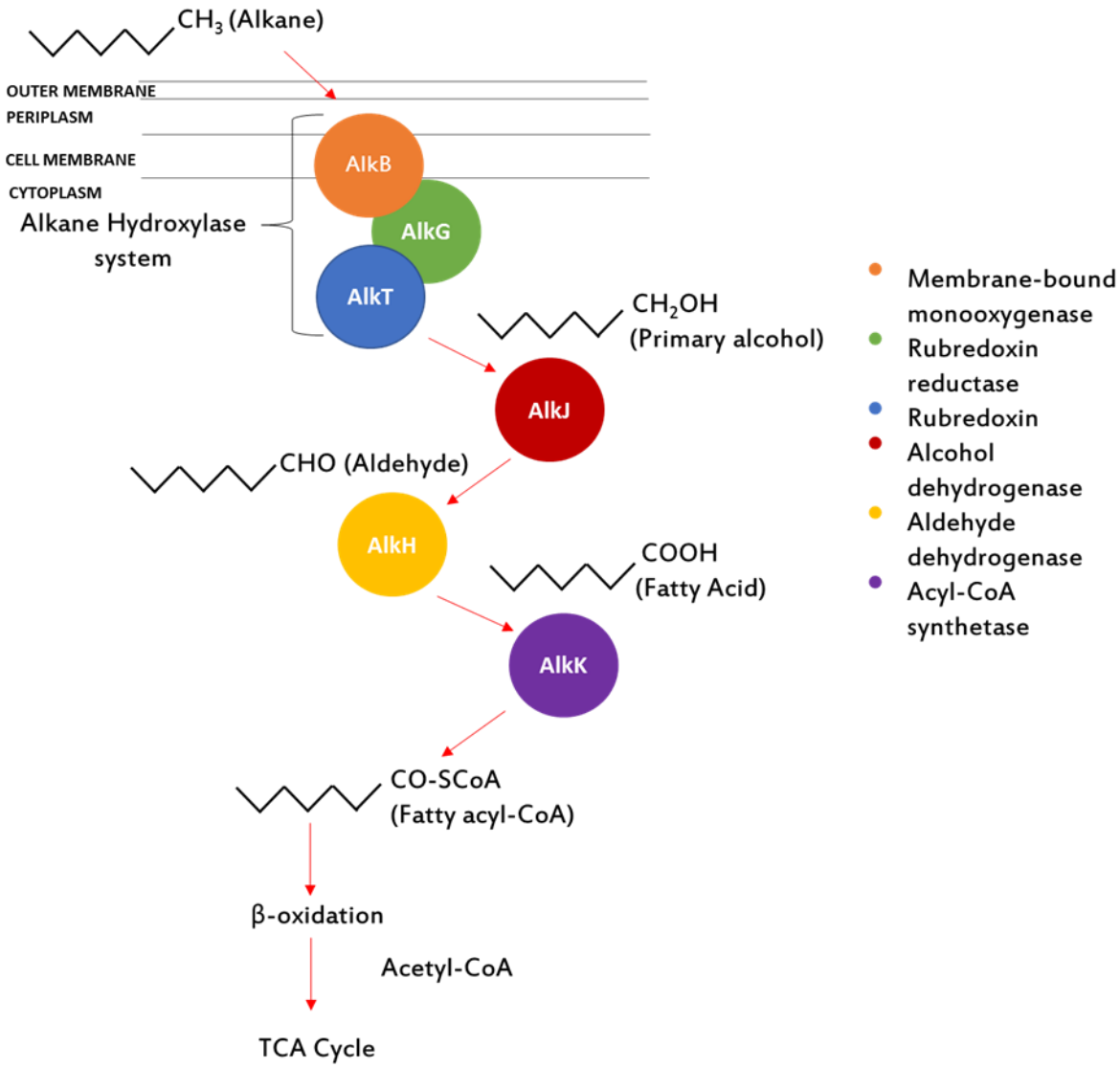


Figure 6: **Diagrammatic representation detailing the basic steps of the alkane hydrocarbon degradation pathway, first discovered in the bacterium *Pseudomonas putida* Gpo1.** This process highlights the main points in the transformation of an alkane → primary alcohol → aldehyde → fatty acid → fatty acyl-CoA → acetyl CoA which is finally processed in the tricarboxylic acid (TCA) cycle to yield energy.

Other notable genes contained on the OCT plasmid, which may play a part of the alkane degradation process, include the *alkF*, *alkL* and *alkN* genes. It has been discovered that the product of the *alkF* gene is a rubredoxin protein, however, its actual function remains unknown (van Beilen *et al*., 2002). The product of the *alkL* gene is an outer membrane protein that is thought to be involved in the uptake of alkanes, however, this has not yet been proven (Abbasian *et al*., 2016; Van Hamme *et al*., 2003). Lastly, the *alkN* gene, which is positioned

between the *alkBFGHJKL* and *alkST* operons, is postulated to produce a protein that may be involved in alkane chemotaxis (Abbasian *et al*., 2016; Van Hamme *et al*, 2003).

Although rubredoxin-dependent monooxygenases are well known, owing to the well-characterized *Pseudomonas putida* Gpo1 alkane degradation pathway, other types of unrelated monooxygenases exist and can catalyse the initial terminal hydroxylation of the *n*-alkane (Wang and Shao, 2013). For example, the cytochrome P450 monooxygenases, encoded by a CYP gene, and orthologues are found in both prokaryotes and eukaryotes (Das and Chandran, 2011). These enzymes also rely on the presence of O_2, NAD(P)H and an electron-transfer system(s) to oxidise a large variety of substrates (Girvan and Munro, 2016). Most of the bacterial P450 enzymes associate with ferredoxin reductase and ferredoxin as an electron-transfer system (Girvan and Munro, 2016). Although they do it through a different means, these enzymes still work to incorporate oxygen into the alkane substrates thus forming a primary alcohol (Das and Chandran, 2011). Hereafter, the same pattern can be observed: primary alcohol → aldehyde → fatty acid (Wang and Shao, 2013). This fatty acid will undergo β-oxidation to form acetyl-CoA which will be processed by the TCA cycle (Wang and Shao, 2013).

It is important to note that the bacterial enzymes responsible for introducing oxygen into a substrate – a step necessary for the commencement of biodegradation – display specificity towards alkanes of different lengths (**Table 3**; Das and Chandran, 2011; Rojo *et al*., 2009). Short-chained alkane degrading bacteria produce enzymes related to methane monooxygenases while medium-chained alkanes are generally catabolized by cytochrome P450s and membrane-bound monooxygenases, such as AlkB (Wang and Shao, 2013).

Table 3: The known oxygen-dependent monooxygenases/hydroxylases responsible of aliphatic hydrocarbon degradation in aerobic bacteria and the alkane lengths they are capable of degrading (Abbasian *et al*., 2015; Rojo, 2009).

Enzyme Type	Alkane Range Degraded
Methane Monooxygenases	C_1-C_5
Alkane Monooxygenases (AlkB-related)	C_5–C_{17}
Bacterial P450 oxygenase system (CY153-related)	C_5–C_{17}

The bacteria capable of degrading C_{10}–C_{20} alkanes generally encode enzymes that are related to the abovementioned AlkB or cytochrome P450 monooxygenases (Rojo, 2009). Though extensive studies are yet to be done, a few bacterial strains have been discovered to utilise alkanes larger than twenty carbons. In contrast, enzymes capable of degrading larger alkanes (>18) seem to be to completely unrelated to AlkB or CYP450 monooxygenases (Rojo, 2009; Wang and Shao, 2013). Examples of these unrelated monooxygenases include the flavin-binding monooxygenase, AlmA, found in the *Acinetobacter* strain DSM 17874 and the two-component flavin-dependent hydroxylase, LadA, discovered in the thermophile *G. thermodenitrificans* NG80-2 (Feng *et al*., 2007; Rojo, 2009; Throne-Holst *et al*., 2007; Wang and Shao, 2013).

AlmA displays great potential in its ability to oxidize alkanes as it is capable of metabolising alkanes longer than thirty-two carbons (>C_{32} alkanes) (Throne-Holst *et al*., 2007; Wang and Shao, 2013). The LadA enzyme can oxidize alkanes between the sizes of C_{15}–C_{36} (Feng et al., 2007; Rojo, 2009; Wang and Shao, 2013). This is a noteworthy discovery in long chained alkane degradation as these hydrocarbons are especially difficult to remove from the environment, owing to their poor water solubility and bioavailability, which causes them to form hydrophobic solids (Abbasian *et al*., 2015; Maletić *et al*., 2011, Nozari *et al*., 2018).

1.5.3. Anaerobic degradation of hydrocarbons

It is important to acknowledge that anaerobic biodegradation of petroleum hydrocarbons is a significant process employed by bacteria in anoxic conditions (Peixoto, *et al*., 2011). For instance, anoxygenic photosynthetic bacteria undergo anaerobic petroleum hydrocarbon degradation via Fe(III)-reducing and sulphate-reducing pathways (Peixoto *et al*., 2011; Van Hamme, *et al*., 2003). In this situation, the ferric cation (Fe^{3+}) and the sulphate anion ($SO4^{-2}$) replace oxygen as the terminal electron acceptor (Lagenhoff *et al*., 1996). Anaerobic biodegradation plays an important role in situations where oxygen is lacking e.g., in aquifers, mangroves and sludge digesters (Peixoto *et al*., 2011). Furthermore, multiple studies have shown that xylene and benzene (MAHs), phenanthrene and naphthalene (PAHs), branched alkanes, C_6 n-alkanes and many other hydrocarbons are capable of being degraded by bacteria under anaerobic conditions (Van Hamme *et al*., 2003).

1.6. Factors influencing petroleum hydrocarbon degradation

Oxygen and temperature are two of the most important factors that impact on the efficacy and rate of decomposition of hydrocarbons (Das and Chandran, 2011; Leahy and Colwell, 1990; Sihag *et al*., 2014). Petroleum hydrocarbons are difficult to degrade in anoxic environments; thus, the rates of degradation by anaerobic bacteria are often deemed negligible in comparison to aerobic bacteria (Atlas and Hazen, 2011; Leahy and Colwell, 1990). The first steps of cyclic, aromatic and aliphatic hydrocarbon catabolism by bacteria, involves the oxidation of the substrate by enzymes called oxygenases, as detailed in section 1.5, which require molecular oxygen to function (Leahy and Colwell, 1990).

Aerobic microorganisms are therefore generally best suited for fast and complete degradation of most hydrocarbon pollutants (Das and Chandran, 2011; Leahy and Colwell, 1990). The aerobic taxa that demonstrate the most potential for hydrocarbon bioremediation include species of the genera *Acinetobacter, Arthrobacter*, *Bacillus, Mycobacterium, Pseudomonas* and *Pseudonocardia* (Rojo, 2009). These bacteria synthesize oxygenases, as well as a variety of oxygen-dependent peroxidases (Abbasian *et al*., 2015; Das and Chandran, 2011; Vidali, 2001), which utilise oxygen as a substrate in the catabolic process of hydrocarbon degradation (Leahy and Colwell, 1990).

The taxa described above are primarily mesophilic in nature: i.e., they are optimally active at ambient temperatures between 20°C and 45°C (Malik, *et al*., 2013). However, it has been observed that the rate of hydrocarbon biodegradation is greater at elevated temperatures (Das and Chandran, 2011). This is because higher temperatures influence both the physical nature and chemical composition of the oil – it does so by decreasing the viscosity of the oil thereby increasing its bioavailability to microbial degradation (Leahy and Colwell, 1990). Furthermore, higher temperatures enhance the volatility of toxic, low molecular weight, short-chain alkanes by decreasing their water solubility and increasing the onset of biodegradation (Das and Chandran, 2011). It may therefore be beneficial to utilise thermophilic bacteria, which naturally thrive at higher temperatures (>50˚C), in bioremediation strategies (Mohammad *et al*., 2017).

1.7. Thermophilic microorganisms and their presence in bioremediation

Thermophilic microorganisms are frequently isolated from high temperature environments, which include oil wells and subterranean oil reservoirs (Kato *et al.*, 2001; Sorkhoh *et al.*, 1993; Zeinali *et al.*, 2008). They can withstand high temperatures (>50˚C) owing to their ability to synthesize thermostable enzymes (Haki and Rakshit, 2003; Mohammad *et al*., 2017). These enzymes are especially attractive in bioindustry as they are generally resistant to proteolysis (Haki and Rakshit, 2003).

Thermostable enzymes are used in bio-industrial processes such as paper production (xylanases used in pulp production), food flavouring (lipases used to produce esters) and pharmaceutical production (Haki and Rakshit, 2003). This is mainly because the reaction kinetics of thermophilic bacteria provide higher diffusion rates, higher mass transfer rates, and faster chemical reaction rates which would result in a higher turnover rate (Nzila, 2018; Zhou *et al*., 2018).

There are thus possible advantages in the usage of thermophilic bacteria, and their enzymes, over mesophilic bacteria in the biodegradation of petroleum-derived contaminants (Xia *et al.*, 2015). However, over the past 20 years, there have been only a handful of studies to support such endeavours (**Table 4**; Nzila, 2018). Furthermore, reviews on thermophilic bacteria and hydrocarbon degradation generally omit information pertaining to the mechanisms of degradation, thorough analyses of the characteristics of the hydrocarbon-degrading thermophilic bacteria, and comparative information between mesophilic and thermophilic bacteria with respect to hydrocarbon degradation (Nzila, 2018).

Table 4: A list of hydrocarbon-degrading aerobic thermophilic bacteria discovered since 1995.

Thermophile Name	Location Sourced	Optimum Temperature	Respiration Type	Hydrocarbon Type Being Degraded
***Thermus aquaticus* ATCC 25104** ***Thermus* sp. ATCC 27978** (Chen and Taylor, 1995)	Hot spring; U.S.A. and Unspecified	70 ˚C and 60 ˚C	Aerobic	Degradation of BTEX
***Geobacillus thermoleovorans* B23 and H41** (Kato *et al*., 2001)	Deep Petroleum Reservoirs; Japan	70 ˚C and 65 ˚C	Facultative Anaerobic	C_{13}–C_{26} n-alkanes
***Pseudomonas aeruginosa* AP02-1** (Perfumo *et al*., 2006)	Sulphataric Hot Spring; Viterbo, Italy	45 ˚C	Aerobic	C_5–C_{19} n-alkanes; MAHs and PAHs (anthracene, naphthalene)
***Geobacillus thermodenitrificans* NG80-2** (Feng *et al*., 2007)	Subterranean oil reservoir; China	65 ˚C	Facultative Anaerobic	C_{15}–C_{36} n-alkanes
***Nocardia otitidiscaviarum* TSH1** (Zeinali *et al*., 2008)	Petro-industrial wastewater soil; Iran	50 ˚C	Aerobic	Naphtalene, Anthracene and Phenanthrene
***Geobacillus* sp. SH-1** (Zhang *et al*., 2012)	Soil from Oil Well; China	60 ˚C	Facultative Anaerobic	Naphtalene and C_{12}–C_{23} n-alkanes
***Geobacillus toebii* B-1024, *Geobacillus* sp. 1017, *Aeribacillus pallidus* 8m3** (Tourova *et al*., 2016)	Dagang oilfield and Thermal Spring; Russia	60 ˚C	Aerobic	C_{10}–C_{30} n-alkanes, C_{13}–C_{19} n-alkanes, C_{11}–C_{29} n-alkanes
***Geobacillus stearothermophilus* A-2** (Zhou *et al*., 2018)	Dagang Petroleum Resevoir	60-65 ˚C	Facultative Anaerobic	>C_{21} n-alkanes

Unfortunately, even though the possible advantages of thermophilic bacteria in hydrocarbon biodegradation have been outlined in some experiments, strong experimental evidence is still lacking (Nzila, 2018). This means that very little is known about the degradation capacity or mechanisms of hydrocarbon degradation amongst thermophilic taxa (Das and Chandran, 2011; Nzila, 2018; Zhou et al., 2018). Some of the genes and pathways found in hydrocarbon-degrading thermophilic bacteria, were first discovered in hydrocarbon-degrading mesophilic bacteria (Nzila, 2018).

Although some novel genes have been discovered in hydrocarbon-degrading thermophilic bacteria (Feng *et al*., 2007; Rojo, 2009; Throne-Holst *et al*., 2007; Wang and Shao, 2013), there is still a lot to discover about the metabolic pathways used by these bacteria. The enzymes and pathways these thermophilic bacteria employ to survive in high temperature environments must therefore be explored and then exploited to improve bioremediation strategies. Such strategies could include a bioreactor-based system where bioremediation can be undertaken by whole-cell bacteria in a parameter-controlled setting (Azubuike *et al.,* 2016). This would allow for maximization of hydrocarbon degradation as oxygen concentration and temperature levels can be altered to a state where bacteria achieve optimal activity (Vieille and Zeikus, 2001).

Alternatively, the production of recombinant mesophilic hosts to carry and express the necessary genes to yield thermophilic protein/enzymes that can retain their thermal properties (Vieille and Zeikus, 2001). The expression of these thermophilic enzymes provides several advantages over the use of mesophilic enzymes. They are easier to purify via heat treatment and have a greater resistance to chemical denaturants (Vieille and Zeikus, 2001). Furthermore, the ability of the recombinant bacteria to react at higher temperatures allow for greater kinetic reaction rates as well as a lower risk of contamination (Vieille and Zeikus, 2001). All in all, the use of aerobic thermophilic bacteria in hydrocarbon bioremediation could provide great versatility, in terms of types of applications.

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Chapter 2

The screening and identification of hydrocarbon-degrading bacterial strains

Abstract

Thermophilic bacteria show great potential in petroleum hydrocarbon bioremediation as they are able to yield thermostable enzymes which possess favourable enzyme kinetics. Additionally, some thermophilic bacteria secrete biosurfactants to enhance the dispersion and bioavailability of hydrophobic hydrocarbons. Here, a collection of 138 aerobic thermophilic bacteria were screened for their capacity to degrade oil hydrocarbons. This was done through liquid assays and plate-based biosurfactant production. From a total of 65 strains which were positive for hydrocarbon degradation, just three strains, *Bacillus licheniformis* But5A1A, But5A1B and Wam9A3, were selected for their superior degradation capacities. Moreover, *Bacillus licheniformis* But5A1A which was found to be the best degrader according to the liquid assays, yielded substantial amounts of biosurfactant as observed in plate-based assays where extensive oil displacement occurred. This suggests that the secretion of this biosurfactant had a substantive influence on the bacteria's ability to degrade the petroleum hydrocarbons.

2.1. Introduction

Thermophilic bacteria include a broad range of aerobic and anaerobic taxa (**Table 1**) that are able to withstand high temperatures (Satyanarayana *et al*., 2013; Zeikus, 1979a). These thermophiles have been isolated from a wide range of both natural and man-made environments (Canganella and Wiegel, 2014; Satyanarayana, *et al*., 2013). Natural thermal habitats include volcanic sites, petroleum reserves, sun-heated soils, deep-sea hydrothermal vents, terrestrial hot springs and geothermal areas (Canganella and Wiegel, 2014; Satyanarayana *et al*., 2013). Man-made environments include biological wastes, coal refuse piles, acid mine effluents and compost heaps (Canganella and Wiegel, 2014; Satyanarayana *et al*., 2013).

Table 1: Examples of aerobic and anaerobic thermophilic bacterial species, the temperature optima (˚C) at which they operate and their primary niche.

Aerobe vs anaerobe	Species	Temperature optima (˚C)	Primary habitat of isolation
Aerobe/facultative aerobe	*Geobacillus stearothermophilus*	70-85	Soils (Gibson and Gordon, 1974)
	Thermus Aquaticus	79	Thermal springs (Brock and Freeze, 1969)
	Thermomicrobium roseum	85	Thermal springs (Jackson *et al*., 1973)
	Alicyclobacillus acidocaldarius	70	Acidic soils (Darland and Brock, 1971)
Obligate anaerobe	*Clostridium thermohydrosulfuricum*	70	Soils (Wiegel *et al*., 1979)
	Clostridium thermocellum	70	Soils, manure (Ng *et al*., 1977)
	Thermoanaerobacter brockii	75	Thermal springs (Zeikus *et al*., 1979b)
	Desulfovibrio thermophilus	85	Geothermal waters (Rozanova and Nazina, 1979)

Thermophilic bacteria have garnered extensive interest, particularly in the field of biotechnology, where these organisms, or the enzymes they produce, can be applied in a broad range of biotechnological and industrial applications (Haki and Rakshit, 2003). This interest stems from the numerous physiological and structural features that allow thermophiles to tolerate extreme temperatures (Canganella and Wiegel, 2014). Thermophiles synthesise a range of thermostable biomolecules called thermozymes. These thermostable enzymes are generally more resistant to denaturation and proteolysis and show more favourable enzyme kinetics than their mesophilic counterparts and are thus of value in high temperature industrial applications (Canganella and Wiegel, 2014; Haki and Rakshit, 2003). Furthermore, the use of thermophiles themselves in biotechnological processes operated at elevated temperatures reduces the risk of contamination, increases the bioavailability of substrates and reduces the energy costs associated with these processes (Haki and Rakshit, 2003).

One area of thermophile research that is gaining attention is the use of thermophiles for the bioremediation of petroleum hydrocarbons or crude oils (Nzila, 2018). A broad range of

thermophilic microorganisms, including members of the genera *Geobacillus, Bacillus, Thermus* and *Aeribacillus*, have been shown to possess efficient metabolic capabilities for the degradation of hydrocarbons (Nzila, 2018). Thermophilic bacteria have also been found to secrete biosurfactants which play an important role in enhancing the dispersion and bioavailability of hydrophobic hydrocarbons (Das and Chandran, 2011; Uzoigwe *et al*., 2015).

Here, with the benefits of bioindustrial application of thermophiles in bioremediation strategies in mind, we have screened a large collection of thermophilic bacteria for their ability to degrade hydrocarbons. A substantial number of the tested strains showed extensive capacities for the degradation of oil hydrocarbons. Some of these strains appear to produce a biosurfactant or bioemulsifying compound which may play a role in hydrocarbon degradation. We also evaluated the effect of culture age on hydrocarbon degradation activities and show that older cultures generally display a greater oil degrading phenotype. Overall, we have established that thermophilic show great potential for hydrocarbon degradation which may be employed in future bioremediation applications.

2.2. Materials and Methods

2.2.1. Culturing of thermophilic bacteria

A culture collection comprising 166 unclassified aerobic thermophilic bacteria, was provided by Professor Don Cowan, Centre for Microbial Ecology and Genomics at the University of Pretoria, South Africa. Bacteria in this collection were previously isolated from various high temperature sites (50-80°C), over a period of 15 years and were maintained as lyophilised stocks. The lyophilised cultures were re-constituted on Luria Bertani (LB) agar: 15 g bacteriological agar, 5 g sodium chloride (NaCl), 10 g casein hydrolysate acid, 5 g yeast extract powder and 1L distilled water and 1.25 mL sodium hydroxide (NaOH, 10 w/v%); autoclaved at 120˚C for 20 minutes. These cultures were further preserved in 25% v/v glycerol stocks stored at -80°C.

2.2.2. Screening the bacteria to determine petrochemical degrading capability

2.2.2.1. Identifying potential hydrocarbon degraders via liquid assays

Liquid assays were conducted using an adaptation of the strategy of Rahman *et al*. (2002). In brief, isolated colonies of the bacterial strains grown on LB agar were inoculated in sterile MacConkey bottles containing 10 mL of LB broth: 5 g sodium chloride (NaCl), 10 g casein hydrolysate acid, 5 g yeast extract powder in 1L distilled water and sodium hydroxide (NaOH, 10 w/v%); autoclaved at 120˚C for 20 minutes. The inoculated LB broth, stored in MacConkey bottles, were incubated at 56˚C for 20 hours under continuous agitation (150 rpm). A volume (200 µL) of autoclaved motor oil was then added to each inoculum and incubated at 56˚C for 20 hours under continuous agitation.

The oil-cultures were evaluated for the oil emulsifying power of the bacteria 20 and 120 hours post-inoculation (Liu *et al*., 2016). Motor and crude oil are water immiscible, forming a single globular mass at the water-air interface. However, degradation of the oil hydrocarbons by bacteria will lead to dispersed mixing of the oil with the water-based medium, which can be observed in the liquid medium upon agitation. (Liu *et al*., 2016). Two rounds of screening were conducted using the above liquid assays. In the first round, the hydrocarbon degradation capacities of 138 viable strains were evaluated using a single assay. From these results twenty strains were sub-selected and these were screened in triplicate.

Controls consisting of only LB broth and 200 µL of autoclaved motor oil were included in all screening procedures to exclude motor oil contamination and provide a comparative basis for the experiments. Furthermore, *Geobacillus thermodenitrificans* DSM 465^{T} was used as an additional negative control as this strain is naturally unable to degrade alkane hydrocarbons (Yao *et al*., 2013).

2.2.2.2. Petrochemical degrading ability versus age of inoculum

To assess the effect of inoculum age on hydrocarbon degradation, the same liquid assay protocol was utilised on older bacterial cultures. Pre-cultures of the twenty strain sub-selection were grown in triplicate for either 20 or 192 hours, prior to the addition of 200 µL of autoclaved oil. These results were also visually evaluated according to their emulsification powers.

2.2.3. Screening for biosurfactant production

Freshly prepared LB agar plates were coated with 500 µL autoclaved motor oil using a sterile glass spreader as per Ahmed *et al.* (2010). A sterile inoculating loop was then used to streak single colonies of each bacterial strain (in triplicate) onto the oil-coated LB agar plates. The oil-coated plates were incubated 56 ˚C for 20 hours. After this incubation period, zones of clearance around the streaks were visually assessed and compared. A scoring system was designed according to whether there were no zones of clearance (N), small zones of clearance or large zones of clearance (L) around the bacterial streaks.

To validate the results of the streak plates, a second assay was developed in-house where LB agar plates were first coated, using a sterilised glass spreader, with 500 µL autoclaved motor oil. A sterile inoculating needle was used to stab a single colony of the evaluated bacteria directly from the culture plate into the oil-coated agar. This assay was performed in triplicate for each sample and the plates were incubated at 56˚C for 20 hours. After this incubation period, zones of clearance around the stab sites were visually assessed for biosurfactant production while the diameter of these zones were measured for comparative purposes.

2.3. Identification of selected strains

2.3.1. DNA extraction

Genomic DNA (gDNA) was extracted from the twenty strains included in the second round of screening using the *ABIOpure*™ Total DNA Blood/Cell Extraction Kit version 2.0 (AllianceBio, U.S.A.) following the Gram-positive work-flow. In brief, a generous amount of bacterial culture was suspended in 200µL dH_2O and thereafter pelleted by centrifugation at a maximum speed of 13,000 rpm for 1 minute. The supernatant was discarded, and the pellet was thoroughly resuspended in 180 µL of 30mg/mL prepared enzyme mixture (a combination of lysozyme and GP buffer provided) and incubated in a heating block at 37˚C for one hour. Subsequently, 20 µL of Proteinase K solution (20 mg/mL) and 200 µL of BL buffer (both provided by kit) were added and the lysates were vortexed briefly. The samples were then incubated in a heating block at 56˚C for 30 minutes and then at 75˚C for a further 30 minutes. The samples were then briefly centrifuged in a microfuge to make sure that no sample remained on the inside of the lid of the microcentrifuge tube. Hereafter, 200 µL of ice-cold absolute

ethanol was added to the mixture which was then pulse-vortexed and centrifuged at low speed. The sample was finally transferred to a mini-column and centrifuged at 10, 000 rpm for 1 minute. Two washes, with BW buffer and LTW buffer (*ABIOpure*™ Total DNA Blood/Cell Extraction Kit) were followed with centrifugation for 1 min at maximum speed. Finally, the gDNA was eluted by adding 35 µL of AE buffer (*ABIOpure*™ Total DNA Blood/Cell Extraction Kit), allowing it to rest for 3 minutes and then finally centrifuging at maximum speed for 1 minute. The DNA extractions were resolved on a gel (1% w/v agarose gel in 1xTBE buffer) to confirm the presence of DNA prior to gene amplification.

2.3.2. 16S rRNA gene amplification and sequencing

A ~1490 bp fragment of the 16S rRNA was amplified using polymerase chain reaction (PCR) amplification with the MyTaq™ Polymerase Kit (Bioline, U.S.A.) following the manufacturer's instructions. Amplification was done with 0.5 µL (in 25 µL final volume) of the universal 16S rRNA primers 8F (5'-AGAGTTTGATCCTGGCTA-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Brosius *et al*., 1981; Lane, 1991), respectively. PCR amplification was performed using the Biorad MyCycler^{TM} thermal cycler (Biorad, U.S.A.) with the cycle program as follows: initial denaturation at 95˚C for 5 minutes; 30 cycles of 30 seconds at 95˚C, 30 seconds at 55˚C and 90 seconds at 72˚C; final extension at 72 ˚C for 10 minutes. The PCR amplicons were evaluated under UV light after gel electrophoresis (1% w/v agarose gel in 1xTBE buffer) for 25 min at 100V. The amplicons were sequenced using a standardized Sanger sequencing protocol at Inqaba Biotec., Pretoria, South Africa.

The raw sequences were trimmed and edited using BioEdit v. 7.2.5 (Hall, 1999). The sequences were classified by comparison against the Ribosomal Database Project (RDP) using the SeqMatch tool and BlastN analysis against the NCBI 16S ribosomal RNA sequences (Bacteria and Archaea) database.

On the basis of the Seqmatch and BlastN analyses, reference 16S rRNA sequences which represented closest matches to the test strains were obtained from the NCBI nucleotide database. The test strain and reference sequences were aligned using the MAFFT server (Katoh and Standley, 2013) and trimmed using BioEdit v. 7.2.5. The 274 nucleotide position alignment was used to generate a Maximum-Likelihood phylogeny using PHYML-SMS (Lefort *et al*., 2017) with bootstrap support (n = 1,000 replicates).

2.4. Results and Discussion

2.4.1. Petrochemical degradation capabilities

2.4.1.1. Evaluation of the hydrocarbon degradation capacity of thermophilic bacteria

A collection comprising of 166 thermophilic strains isolated from a diverse range of high temperature environments was incorporated in this study. Of these, 138 strains were viable, producing growth on LB agar plates. These strains were subjected to liquid oil degradation assays and evaluated on the basis of the oil emulsifying capabilities/power of each bacterial isolate. The emulsifying power can be described as the degree to which the hydrophobic oil disperses into the water-based medium upon mixing (Liu *et al*., 2016) (**Figure 1**).

Of the 138 strains, a total of 63 strains showed definitive signs of oil degrading activity while the remaining 75 strains showed very little to no activity (**Figure 2**). Of the 63 strains able to degrade oil, thirteen produced strongly positive reactions while the remaining fifty yielded moderately positive reactions (**Figure 2**).

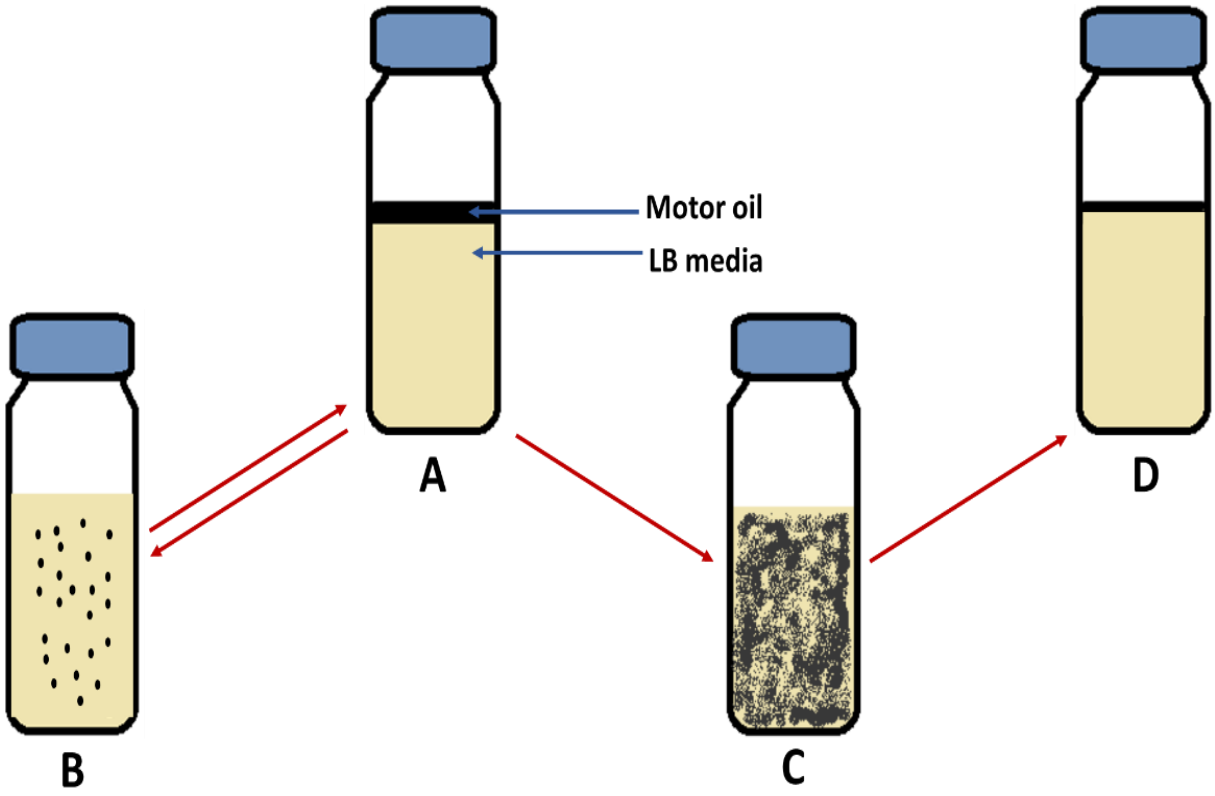

Figure 1: Schematic representation of the oil bio-emulsification activity of bacterial strains. Sample A shows the water-immiscible oil above the liquid column. Upon agitation, the oil droplets in the negative control and strains which showed no oil degradation activity travelled into the liquid column (Sample B), but rapidly reverted to the surface (Sample A). By contrast, upon agitation when a hydrocarbon degrading strain is present (positive result - Sample C), no distinct oil droplets could be observed in the liquid column, but small asphaltenes could be observed. After agitation, the oil droplets in samples containing test strains which were positive for oil degradation again reverted to the immiscible layer at the top of the liquid column (Sample D), but substantially less oil was observed at the top of the liquid column. Of note is that it took substantially longer for oil to return to the water-immiscible phase above the liquid column in positive samples (Sample D) than negative and control samples (Sample B).



Figure 2: Examples of the results obtained from the liquid assays. Upon agitation, large oil droplets dispersed into the liquid medium in negative controls and samples where the test strains did not degrade the oil (Sample 1). Sample 2 shows a moderately positive reaction, with the oil droplets returning to the immiscible oil layer above the liquid column within minutes of agitation. Sample 3 shows a strong positive reaction, where a smaller oil layer reformed at the top of the medium and the medium remained cloudy after agitation.

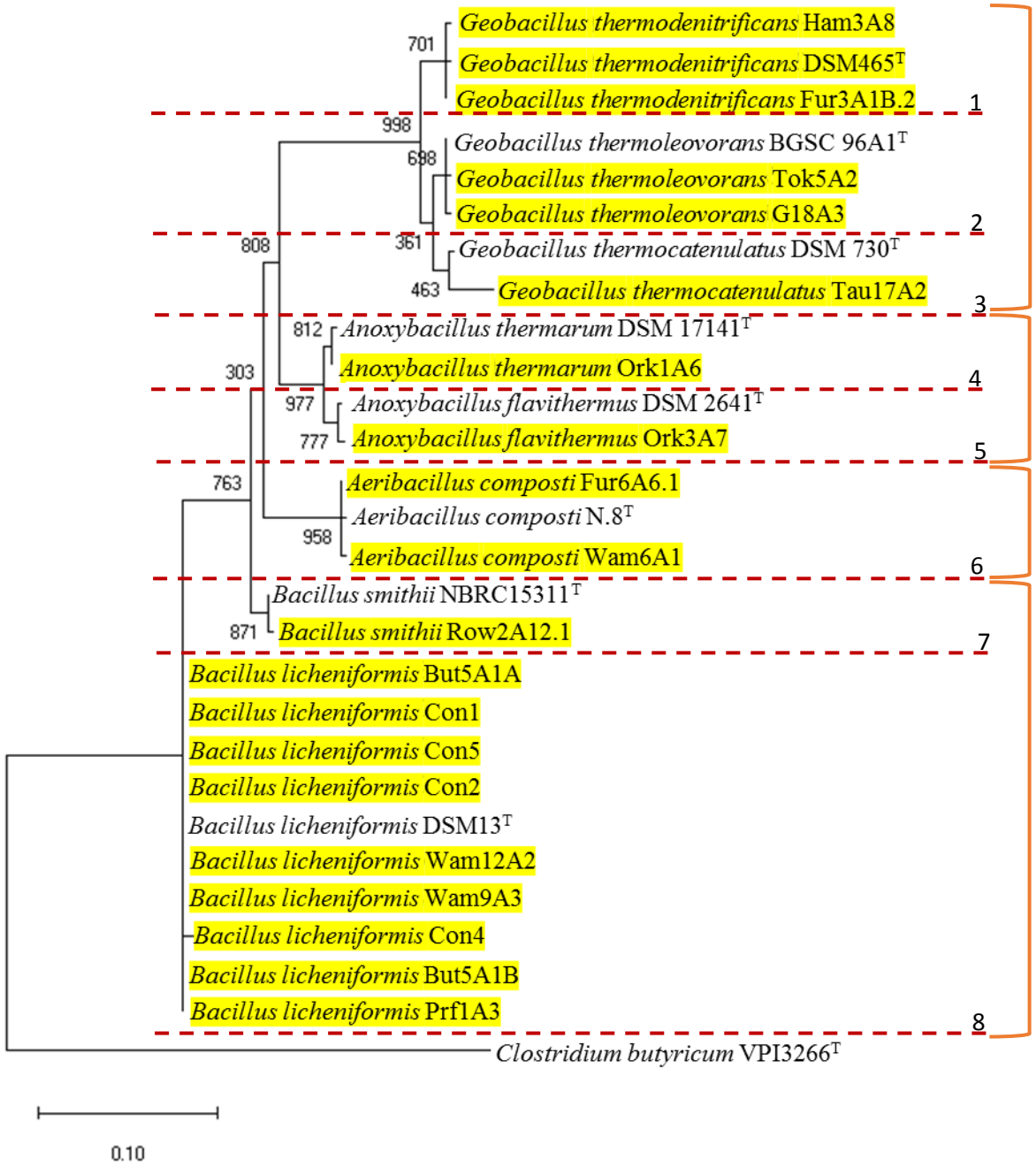

Figure 3: 16S rRNA phylogenetic tree of the twenty strains used in the sub-set (highlighted in yellow). This Maximum-Likelihood tree (1000 bootstraps) was created using PHYML-SMS. Here, the type strain *Clostridium butyricum* VPI3266^T was used as an outgroup. All the strains in the subset belong to the family *Bacillaceae* with a total of four genera (indicated by the brackets) and eight species (numbers 1-8) represented within the subset.

A sub-selection of twenty strains was made on the basis of distinct colony morphologies on LB agar and on the oil emulsification reaction in the liquid assays. These strains were identified on

the basis of their 16S rRNA gene sequences (**Figure 3**). All twenty strains belong to the family *Bacillaceae*, with the majority belonging to the genus *Bacillus* (**Figure 3, Table 2**). Nine of these strains share 98.28-99.87% nucleotide identity with the type strain of *Bacillus licheniformis* DSM13^{T} and could thus be ascribed to this species, while one further strain, *Bacillus smithii* Row2A12.1, shares 99.81% sequence identity with *Bacillus smithii* NBRC 15311^{T}.

The remaining nine strains (excluding *G. thermodenitrificans* DSM 465^{T}) belong to three distinct genera, namely *Anoxybacillus, Aeribacillus* and *Geobacillus* (**Table 2**). Two strains, Wam6A1 and Fur6A6.1, were assigned to the species *Aeribacillus composti*, sharing 98.89% and 99.63% nucleotide identity with *A. composti* N8^{T}. A further two strains were allocated to two different species in the genus *Anoxybacillus* with Ork1A6 and Ork3A7 sharing 99.44% and 98.88% sequence identity with the type strains of *A. thermarum* and *A. flavithermus*, respectively. The remaining five strains belong to three different species in the genus *Geobacillus*, namely *G. thermodenitrificans* (Ham3A8, Fur3A1B.2), *G. thermoleovorans* (Tok5A2, G18A3), and *G. thermocatenulatus* (Tau17A2) (**Figure 3, Table 2**).

Table 2: A summary species, sequence identity and isolation source of the twenty sub-selected strains.

Strain code	Species	Sequence Identity	Isolation Source	Colony Morphology (LB agar at 56˚C for 20 hours)
1. Fur6A6.1	*Aeribacillus composti*	*Aeribacillus composti* N.8^T (99.63%)	Furnas, Azores	Small, raised, shiny, circular, cream white
2. Wam6A1	*Aeribacillus composti*	*Aeribacillus composti* N.8^T (98.89%)	Waimangu NZ	Small-medium, raised, shiny, circular, cream white, entire
3. Ork3A7	*Anoxybacillus flavithermus*	*Anoxybacillus flavithermus* (98.88%)	Orakei Korako, NZ	Small, circular, entire, yellow cream, raised, shiny
4. Ork1A6	*Anoxybacillus thermarum*	*Anoxybacills thermarum* DSM 17141^T (99.44%)	Orakei Korako, NZ	Medium, raised, circular/undulate, shiny, mustardy yellow
5. But5A1A	*Bacillus licheniformis*	*Bacillus licheniformis* DSM 13^T (99.87%)	n/a	Large, curled, irregular, cream/translucent, large amounts of EPS
6. But5A1B	*Bacillus licheniformis*	*Bacillus licheniformis* DSM 13^T (99.53%)	n/a	Large, curled, irregular, cream/translucent
7. Con1	*Bacillus licheniformis*	*Bacillus licheniformis* DSM 13^T (99.57%)	n/a	Large, curled, cream translucent, undulate
8. Con2	*Bacillus licheniformis*	*Bacillus licheniformis* DSM 13^T (99.42%)	n/a	Large, curled, irregular, filliform, undulate, cream white, raised
9. Con4	*Bacillus licheniformis*	*Bacillus licheniformis* DSM 13^T (98.28%)	n/a	Large, curled, irregular, filliform, undulate, creamy mustard yellowy white, raised
10. Con5	*Bacillus licheniformis*	*Bacillus licheniformis* DSM 13^T (99.57%)	n/a	Large, curled, cream translucent yellow, undulate

Table 2 contd.: A summary species, sequence identity and isolation source of the twenty sub-selected strains.

Strain code	Species	Sequence Identity	Isolation Source	Colony Morphology (LB agar at 56˚C for 20 hours)
11. Prf1A3	*Bacillus licheniformis*	*Bacillus licheniformis* DSM 13^T (99.28%)	Praia do Fogo, Azores	Large, cream, curled, irregular, undulate raised
12. Wam9A3	*Bacillus licheniformis*	*Bacillus licheniformis* DSM 13^T (99.67%)	Waimangu NZ	Curled, undulate irregular, cream white, flat
13. Wam12A2	*Bacillus licheniformis*	*Bacillus licheniformis* DSM 13^T (99.14%)	Waimangu NZ	Large, curled, irregular, cream white, flat/crateriform
14. Row2A12.1	*Bacillus smithii*	*Bacillus smithii* NBRC 15311^T (99.81%)	n/a	Medium, cream, irregular, lobate, raised
15. Tau17A2	*Geobacillus thermocatenulatus*	*Geobacillus thermocatenulatus* DSM 730^T (98.88%)	Taupo, NZ	Small-medium, flat, shiny, circular, entire margin, creamy yellow
16. Fur3A1B.2	*Geobacillus thermodenitrificans*	*Geobacillus thermodenitrificans* BGSC 94A1^T (99.09%)	Furnas, Azores	Medium, irregular, undulate flat, cream white
17. Ham3A8	*Geobacillus thermodenitrificans*	*Geobacillus thermodenitrificans* BGSC 94A1^T (99.28%)	n/a	Large, cream, irregular, lobate, flat
18. DSM 465^T	*Geobacillus thermodenitrificans*	*Geobacillus thermodenitrificans* DSM 465^T (100%)	Sugar beet juice, Australia	Medium, undulate irregular, cream, raised
19. G18A3	*Geobacillus thermoleovorans*	*Geobacillus thermoleovorans* BGSC 96A1^T (99.81%)	Sterilised Media	Small-medium, circular, entire, translucent cream white, shiny
20. Tok5A2	*Geobacillus thermoleovorans*	*Geobacillus thermoleovorans* BGSC 96A1^T (99.41%)	Tokaanu NZ	Medium-large, curled, circular, raised, beige

Thirteen of the selected strains were able to degrade motor oil, as could be observed in terms of the dissolution of the oil throughout the LB medium upon agitation (**Table 3**). These included nine *B. licheniformis* strains (But5A1A, But5A1B, Con1, Con2, Con4, Con5, Prf1A3, Wam9A3 and Wam12A2), two *G. thermoleovorans* strains (G18A3 and Tok5A2), *A. thermarum* Ork1A6 and *G. thermocatenulatus* Tau17A2.

These strains produced oil morphologies akin to those seen in **Figure 2** upon agitation. Numerous black specks could be observed which may represent asphaltenes, one of the four components (along with saturates, aromatics and resins) that constitute crude oil (Soleymanzadeh *et al*., 2019), which remain once oil is degraded. Asphaltenes comprise the polyaromatic hydrocarbon (PAH) component of crude oil and are considered to be the heaviest, most polar and recalcitrant of the four components of crude oil (Hernández-López *et al*., 2015; Soleymanzadeh *et al*., 2019; Svalova *et al*., 2017). Asphaltenes have been observed to precipitate upon the addition of low molecular weight n-alkanes e.g. pentane, hexane, heptane (Soleymanzadeh *et al*., 2019; Svalova *et al*., 2017). Therefore, the asphaltene specks observed may be the result of an increased presence of low molecular weight alkanes within the sample as the longer chain alkanes are degraded into smaller, low molecular weight alkane constituents.

For a further seven strains, namely *G. thermodenitrificans* Fur3A1B.2 and Ham3A8, *A. composti* Fur6A6.1 and Wam6A1, *A. flavithermus* Ork3A7 and *B. smithii* Row2A12.1 the results mimicked those of the negative control (oil in LB medium only, no strain added) as well as the negative bacterial control *G. thermodenitrificans* DSM 465^{T}.

The rate of oil degradation was assayed by monitoring the emulsifying power of the bacteria 20 and 120 hours post-inoculation. For five of the strains, namely *B. licheniformis* Con1, Con2, Con4, Con5 and Prf1A3 oil degradation could be observed after just 20 hours. The remaining eight strains took a longer period of time to display signs of motor oil degradation. However, 120 hours post-inoculation several strains, including *B. licheniformis* But5A1A, But5A1B and Wam9A3, as well as *G. thermoleovorans* Tok5A2, which showed no signs of oil degradation 20 hours post-inoculation, displayed greater emulsifying power than many of those strains which degraded oil already after 20 hours.

2.4.1.2. The effect of culture age on thermophilic oil degradation

The liquid assays described above were repeated, but the pre-cultures were maintained for 192 hours (instead of 20 hours), before oil was added. Using these older cultures, fourteen showed signs of oil emulsification and degradation, while six strains did not (**Table 3**). In general, the 192 hour pre-cultures performed better than the younger pre-cultures. This may be attributed to the older bacterial cultures utilising all of the readily available nutrients in the LB broth, and subsequently using the motor oil hydrocarbons as a primary carbon substrate.

Two strains, *A. flavithermus* Ork3A7 and *B. smithii* Row2A12.2, which were negative when 20 hour cultures were used, displayed oil degradation when the 192 hour cultures were used. By contrast, the 20 hour pre-cultures of *A. composti* Fur6A6.1, *A. thermarum* Ork1A6 and *G. thermocatenulatus* Tau17A2 were able to degrade the motor oil, while their older counterparts were unable to. This revealed that culture age is an important factor in determining the oil degradation capacities of the evaluated thermophilic bacteria. Of note is that in several of the bacterial strains incubated for 192 hours, the oil was observed to be retained in "ball-like" structures near the surface of the medium. This was particularly evident in *G. thermodenitrificans* Ham3A8 (**Figure 4**). These oil-ball structures were not disrupted upon agitation, suggesting that the bacterium or an extracellular bacterial component may be encapsulating the oil.

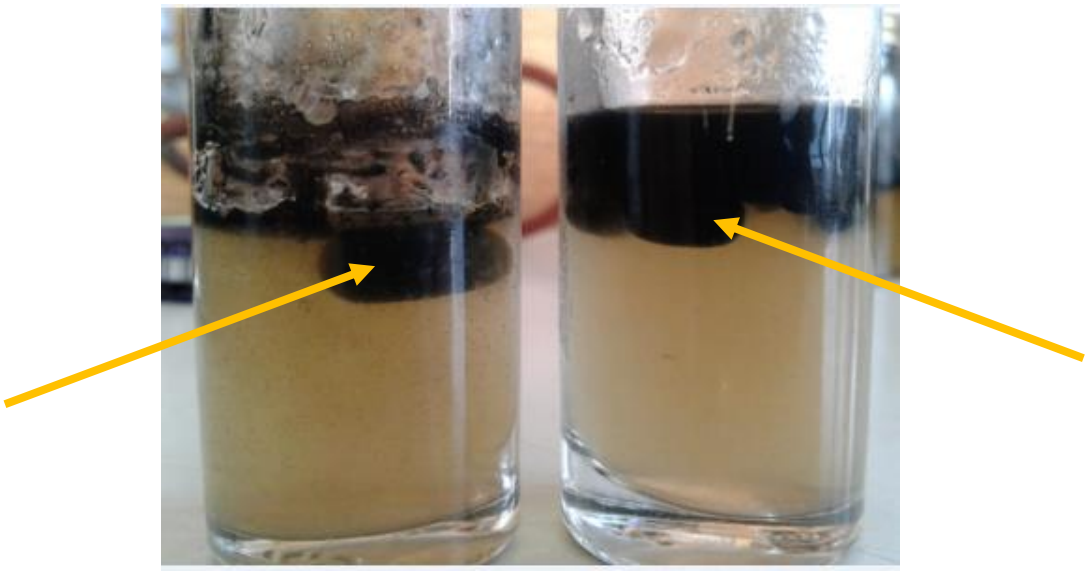

Figure 4: The *Geobacillus thermodenitrificans* Ham3A8 cultures after five days of incubation. The oil was observed to aggregate in ball-like structures (as indicated by the orange arrows) at the liquid-surface interface, which may be due to the production of biosurfactants.

Potential candidates of such an extracellular molecule are the biosurfactants (Bhattacharya *et al*., 2015; Liu *et al*., 2016; Panda *et al*., 2013). These molecules gather at the interface between fluids of opposing polarities (such as non-polar oil and polar water) where their amphiphilic nature allows them to reduce the repulsive forces/interfacial tension between the different phases (El-Sheshtawy *et al*., 2015; Pacwa-Płociniczak *et al*., 2011). By this means, oil emulsification can occur more readily (Pacwa-Płociniczak *et al*., 2011). As such, further assays were undertaken to elucidate whether a bio-emulsifier or biosurfactant is produced and plays a potential role in oil degradation in the thermophilic bacteria.

2.4.2. Plate assays and biosurfactant production

Twenty strains were streaked onto LB agar plates coated with motor oil to observe any zones of clearance they may yield.

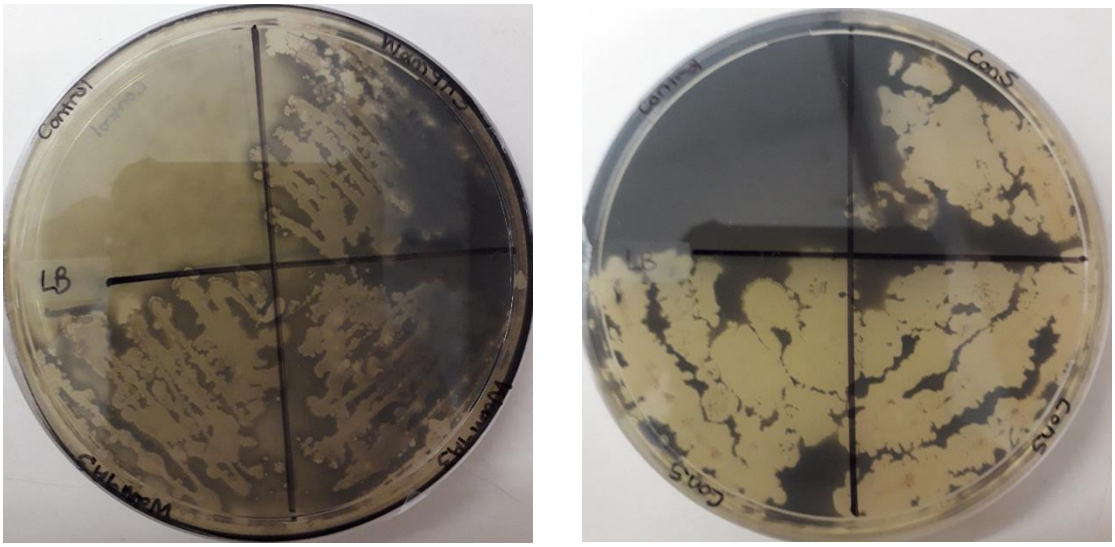

Figure 5: A non-biosurfactant producer (negative; left) and a biosurfactant-producer (positive; right) from the streak plate assays. Both of these bacteria, when streaked, were able to grow on the LB agar plates, however, only one (right) produced a zone of clearance around the streak.

Zones of clearance around the oil could be observed around the growing colonies of nine thermophilic isolates, *B. licheniformis* But5A1A, Con1, Con2, Con4, Con5, Prf1A3 and Wam12A2, as well as *Geobacillus thermodenitrificans* Fur3A1B.2 and Ham3A8 (**Table 3**). The zones of clearance around the colonies indicate that these strains produce a biomolecule which results in displacement of the oil (**Figure 5**). By contrast, in the remaining eleven strains, the oil was observed directly around the colonies, and these are considered non-biosurfactant producers.

To validate the results from the streak plates, single colonies of each of the twenty strains were also stab-inoculated into oil coated agar (**Figure 6**). Aside from the above mentioned nine positive strains, two further taxa, namely *B. licheniformis* Wam9A3 and *A. composti* Fur6A6.1 also produced zones of clearance around the site of inoculation (**Table 3**). The latter strains, however, only caused minimal clearance around the inoculation site and may thus produce little biosurfactant or emulsifying agent.

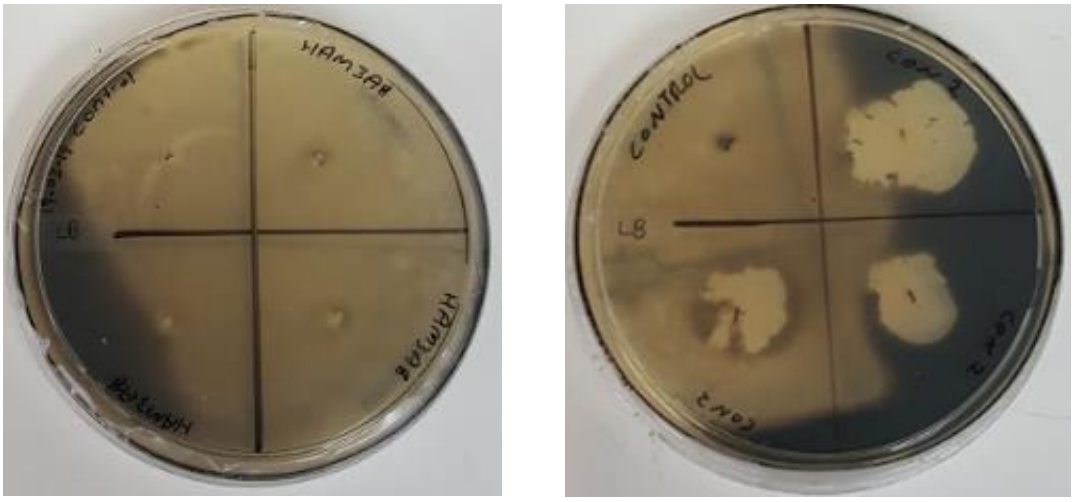

Figure 6: A non-biosurfactant producer (negative; left) and a biosurfactant-producer (positive; right) from the stab plate assays. These stab plates were done to validate the results from the streak plates. Biosurfactant produced caused a displacement in the oil around the origin of the stab.

Most of the strains that caused oil displacement also showed a capacity to degrade motor oil. This included *B. licheniformis* But5A1A, Con 1, Con 2, Con 4, Con 5, Prf1A3, Wam12A2 and Wam9A3 as well as *A. composti* Fur6A6.1. However, several strains, notably *B. licheniformis* But5A1B, *G. thermoleovorans* G18A3 and Tok5A2, *A. thermarum* Ork1A6, *A. flavithermus* Ork3A7, *B. smithii* Row2A12.1 and *G. thermocatenulatus* Tau17A2, did not produce evidence of oil displacement on the streak or stab plates but were positive for oil degradation. This finding suggests that the production of biosurfactant or other emulsifying biomolecules, although advantageous to the oil degrading capacity of the bacterium, is not essential (Xu *et al*., 2018).

The ability of a biosurfactant to enhance the bioremediation of hydrocarbon pollutants is largely dependent on three factors: the pollutant type, the physico-chemical characteristics of the biosurfactant and the physiological properties of the bacteria present (Xu *et al*., 2018; You *et al*., 2018). These factors may have attributed to why strains such as *G. thermodenitrificans* Ham3A8 and *G. thermodenitrificans* Fur3A1B.2, which caused zones of clearance on the streak plates, were unable to degrade the motor oil within the liquid assays. Furthermore, it may explain why a strain such as *B. licheniformis* Prf1A3 (who produced an abundant amount of biosurfactant) did not degrade oil to as great of an extent as the strain *B. licheniformis* Wam9A3 which yielded less biosurfactant.

Below (**Table 3**) is a summary of the results obtained from each of screening tests conducted. This includes the petrochemical-degradation capability, the age of the inoculum versus its ability to degrade and finally the ability of the bacterium to produce a biosurfactant screenings.

Table 3: **Summary of the results obtained from the tests conducted on the bacterial subset during their screening for petrochemical degrading capability.** Tests were conducted in triplicate; green blocks containing (**+**) are indicative of positive reactions while red blocks containing (**-**) are indicative of negative reactions.

Bacterial reference name	Liquid screening (20 hour old culture) Day 1			Liquid screening (20 hour old culture) Day 5			Surfactant produced on stab plates			Bacterial growth on streak plates			Zones of clearance around streak			Liquid screening (192 hour old culture) Day 1			Liquid screening (192 hour old culture) Day 5		
	Sample number																				
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Control	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1. But5A1A	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+
2. But5A1 B	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	+	+	+
3. Con1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4. Con2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5. Con4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+
6. Con5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7. Fur3A1B.2	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-
8. Fur6A6.1	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+

Table 3 contd.: Summary of the results obtained from the tests conducted on the bacterial subset during their screening for petrochemical degrading capability.

Bacterial reference name	Liquid screening (20 hour old cultures) Day 1			Liquid screening (20 hour old cultures) Day 5			Surfactant produced on stab plates			Bacterial growth on streak plates			Zones of clearance around streak			Liquid screening (192 hour old culture) Day 1			Liquid screening (192 hour old culture) Day 5		
	Sample number																				
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
9. G18A3	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	+	+	+
10. Ham3A8	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-
11. Ork1A6	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
12. Ork3A7	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+	+	+	+	+	+
13. Prf1A3	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
14. Row2A12.1	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	+	+	+
15. T4	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
16. Tau17A2	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
17. Tok5A2	-	-	-	+	+	+	-	-	-	+	+	+	-	-	-	-	-	-	+	+	+
18. Wam6A1	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
19. Wam9A3	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+
20. Wam12A2	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+

2.5. Conclusion

A total of 138 aerobic thermophilic bacteria were screened for their ability to degrade hydrocarbons. A substantial number of the evaluated taxa (47%) proved to be able to degrade motor oil to varying degrees. This is indicative of the potential of these thermophilic strains in bioremediation studies. A sub-selection of twenty strains were then subjected to further oil-degradation liquid assays, where the strains were assessed based on their ability to degrade motor oil, the extent to which they degrade motor oil and the rate at which they the degrade motor oil. Of this subset, the strains *B. licheniformis* But5A1A, *B. licheniformis* But5A1B, *B. licheniformis* Con1, *B. licheniformis* Con2, *B. licheniformis* Con5, *A. composti* Fur6A6.1, *B. licheniformis* Prf1A3, *G. kaustophilus* Tok5A2 and *B. licheniformis* Wam9A3 showed the greatest promise.

Strains were also screened for their ability to produce biosurfactants/bio-emulsifiers. Bio-emulsifiers/biosurfactants are reported to have a direct impact on enhancing oil bioavailability by increasing the emulsification power of the oil and also, by ensuring adhesion of the hydrophobic compound to the bacterial cell, thus leading to enhanced uptake and accelerated degradation of the oil (Bhattacharya *et al*., 2015; Liu *et al*., 2016; Panda *et al*., 2013; You *et al*., 2018).

From this study, it was discovered that elevated levels of biosurfactant were produced in aged cultures. This was observed through the production of encapsulated oil ball-like structures at the gas-medium interface. This was confirmed through streak and stab assays on oil-coated agar plates. The production of biosurfactants is not always advantageous nor are they necessary, in assisting in the biodegradation of oil. For example, *G. thermodenitrificans* Ham3A8 yielded large quantities of biosurfactant but was incapable of degrading the oil present. Moreover, *B. licheniformis* But5A1B, a predicted non-biosurfactant producing strain, displayed a greater capacity for motor oil degradation than some of the strains who are predicted to produce biosurfactant. This may be the result of its microbial adhesion to hydrocarbons (MATH) factor.

MATH is the affinity of a microbial cell to adhere to a hydrophobic interface – a property which bacteria alter according to the hydrocarbon types present in their environment (Kaczorek *et al*., 2018; Rosenburg *et al*., 1980). The bacterial cells alter the amount of saturated alcohols,

lipopolysaccharides, intramolecular hydrogen, phosphoryl, amine, and carboxyl groups expressed on their cell surface to enhance cell surface hydrophobicity which allows biodegradable hydrocarbons to adhere to their surface more readily (Kaczorek *et al*., 2018; Van Hamme *et al*., 2003). In this instance, although it was unable to produce a biosurfactant, *B. licheniformis* Wam9A3 may have been able to alter its cell surface components to optimize hydrocarbon bioavailability. This evidence shows that the ability to produce a biosurfactant is not always necessary but may be beneficial in the degradation of oil.

Overall, our analyses identified several thermophilic bacteria with a good capacity for the degradation of crude oil. Three strains were subsequently selected for further analysis through genome sequencing and GC-MS analysis. These strains are namely: *Bacillus licheniformis* But5A1A, But5A1B and Wam9A3.

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Chapter 3

Identifying and assessing the degradation capabilities of the selected bacteria

Abstract

The use of thermophilic *Bacillus licheniformis* strains in the bioremediation of petroleum hydrocarbons has been reported by just a handful of studies; this leaves a large gap in knowledge that needs to be filled. Here, this gap is addressed by studying the genome sequences of three *B. licheniformis* strains found to degrade motor oil. Although a handful of enzymes known to be involved in oil degradation were found, the presence of a lichenysin-encoding gene cluster was a stand-out discovery. Lichenysin is thought to be the biosurfactant produced by the bacterium *B. licheniformis* But5A1A, in large quantities, which led to its superior hydrocarbon degradation capacity. GC-MS analysis was undertaken to semi-quantitatively analyse the hydrocarbon degradation capacities amongst the bacterial strains. These strains were found to not only degrade alkanes ($C_{9\text{-}14,\ 16}$) but also appear to be synthesizing alkanes ($C_{18,\ 27}$). These alkanes are thought to be secondary metabolites which are yet to be characterized. Overall, the strains *Bacillus licheniformis* But5A1A, But5A1B and Wam9A3 demonstrate great potential in the degradation of petroleum hydrocarbons and should be applied to future bioremediation studies.

3.1. Introduction

Bacillus licheniformis is a species of Gram positive, endospore-forming, facultative anaerobic, motile rods belonging to the family Bacillaceae and the order Bacillales (De Vos *et al*., 2009). When grown on LB agar plates, strains of this species present as whitish or creamy/brown colonies exhibiting lichen-like extensions (De Vos *et al*., 2009). Strains of *B. licheniformis* have been isolated from a wide range of habitats, including plant material, various food-stuffs (resulting in food poisoning) and clinical specimens (De Vos *et al*., 2009, Salkinoja-Salonen *et al*., 1999; Veith *et al*., 2004). However, they are particularly prevalent in soils and they tend to dominate in environments such as deserts and moors where soils are often deficient in nutrients (De Boer *et al*., 1994; De Vos *et al*., 2009). *B. licheniformis* is largely described as a mesophilic

species, however, some members of this species have been observed to grow at higher temperatures, with the maximum temperature (T_{max}) recorded as 57˚C thus classifying these strains as moderate thermophiles (Kotzekidou, 2016; Mohammad *et al*., 2017).

Thermophilic *B. licheniformis* strains yield heat-tolerant enzymes which are of great potential in the biotechnological industry (Abdel-Fattah *et al*., 2013; Sellami-Kamoun *et al*., 2008). Enzymes, such as *B. licheniformis* alkaline proteases, have found use in laundry detergents while their thermostable α-amylases are used in brewing, starch processing and sugar production (Abdel-Fattah *et al*., 2013; Sellami-Kamoun *et al*., 2008). Several members of the *Bacillaceae* and the genus *Bacillus* have shown promise for the biodegradation of long-chain alkanes and various polycyclic aromatic hydrocarbons (PAH) (García-Alcántara *et al*., 2016; Feitkenhauer *et al*., 2003). However, information relating to the biodegradation of these compounds by *B. licheniformis*, particularly heat-resistant members of the species, is limited (García-Alcántara *et al*., 2016). Mnif *et al*. (2011) described a strain of *B. licheniformis* isolated from the Sercina oilfield in Tunisia which degraded up to 60% of the aliphatic fraction of crude oil within 20 days at a temperature of 55˚C (Mnif *et al*., 2011). In experiments with *B. licheniformis* A10, isolated from oil-contaminated soil at an oil refinery, it was observed that this strain degraded up to 80% of diesel oil and aromatic hydrocarbons at a temperature of 55˚C during a thirty-day incubation (Basu *et al*., 2014). *B. licheniformis* M2-7, which was isolated from a hot spring in southern Mexico, showed efficient degradation of the carcinogenic PAH benzo[a]pyrene within three to 24 hours (Guevara-Luna *et al*., 2018). Furthermore, strains of this species have been implicated in oil degradation in both mixed and in pure bacterial culture (Gkorezis *et al*., 2016).

In the current study, three *B. licheniformis* strains, namely Wam9A3, But5A1A and But5A1B were selected on the basis of their oil degradation capabilities as well as their phylogeny (**Chapter 2**). Strains who displayed the greatest degree, not specifically the fastest rate, of motor oil degradation, and who were from a single phylogenetic lineage were selected for comparative analysis. Of the three strains, *B. licheniformis* But5A1A showed the greatest capacity for oil degradation (**Figure 1**) as well as the most prominent production of biosurfactant (**Figure 2**), while But5A1B and Wam9A3 showed slightly lower oil degradation capacities.

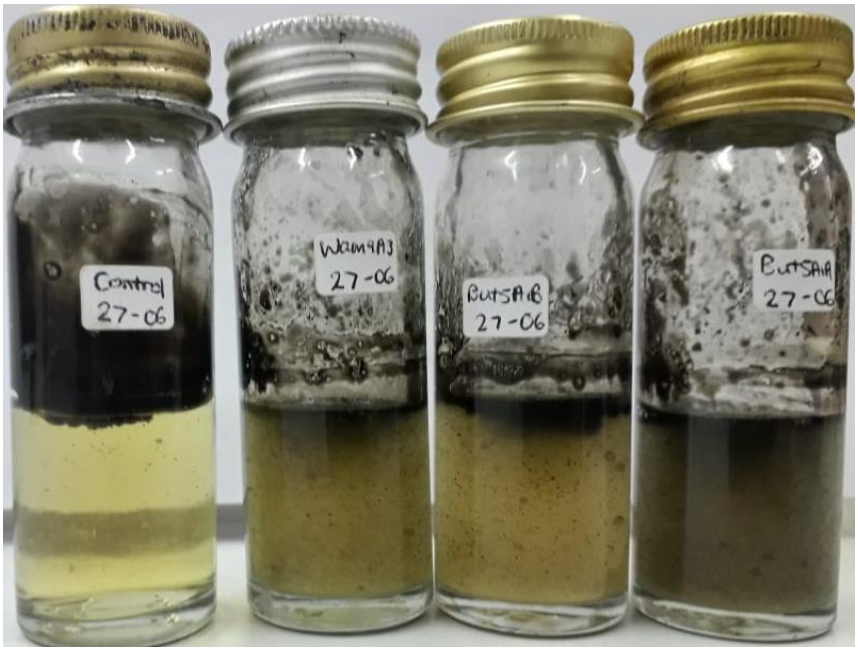

Figure 1: The liquid assays of the strains *B. licheniformis* But5A1A, But5A1B and Wam9A3, labelled as per their strain code, and the negative control. These cultures were grown for eight days prior to adding motor oil and were left to incubate for a further five days. This image depicts a gradient of the emulsification power and therefore also depicts the degree of motor oil degradation at a temperature of 56˚C.

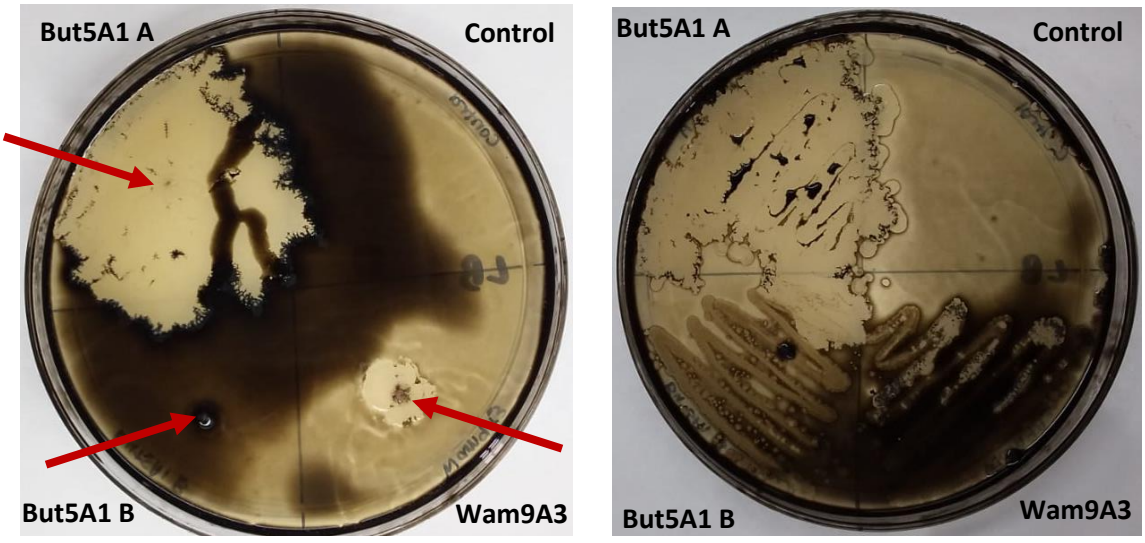

Figure 2: Plate assay of the strains *B. licheniformis* But5A1A, But5A1B and Wam9A3, labelled as per their strain code. LB plates were lined with 500µL of used motor oil prior to stabbing (left; location of initial stab indicated by the red arrows) and streaking (right). These samples were then left at 56˚C for a period of one day.

The complete genomes of all three strains were sequenced and compared, with >99% of the proteins encoded on each genome shared by all three strains. Furthermore, the oil degradation capacities of the three strains were analysed qualitatively and semi-quantitatively using Gas Chromatography-Mass Spectrometry. GC-MS analysis was used to measure the n-alkane content and has been used in previous oil degradation studies (e.g. Liu *et al*. 2016) to show the ability and capacity of the bacterium to degrade crude oil/petroleum hydrocarbons. These results supported the observations made during liquid assays and demonstrated the efficient degradation of smaller chain alkanes (C_9-C_{16}) by the *B. licheniformis* strains, especially. Furthermore, the GC-MS analysis showed that the *B. licheniformis* strains evaluated may also possess the capacity to synthesise alkane hydrocarbons. Overall, all three *B. licheniformis* strains demonstrated excellent motor oil degradation capabilities and further research should focus on their incorporation, either individually or as part of a consortium, for bioindustrial bioremediation approaches.

3.2. Materials and Methods

3.2.1. Genome sequencing of hydrocarbon degrading *B. licheniformis* strains

3.2.1.1. DNA extraction

Genomic DNA (gDNA) was extracted from *B. licheniformis* But5A1A, But5A1B and Wam8A3 using the *ABIOpure*™ Total DNA Blood/Cell Extraction Kit (version 2.0) using the Gram-positive protocol (**Section 2.3.1. of Chapter 2**). Five separate extractions of each strain were done, and the DNA pooled together in order to maximise the concentration of the final gDNA sample. The purity and quantity of gDNA was assessed using the NanoDrop® ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA). The gDNA was then shipped to MRDNA (Texas, USA) for sequencing using the Illumina NovaSeq 6000 platform with paired-end read conformation.

3.2.1.2. Genome assembly and annotation

The raw genome sequences were assembled using several tools in the Illumina BaseSpace suite (Milicchio *et al*., 2016). Initial DNA quality was evaluated using FastQC (Andrews, 2010), followed by trimming of low-quality reads with the FastQ toolkit version 2.2.0 (Illumina, 2019). Reads with an overall Q score <28 and those shorter than 100 bp in length were trimmed.

Subsequently, the trimmed read sets were *de novo* assembled using SPAdes version 3.9.0. (Nurk *et al*., 2013). Reference-based assembly was performed to improve initial *de novo* assemblies using Mauve version 2.4.0 (Rissman *et al*., 2009) and the MeDuSa genome scaffolder (Bosi *et al*., 2015), with the complete genome of the type strain of *B. licheniformis* (DSM 13^T – NCBI Accession number CP000002.3) serving as reference.

The assembled genomes were classified using the online Genome-to-Genome Distance Calculator (GGDC), which serves as an *in silico* approach analogous to DNA-DNA hybridization (dDDH) (Auch *et al*., 2010). GGDC values of >70% are considered to be the equivalent of a positive result obtained during a wet-lab DNA-DNA hybridization test and can therefore be used to delineate two compared genomes to the same species (Auch *et al*., 2010). Finally, the high quality draft genome assemblies were structurally and functionally annotated using the Rapid Annotations using Subsystems Technology (RAST) server (Aziz *et al*., 2008).

3.2.1.3. Comparative genomic analysis

The protein datasets encoded on the genomes of the three *B. licheniformis* strains sequenced in this study, as well as those encoded on the genome of the type strain *B. licheniformis* DSM 13^T, were compared using Orthofinder version 2.3.3 (Emms and Kelly, 2015). This algorithm infers orthologous proteins shared between pair-wise compared genomes on the basis of bi-directional BLASTp analysis (Emms and Kelly, 2015). This allowed for the identification of proteins shared by all strains (core genome), those shared by two or three of the compared genomes (accessory genome) and those unique to a single strain. The functions of proteins in each genome compartment (core, accessory and unique) was inferred on the basis of the RAST functional annotations.

In addition, the CGview server was used to generate a graphical representation of the genomes and their shared/unique genomic fractions (Grant and Stothard, 2008). The CGview server uses BLAST to compare a primary genome sequence to a maximum of three other genomes (Grant and Stothard, 2008). Both sets of comparative genomic analyses were done against *B. licheniformis* DSM13^T.

3.2.1.4. *in silico* identification of oil degradation and secondary metabolite pathways

The nucleotide and protein sequences of twenty genetic elements with a known role in hydrocarbon degradation were searched against the genomes of *B. licheniformis* But5A1A, But5A1B and Wam9A3 using localized BLASTp analysis in Bioedit v 7.2.5 (Hall, 1999). These include the nine *alk* genes/proteins of the alkane degradation pathway and the genes/proteins which have been reported to play a role in alkane degradation in members of the genus *Bacillus*. Finally, some of the oil-degrading proteins inferred by automated annotation in the RAST server were also subjected to a BLAST search against the NCBI non-redundant protein database to confirm function. The antiSMASH (antibiotics and Secondary Metabolite Analysis Shell) server was used to locate and annotate secondary metabolite gene clusters encoded in the genomes of the *B. licheniformis* strains (Medema *et al*., 2011). These gene clusters generally comprise of physically co-localized genes which, as a unit, encode the enzymes responsible for the biosynthesis of secondary metabolites such as antibiotics and biosurfactants (Chavali and Rhee, 2018; Medema *et al*., 2011).

3.2.2. Qualitative and semi-quantitative assessment of *B. licheniformis* hydrocarbon degradation by Gas Chromatography-Mass Spectrometry analysis

3.2.2.1. Sample preparation prior to Gas Chromatography-Mass Spectrometry

Sample preparation was performed using an adapted version (Patowary *et al*. 2016) of the protocol developed by Rahman *et al.* (2002). In brief, pre-cultures of the three evaluated strains were prepared in duplicate in Erlenmeyer flasks containing 30 mL LB broth and incubated for 20 hours at a 56˚C on a shaker (150 rpm). Strain growth was evaluated by spectrophotometric analysis to determine the absorbance (OD_{600}) of each sample. The samples were diluted, in sterile microcentrifuge tubes, to a uniform OD_{600} of 0.5 across all samples.

A total volume of 1 mL of the diluted inoculum was transferred into MacConkey bottles containing 9 mL of autoclaved LB broth; this was done in triplicate. These final cultures were then incubated for 20 hours at 55˚C and on a shaker (150 rpm). Thereafter, 200 µL of autoclaved motor oil was added to each MacConkey bottle and these were also incubated at 55˚C and on a shaker at 150 rpm for 12 days (288h). Autoclaved motor oil in LB incubated in the same manner as the test samples was incorporated as a negative control in the experiments.

Following incubation, the samples were subjected to a solvent extraction using a modified version of the protocol described by Liu *et al*. (2016). In brief, 10 mL of n-hexane was added to each sample to dissolve any non-degraded motor oil still present in the medium. This non-polar solvent is immiscible in the presence of the water-based LB medium (Dahm and Visco, 2015). Following the addition of the n-hexane, the samples were shaken vigorously to allow for adequate mixing. The samples were then passed through a separating funnel and the aqueous solution was eluted and discarded. A second wash with 10 mL of n-hexane was performed prior to evaporation for 48 hours in a fume hood to remove excess n-hexane.

3.2.2.2. Gas Chromatography-Mass Spectrometry

GC-MS was undertaken according to the method of Liu *et al*. (2016). GC-MS was performed using a Pegasus 4D GCxGC-TOFMS Gas Chromatograph equipped with a Pegasus III Time-of flight Mass Spectrometer (TOFMS) (Leco, U.S.A) at the School of Chemistry, University of the Witwatersrand. The temperature regime implemented for the detection of n-alkanes comprised of: 80˚C for 2 minutes; increment of 30 ˚C/min to 240˚C and increment by 3˚C/min to 310˚C; hold for 2 minutes. Helium (at a rate of 1.5 mL/min) was used as a carrier gas.

3.3. Results and Discussion

3.3.1. General genome characteristics

Genome sequencing yielded 13.5, 11.2 and 8.6 million reads, with a total yield of 3.67, 3.11 and 2.38 gigabases of sequence data for *B. licheniformis* But5A1A, But5A1B and Wam9A3, respectively. Initial *de novo* genome assembly with SPAdes v 3.9.0. yielded 58, 56 and 58 contigs (>1,000 bp) for the three organisms, respectively. Reference-based assembly allowed for most gaps to be closed, with the final high quality draft genomes comprising of seven, four and seven contigs for *B. licheniformis* But5A1A, But5A1B and Wam9A3, respectively.

Table 1: Genomic properties of *Bacillus licheniformis* But5A1A, But5A1B, Wam9A3 and the type strain DSM13^T.

Bacterium name	Genome size (bp)	G+C content (%)	Number of Proteins	Number of rRNAs encoded	Number of Plasmids
Bacillus licheniformis DSM13^T	4,222,597	46.2	4577	93	0
Bacillus licheniformis But5A1A	4,463,770	45.7	4990	99	1
Bacillus licheniformis But5A1B	4,469,812	45.7	4987	100	1
Bacillus licheniformis Wam9A3	4,470,870	45.7	4996	87	1

The genomes of the three strains are similar in size (~4.47 Mb), but are ~ 245 kilobases larger than that of the type strain (**Table 1**). This can be attributed, in part, to the presence of an identical 80 kilobase plasmid in the three strains from the current study. Between 4,987 (*B. licheniformis* But5A1B) and 4,996 (*B. licheniformis* Wam9A3) proteins are encoded on the current genomes. By contrast, the type strain genome only codes for 4,577 proteins. The G+C content of the *B. licheniformis* But5A1A, But5A1B and Wam9A3 genomes is also 0.5% below that of *B. licheniformis* DSM13^T (46.2%). Regardless, their G+C contents lie within the 42.9% and 49.9% G+C content range observed for the species (De Vos *et al*., 2009).

To validate that the sequenced strains do indeed belong to the species *B. licheniformis*, digital DNA-DNA (GGDC) was performed. This analysis showed that for all three strains, the dDDH values were far above the 70% threshold value for species delineation (**Figure 3)**. The dDDH values between the three *B. licheniformis* strains in this study were >99.9%, which suggests that they are near identical clones of the same strain. This may very well be the case for the *B. licheniformis* But5A1A and But5A1B strains which were initially obtained from a single glycerol stock and were separated on the basis of distinct colony morphologies. This may also reflect a common misinterpretation inherent to *B. licheniformis* where colony morphology is variable among strains of the species (De Vos *et al*., 2009), and may therefore have resulted in

the appearance of this representing a mixed culture of two distinct organisms. However, all three strains yielded distinct results, in terms of their oil degradation capacities.

B. licheniformis DSM13^T	100			
B. licheniformis But5A1A	97.2	100		
B. licheniformis But5A1B	97.2	99.7	100	
B. licheniformis Wam9A3	97.1	99.7	99.9	100
	B. licheniformis DSM13^T	*B. licheniformis* But5A1A	*B. licheniformis* But5A1B	*B. licheniformis* Wam9A3

Figure 3: Digital DNA-DNA hybridization (dDDH) values obtained using the Genome to Genome Distance Calculator.

3.3.2. Comparative genomic analysis of the oil degrading *B. licheniformis* strains

The high quality draft genome sequences of *B. licheniformis* But5A1A, But5A1B and Wam9a3 were mapped against the complete genome of *B. licheniformis* DSM13^T using CGview (Grant and Stothard, 2008). The figure shows that the *B. licheniformis* genomes are highly conserved (**Figure 4**), with only small regions in *B. licheniformis* DSM13^T not shared by the three strains analysed in this study. To gain deeper insights into potential differences in the protein content of the strains, orthologous proteins were identified using Orthofinder version 2.3.3 (Emms and Kelly, 2015).

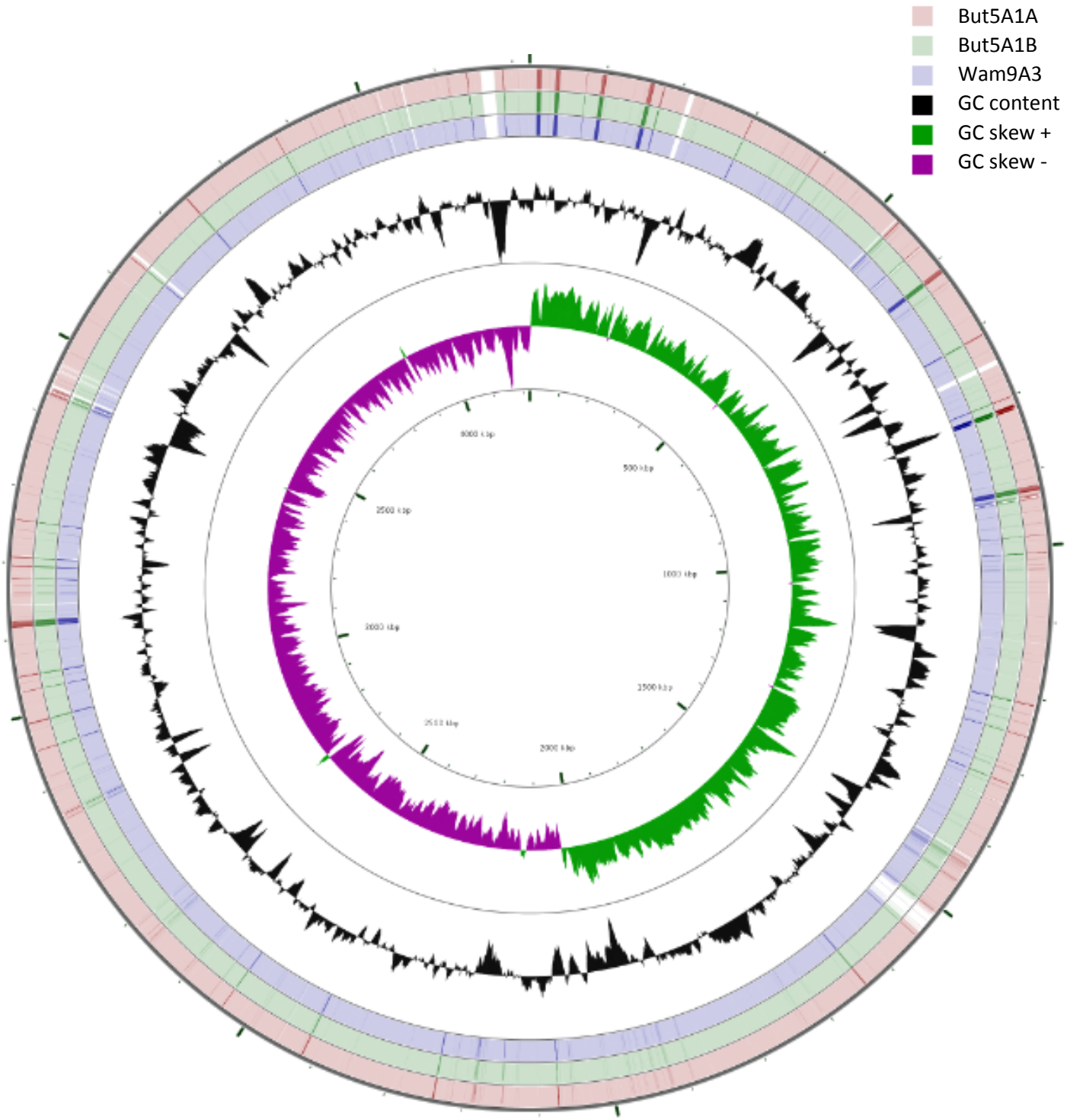

Figure 4: Circular map of the full genomes of But5A1A (Red lines), But5A1B (Green lines) and Wam9A3 (Blue lines) against the genome of *B. licheniformis* DSM13. The black graph represents the GC content within the genomes. The GC skew (Purple for negative GC skew; Bright green for positive GC skew) indicates under-representation or overabundance, respectively, of cytosine nucleotides in relation to guanosine nucleotides in certain regions of DNA (Arakawa and Tomita, 2007).

The *B. licheniformis* core genome consists of 4,182 proteins (**Figure 5**) shared amongst the four strains. While the genome of *B. licheniformis* DSM13^{T} encodes two unique proteins, the three test subjects have zero proteins that are unique to their genome – this observation reiterates their near identical nature.

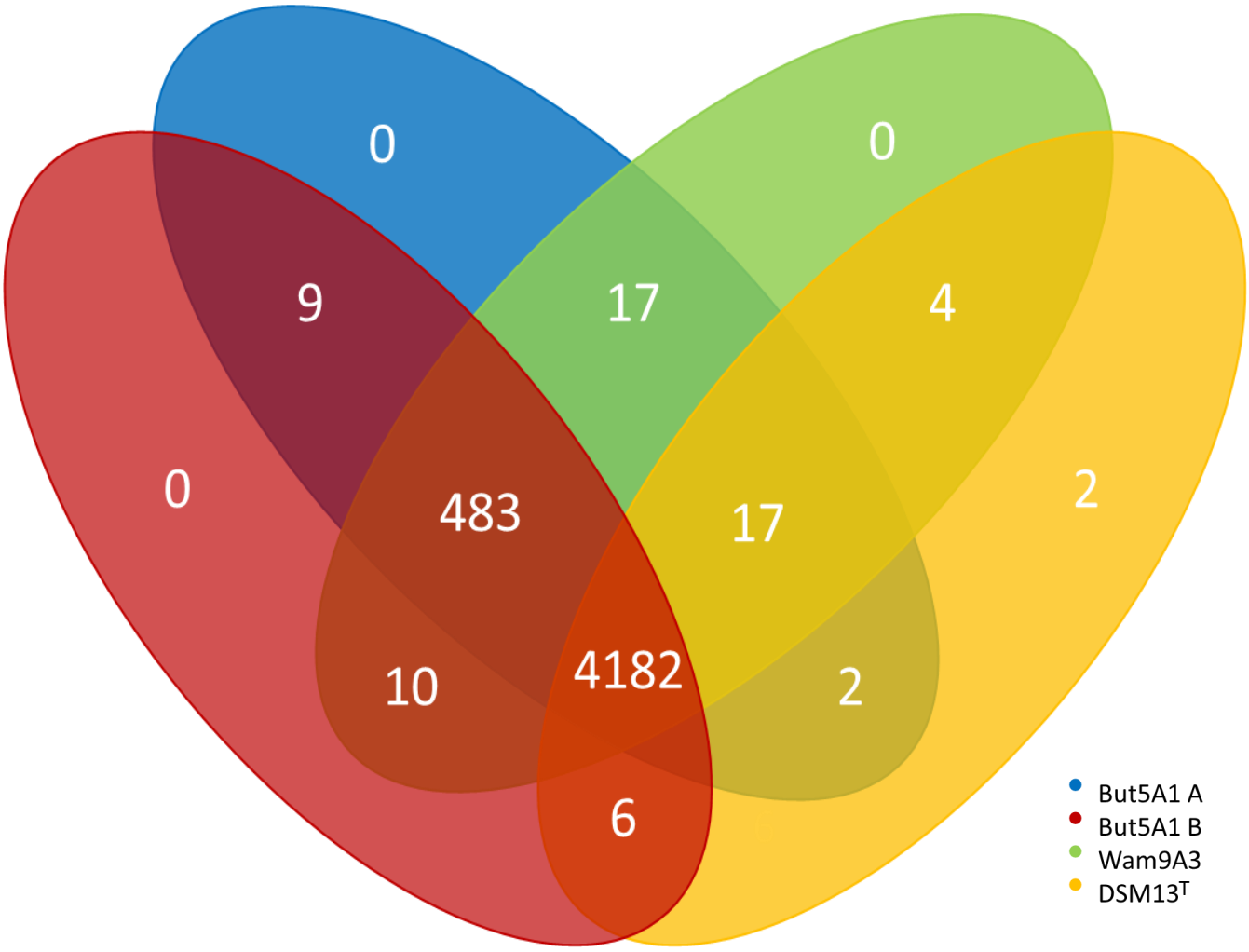

Figure 5: Venn diagram of orthologous proteins shared among the compared *Bacillus licheniformis* strains. Values that lie in the intersecting ovals represent the number of orthologous proteins shared amongst the strains But5A1 A (Blue), But5A1 B (Red), Wam9A3 (Green) and the type strain DSM13^{T} (Orange). The value that lies in the exterior of each oval represents the number of genes that are unique to that individual strain of *B. licheniformis*.

However, the genomes of the three *B. licheniformis* strains sequenced in this study incorporate 483 proteins which are absent from *B. licheniformis* DSM13^{T}. It is plausible that hydrocarbon degradation may be determined by this protein set. However, no information pertaining to the capability for hydrocarbon degradation in the type strain of *B. licheniformis* is currently available.

The RAST server was subsequently used to annotate the proteins encoded on the genome and classify them according to their functional categories (Subsystems). Because the genomes of the three strains were nearly identical, the proportions of proteins assigned to the defined subsystems were near identical (**Table 2**). Some differences were observed for *B. licheniformis* DSM13^{T} , which incorporates a larger number of proteins involved in protein metabolism, and slightly lower protein numbers involved in other categories including regulation and cell signalling, DNA metabolism, dormancy and sporulation and phages, prophages, plasmids and transposable elements. This can be correlated to the fact that the genome of this strain coded for between 410-420 proteins less than the other three strains. Furthermore, the type strain lacks a plasmid which is observed in *B. licheniformis* But5A1A, But5A1B and Wam9A3.

Table 2: A summary of the subsystem feature counts for the species *Bacillus licheniformis* But5A1A, But5A1B, Wam9A3 and the type strain *B. licheniformis* DSM13^T. These protein annotations were obtained through the RAST server.

Subsystem	*Bacillus licheniformis* DSM13^T	*Bacillus licheniformis* But5A1A	*Bacillus licheniformis* But5A1B	*Bacillus licheniformis* Wam9A3
Cofactors, vitamins, prosthetic groups, pigments	164	164	164	164
Cell wall and capsule	57	62	62	62
Virulence, disease and defence	44	43	43	43
Potassium metabolism	2	2	2	2
Phages, prophages, plasmids transposable elements	28	37	35	35
Membrane transport	72	70	70	70
Iron acquisition and metabolism	43	44	44	44
RNA metabolism	60	63	63	63
Nucleosidesand nucleotides	98	104	104	104
Protein metabolism	208	186	188	186
Cell division and cell cycle	4	4	4	4
Motility and chemotaxis	43	43	43	43
Regulation and cell signalling	33	32	30	30
Secondary metabolism	12	12	12	12
DNA metabolism	75	77	76	76
Fatty acids, lipids and isoprenoids	41	41	41	41
Nitrogen metabolism	24	24	24	24
Dormancy and sporulation	97	103	102	101
Respiration	45	45	45	45
Stress response	41	41	41	41
Metabolism of aromatic compounds	11	11	11	11
Amino acids and derivatives	362	364	364	364
Sulphur metabolism	9	9	9	9
Phosphorous metabolism	26	25	25	25
Carbohydrates	358	355	355	355
Miscellaneous	28	29	29	29

Because *B. licheniformis* But5A1A displayed the greatest levels of oil degradation, amongst the three strains, greater interest was placed on the genes present in its subsystems. From the RAST results, it could be observed that this strain has two extra phage tail-encoding genes (phages, prophages, plasmids transposable elements subsystem), an additional RecD-like DNA helicase YrrC gene (involved in DNA repair; DNA metabolism subsystem), two extra genes encoding the programmed cell death toxin YdcE (regulation and cell signalling subsystem) and an extra gene involved in dormancy and sporulation compared to *B. licheniformis* But5A1B. Upon consideration, it is highly unlikely that these gene products are responsible for the higher degradation capacity observed in this organism.

Notable amongst the RAST subsystems, are those involved in the degradation of aromatic compounds, although these are universally present among all four compared *B. licheniformis* strains. These incorporate proteins involved in benzoate (benzoate Major Facilitator Superfamily (MFS)) transporter BenK and p-hydroxybenzoate hydroxylase (EC 1.14.13.2), quinate (3-dehydroquinate dehydratase I and II (EC 4.2.1.10), aromatic amine (3,4-dihydroxyphenylacetate 2,3-dioxygenase (EC 1.13.11.15) and nitrilotriacetate monooxygenase component B (EC 1.14.13) and gentisate (fumarylacetoacetate hydrolase family protein) degradation. Furthermore, all four strains encode an orthologue of a hydroxyaromatic non-oxidative decarboxylase proteins B, C and D (EC 4.1.1), which are involved in anaerobic degradation of aromatic compounds. The enzymes listed here are involved in the degradation of small aromatic compounds, but are not known to play a role in long-chain alkane degradation (which is not subsumed in any RAST subsystem) and as such, BLASTP and BlastN searches with the amino acid/nucleotide sequences of enzymes with known roles in hydrocarbon and long chain alkane degradation from other bacterial taxa were performed.

3.3.3. Identification of hydrocarbon degradation enzymes in oil degrading *B. licheniformis* strains

Analysis of the RAST annotated genomes and Blast comparisons with known hydrocarbon degradation factors identified six candidate genes coding for proteins with putative roles in hydrocarbon degradation (**Table 3**).

Table 3: Common enzymes involved in the hydrocarbon degradation

Enzyme name	Locus tag		Length (amino acids)	Reference bacterium	Max score	Query cover (%)	E-value	Amino acid identity (%)
	Wam9A3_CDS	But5A1B_CDS	But5A1A_CDS					
Aldehyde dehydrogenase (AlkH)	652	2941	650	1382	*P. putida* Gpo1	348	86	$1e^{-116}$	44.87
					B. subtilis subsp. subtilis 168	636	99	0.0	64.47
Fatty acid CoA-ligase/synthetase (AlkK)	2132	1466	2128	1541	*P. putida* Gpo1	207	92	$1e^{-26}$	29.04
					B. subtilis subsp. subtilis 168	768	99	0.0	72.21
FMNH2-dependent alkanesulfonate monooxygenase	1956	1641	1951	1130	Multispecies [*Bacillus*]	775	100	0.0	99.73
Nitronate monooxygenase	4765	3704	4766	998	*Bacillus licheniformis*	682	100	0.0	100
Alpha/beta hydrolase	3111	506	3107	629	Multispecies [*Bacillus*]	434	100	$1e^{-152}$	100
Catechol-2,3-dioxygenase	1891	1705	1886	680	Multispecies [*Bacillus*]	588	100	0.0	100

Blast searches with the AlkB (monooxygenase/alkane hydroxylase), AlkT (rubredoxin reductase), AlkG (rubredoxin), AlkF (rubredoxin) and AlkJ (Alcohol dehydrogenase) proteins, which are central to the well-characterized alkane degradation pathway (*alkTBFGHIJKL*) of *Pseudomonas putida* Gpo1 (**Figure 4**) yielded no Blast hits. However, an orthologue of the aldehyde dehydrogenase AlkH, was identified in all three *B. licheniformis* genomes, sharing 44.63% amino acid identity with the *P. putida* protein (NCBI Acc. # WP_022963143.1) (**Table 3**). This aldehyde dehydrogenase furthermore shares 64.47% average amino acid identity with the YwdH-like aldehyde dehydrogenase (NCBI Acc. # CAA51614.1) of *Bacillus subtilis* 168. Similarly, orthologues of the *P. putida* AlkK long-chain fatty acid-CoA ligase/synthetase protein (NCBI Acc. # NP_388908.2), sharing 29.04% average amino acid with the former protein, are encoded on all three *B. licheniformis* genomes. The *B. licheniformis* orthologues furthermore share a 72.21% average amino acid identity with the long chain fatty-acid CoA-ligase/synthetase (NCBI Acc. # NP_388908.2) in *B. subtilis* 168.

In *P. putida* Gpo1, AlkH and AlkK are involved in the conversion of the aldehyde intermediate that results from alkane hydrocarbon oxygenation into the precursor molecule of acetyl-CoA which undergoes β-oxidation to form acetyl-CoA prior to entering the TCA cycle (**Figure 6**; Rojo, 2005; Van Hamme *et al*., 2003). Both the AlkH and AlkK orthologues are located on chromosomal contigs of all three strains sequenced in this study and are localised at distinct chromosomal locations. Both of these enzymes are ubiquitous in a broad range of organisms (including eukarya) and play roles in several bioconversion processes (Li *et al*., 2010; Janßen and Steinbüchel, 2014). As such, their exclusive role in alkane degradation in the three *B. licheniformis* strains can be disputed. This is further supported by the lack of other alkane hydroxylation/oxygenation enzymes from the *P. putida* alkane degradation pathway in our strains.

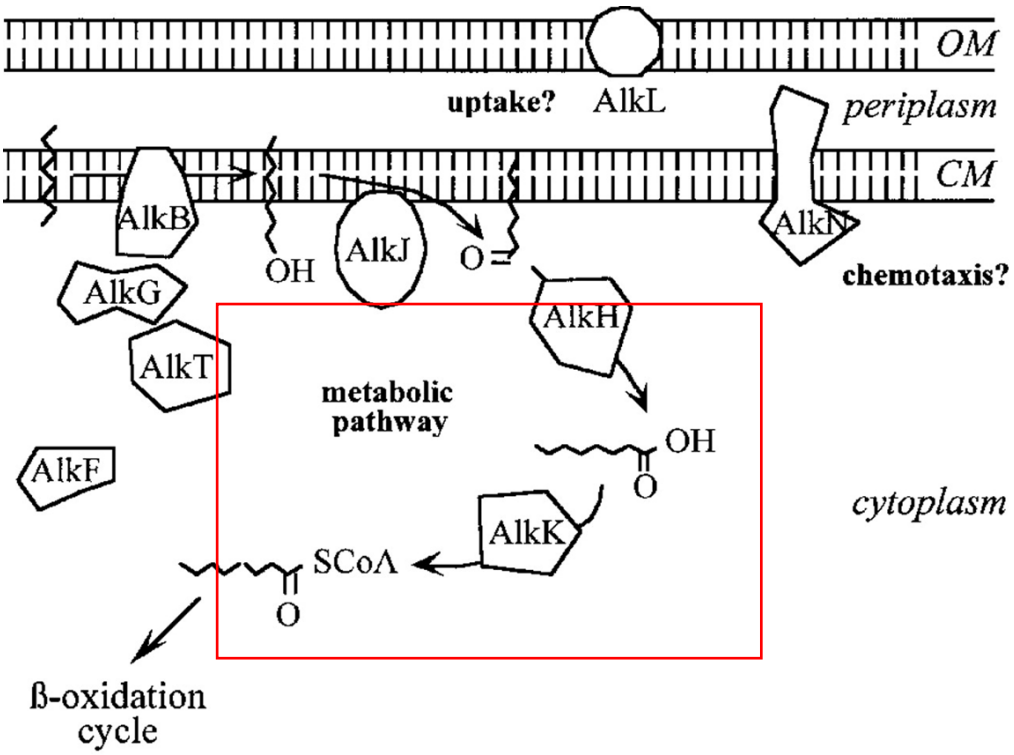

Figure 6: Visual representation of how the enzymes aldehyde dehydrogenase and fatty acyl-CoA ligase/synthetase (red box) produce the precursor substrates to the tricarboxylic acid (TCA) cycle (van Beilen *et al.*, 2001). Once the acyl-CoA is produced, it undergoes β-oxidation before finally entering the TCA cycle.

Bacterial oxygenases, in particular monooxygenases, have an important role in the aerobic biodegradation of both aliphatic and aromatic hydrocarbons (Abbasian *et al*., 2015; Rojo, 2009; Vogt *et al*., 2011; Wang *et al*., 2010). They are generally active in the first few steps of the biodegradation process and are responsible for the addition of an oxygen molecule to the hydrocarbon substrate (Abbasian *et al*., 2015). Using the RAST server annotations and localized BLASTp analyses, four possible alkane monooxygenases were identified. The BLASTp results identify these four proteins as belonging to the family of flavin-dependent oxidoreductases (Commission on Biochemical Nomenclature, 1978; Schomburg *et al*., 2009). Further analysis showed that each of them possess a conserved pfam00296 domain (Pssm ID 274646; Cd length 323) (**Table 4**) for luciferase-like monooxygenases.

Table 4: Summary of the conserved pfam00296 domain information found in the four possible alkane monooxygenase proteins encoded on each of the *Bacillus licheniformis* genomes.

RAST identification	BLASTp identification	Locus tag			Bit score	E-value
		But5A1A_CDS	But5A1B_CDS	Wam9A3_CDS		
Uncharacterized luciferase-like protein YwcH	multispecies LLM class flavin-dependent oxidoreductase [*Bacillus*]	661	2930	664	488.53	$2.70e^{-175}$
Uncharacterized protein YceB	monooxygenase [*B. licheniformis* WX-02]	1360	2232	1364	483.91	$1.22e^{-173}$
Luciferase-like monooxygenase YhbW	multispecies LLM class flavin-dependent oxidoreductase [*Bacillus*]	294	3297	295	549.78	0.0
Oxidoreductase	multispecies LLM class flavin-dependent oxidoreductase [*Bacillus*]	1663	1931	1666	591.91	0.0

One of these monooxygenases was further identified as an FMNH2-dependent alkanesulfonate monooxygenase, SsuD (**Table 3**). This protein is found across species of the genus *Bacillus* and has a conserved PRK00719, alkanesulfonate monooxygenase domain (Pssm-ID: 234821, Cd length: 378, Bit score: 752.56, E-value: 0.0). Within the *B. licheniformis* genomes, the gene coding for the FMNH2-dependent alkanesulfonate monooxygenase SsuD is genomically co-localized with those coding for an ABC transporter (*ssuABC*). These proteins play an important role in aliphatic hydrocarbon degradation (Imperato *et al*., 2019), catalysing the removal of sulphur from sulfonated alkanes by cleaving the carbon–sulphur bond (Abbasian *et al*., 2016; Ellis, 2011). In this oxygenolytic reaction the alkanesulfonate is converted to a sulphite and an aldehyde compound (**Figure 7**; Eichhorn *et al*., 1999).

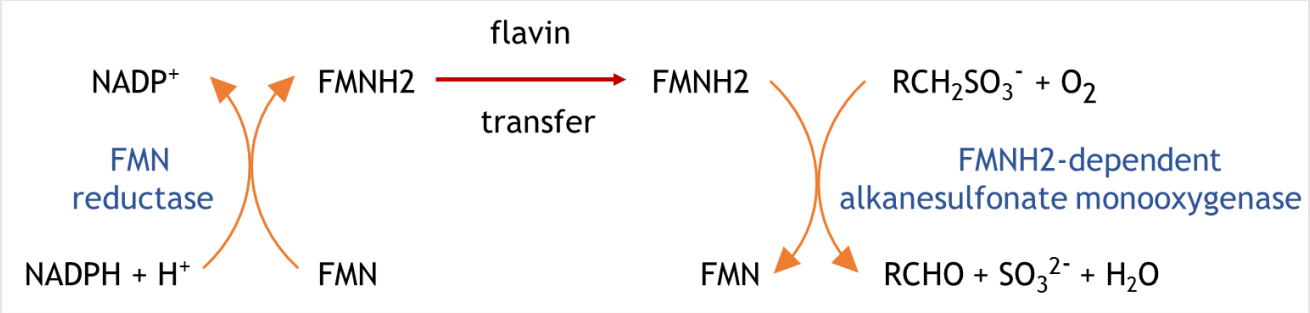

Figure 7: Catalysis of the desulfonation reaction by FMNH2-dependent alkanesulfonate monooxygenase SsuD (Ellis, 2011). FMN reductase SsuE provides reduced flavin (FMNH2) to the alkanesulfonate monooxygenase enzyme which is responsible for the cleavage of the carbon–sulphur bond. This yields an aldehyde and a sulphite molecule.

A second predicted oxygenase represents a predicted nitronate monooxygenase. This monooxygenase has a conserved pfam03060 domain (Pssm-ID: 308601, Cd length: 330, Bit score: 324.08, E-value: $2.17e^{-110}$) and functions by oxidizing nitroalkanes into nitrite and their corresponding carbonyl compounds (Francis *et al*., 2005).

Aside from the above proteins, the RAST annotations identified several other proteins with a putative role in alkane degradation. Orthologues of a putative 'aromatic hydrocarbon catabolism protein' are encoded on the genomes of all three *B. licheniformis* strains in this study. BLASTp analysis against the NCBI non-redundant protein database which produced a 100% amino acid identity to an alpha/beta hydrolase found across multiple species of the genus *Bacillus* (**Table 3**). These hydrolases belong to a family of proteins which are characterised by a distinctive alpha/beta fold (Holmquist, 2000; Ollis *et al.*, 1992). The hydrolase protein catalyses the hydrolysis of substrates with different physicochemical properties (Holmquist, 2000) and therefore may have a catabolic effect on aromatic hydrocarbons. However, no further definitive information could be found on this protein.

A predicted catechol-2,3-dioxygenase was also amongst the proteins identified by the RAST server. This protein incorporates a conserved CatE domain (Pssm-ID: 225312, Cd length: 265, Bit score: 289.23, E-value: $2.31e^{-98}$) and BLASTp analysis against the NCBI nr protein database identifies it as a vicinal oxygen chelate (VOC) family protein, found across multiple species of the genus *Bacillus.* Because it is a member of the VOC family protein (similar to the catechol-2,3-dioxygenase found in *B. subtilis*) and has a conserved CatE domain, this greatly

supports its function in the detoxification of catechol. While it cannot be confirmed that this catechol-2,3-dioxygenase protein has a role in any aromatic hydrocarbon degradation pathways within the bacterium, it has been widely recorded that aromatic hydrocarbons which undergo oxidation (as part of the bacterial biodegradative process) generally yield a catechol molecule as a final product (Abbasian *et al*., 2015; Tao *et al*., 2004; Vogt *et al*., 2011; Wang *et al*., 2010; Van Hamme *et al*., 2003). This molecule must then be further acted on by a catechol dioxygenase enzyme for the aromatic ring to be cleaved into intermediates that will enter the TCA cycle (Wang *et al*., 2010; Van Hamme *et al*., 2003).

Orthologues of several proteins involved in either aliphatic or aromatic hydrocarbon degradation (**Table 3)** were universally identified in all three compared *B. licheniformis* strains. This again suggests that the variation in degradation capacities observed across the three genomes is a result of different gene expression patterns rather than the presence or absence of a particular gene in one of the genomes. Moreover, these genes are also present in *B. licheniformis* DSM13^{T} whose degradation capacity is yet to be studied.

3.3.4. Secondary metabolite production

The antiSMASH server identified ten distinct secondary metabolites on the genomes of the *B. licheniformis* strains. However, only five could be validated on the basis of similar gene clusters in other taxa (**Table 5**).

Table 5: Summary of the secondary metabolite gene clusters identified using the antiSMASH server (Medema *et al*., 2011).

Most similar known cluster	Percentage similarity (%)	Contig and Position			
		Bacillus licheniformis DSM13^T	*Bacillus licheniformis* But5A1A	*Bacillus licheniformis* But5A1B	*Bacillus licheniformis* Wam9A3
Lichenysin	100	NC_006270.3 359,133-424,266	Chrom_1C 176,713-242,162	Chrom_1A 1,897,761-1,962,905	Chrom_1C 176,713-242,162
Lichenicidin VK21A1/ VK21A2	100	NC_006270.3 3,936,283-3,963,244	Chrom_1A 691,869-718,830	Chrom_1A 2,557,617-2,584,578	Chrom_1A 692,081-719,042
Fengycin	53	NC_006270.3 2,075,733-2,104,247	Chrom_1F 713,292-741,806	Chrom_1A 64,503-93,017	Chrom_1F 784,273-812,787
Bacillibactin	53	NC_006270.3 3,698,288-3,748,033	Chrom_1A 691,869-718,830	Chrom_1A 2,772,984-2,822,729	Chrom_1A 453,930-503,675
Butirosin	7	NC_006270.3 1,033,420-1,074,665	Chrom_1C 827,878-869,194	Chrom_1A 1,266,344-1,307,660	Chrom_1C 837,544-878,860

Three secondary metabolite clusters code for the predicted antimicrobial compounds, lichenicidin, fengycin and butirosin. Lichenicidin is a two-component peptide which is produced by strains of *Bacillus* and has antibiotic/bactericidal properties (Begley *et al*., 2009; Caetano *et al*., 2011). These molecules are of particular interest in bioindustry as they are active against several disease-causing bacteria, including vancomycin-resistant *Enterococcus* strains, *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Begley *et al*., 2009; Caetano *et al*., 2011).

Fengycin is an antifungal lipopeptide, generally secreted by the species *B. subtilis*, which inhibits the growth of filamentous fungi (Deleu *et al*., 2008; Vanittanakom *et al*., 1986). The putative orthologous loci in the *B. licheniformis* But5A1A, But5A1B and Wam9A3 strains share a 53% similarity percentage with the fengycin gene cluster from *Bacillus velezensis* FZB42. The final antimicrobial compound, butirosin, is an aminoglycosidic antibiotic complex which displays bactericidal properties towards both Gram positive and Gram negative bacteria (Dion *et al*., 1972). The predicted gene cluster in *B. licheniformis*, however, only shares a 7% similarity percentage with a strain of the species *Bacillus circulans*.

All studied *B. licheniformis* strains also incorporate a gene cluster sharing 53% similarity with the siderophore Bacillibactin in *Bacillus subtilis* subsp. *subtilis* str. 168. This low molecular weight, secondary metabolite is endogenous to *B. subtilis* and functions as an iron-chelator which acts by acquiring and transporting iron to the appropriate enzymes within the bacterial cell (Dertz *et al*., 2006). This iron is a growth-limiting nutrient important for cellular processes such as DNA synthesis, cell proliferation, detoxification and oxygen transport (Dertz *et al*., 2006; Symeonidis and Marangos, 2012).

Most pertinent to the current study is a secondary metabolite cluster which shares 100% cluster similarity with that coding for lichenysin in *B. licheniformis* DSM13^{T}. Lichenysin is an anionic, cyclic lipoheptapeptide molecule (**Figure 8**) with several, species-specific variants of the molecule currently identified: lichenysin A, B, C, D, G and surfactant BL86 (Nerurkar, 2010). This secondary metabolite is synthesized by a lichenysin synthetase - a multienzyme complex encoded by the lichenysin operon, lchA (Nerurkar, 2010). This multidomain enzyme typically consists of the proteins LchAA (*lchAA*), LchAB (*lchAB*), LchAC (*lchAC*) as well as the thioesterase LchA-TE (*lchA TE*), all of which are present within the *B. licheniformis* But5A1A, But5A1B and Wam9A3 strains. Moreover, the structure of this enzyme, and the operon

encoding it, suggests that this is a non-ribosomal peptide synthetase (Nerurkar, 2010) which is able to synthesize peptides without the need for mRNAs or cell ribosomal machinery (Gupta, 2016).

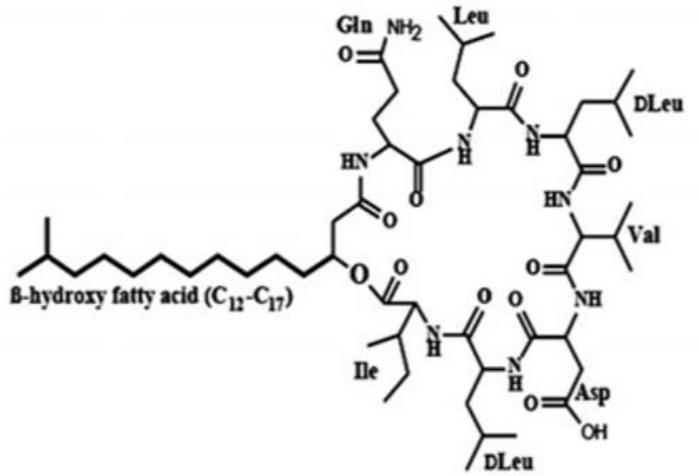

Figure 8: Structure of a lichenysin A molecule, produced by *B. licheniformis* BAS50 (Nerurkar, 2010; Yakimov *et al*., 2000). A β-hydroxy fatty acid (generally ranging from C_{13}-C_{15}) is attached to the heptapeptide sequence L-Gln→L-Leu→D-Leu→L-Val→L-Asp→D-Leu→L-Ile at the first amino acid (amide bond) and to the terminal carboxyl of the seventh residue (lactone bond) to form a lactone ring (Grangemard *et al*., 2001; Konz *et al*., 1999). This heptapeptide sequence differs with each variant (Nerurkar, 2010).

Lichenysins have demonstrated both antibiotic and biosurfactant activity (Nerurkar, 2010; Veith *et al*., 2004). Antibiotic activity was documented by Yakimov *et al*. (1995) who discovered the antibiotic properties of lichenysin A towards several bacterial species including *Escherichia coli* and *B. subtilis*. Although the manner in which these antibiotic molecules function is yet to be clarified, Yakimov *et al*. (1995) did establish that their antibiotic activity is independent of the lactone linkage (cyclic structure) as the open molecule displayed the same antibiotic potential as the closed molecule. Instead, Yakimov *et al*. (1995) showed that the antibiotic activity is dependent on the free polar groups of the molecule.

With regards to its biosurfactant activity, lichenysin works to solubilize hydrophobic substrates by lowering interfacial tension between the substrate and its aqueous environment (Nerurkar, 2010). Lichenysin, like other biosurfactants, are amphipathic in nature - they possess both a hydrophilic domain (carboxylic group of aspartate or glutamate depending on the lichenysin

variant) and a hydrophobic domain (formed by the nonpolar residues in the peptide chain along with the lipid tail) (Nerurkar, 2010). This allows for the accumulation of hydrocarbons within the hydrophobic core leading to higher solubility and enhanced bioavailability to bacterial cells (Das and Chandran, 2011; Uzoigwe *et al*., 2015; Kaczorek *et al*., 2018).

Biosurfactant-producing members of the *Bacillus* spp. are often isolated from oil-contaminated areas (Nerurkar, 2010). Lichenysins have garnered great interest within the oil industry owing to the environmentally friendly and cost-effective manner in which they are used to recover oil from reservoirs (Nerurkar, 2010). In addition, these biosurfactants are currently being studied for a variety of uses; this includes their biofilm control properties which may be implemented in both hospital settings and the food industry against pathogenic bacteria who tend to grow on the equipment used in these sectors (Coronel-Leon *et al*. 2015). The presence of lichenysin-like gene clusters in all three of *B. licheniformis* strains in this study correlates with the oil displacement activity observed in the liquid and plate assays in Chapter 2 (**section 2.4.2. of Results and Discussion**) and suggests that this biosurfactant may play a pivotal role in hydrocarbon degradation. It must be noted that, while oil displacement activity was noted in *B. licheniformis* But5A1A (extensive) and Wam9A3 (moderate), no oil displacement activity was noted for *B. licheniformis* But5A1B. As such, more complex regulatory mechanisms may underlie biosurfactant production and oil displacement and this should be the subject for further investigation.

3.3.5. GC-MS Analysis

GC-MS analysis was undertaken to confirm the results of the liquid assays and additionally, to semi-quantitatively measure the capacity of hydrocarbon degradation across the bacterial strains *B. licheniformis* But5A1A, But5A1B and Wam9A3.

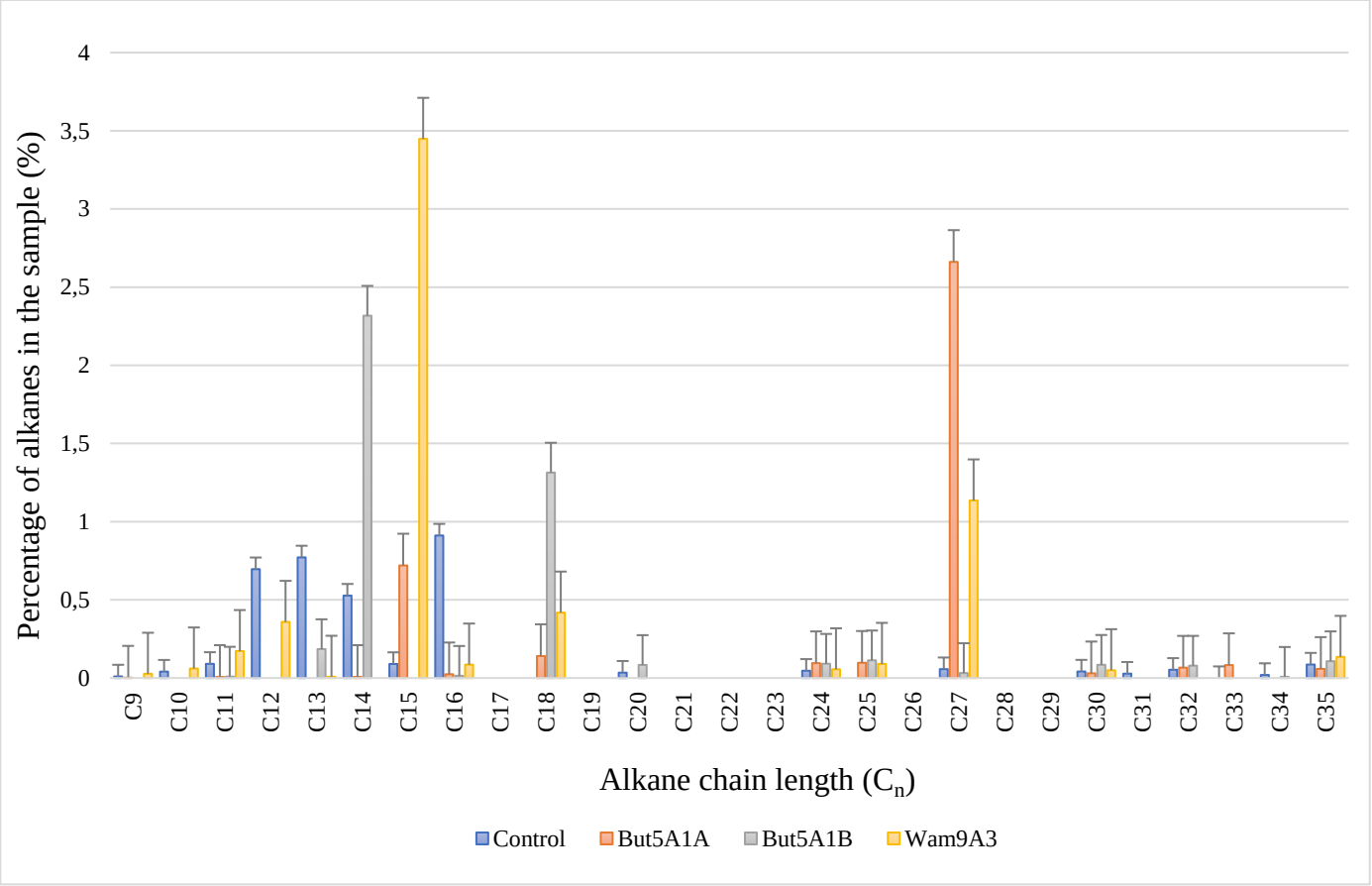

Figure 9: Percentage (%) of alkanes within the biodegraded oil samples, identified through the GC-MS analysis. The graph displays the remaining alkanes, as well as the quantity of each, left within the oil samples following their five day incubation with the three *B. licheniformis* strains. Chain length of the alkanes are denoted as C_n.

All three *B. licheniformis* strains showed a general decrease in the alkanes ranging between C_9-C_{14}, and also C_{16}, when compared to the control (**Figure 9**). According to the liquid assays, *B. licheniformis* But5A1A showed the greatest capacity for oil degradation while *B. licheniformis* Wam9A3, comparatively, produced the lowest level of oil degradation. From the GC-MS results (**Figure 9**), it can be observed that *B. licheniformis* But5A1A degraded the smaller alkanes (C_9-C_{14}, C_{16}) to the greatest extent. Meanwhile *B. licheniformis* Wam9A3, although it did cause some level of degradation, did not perform as well as *B. licheniformis*

But5A1A or *B. licheniformis* But5A1B – this is particularly true for the alkanes C_{10}-C_{12} and C_{16}. These GC-MS results correlate to the liquid assays where *B. licheniformis* Wam9A3 was observed to be the worst oil-degrader amongst the three strains.

The GC-MS data showed a handful of instances where alkanes appear to be in greater volumes in the test samples than that of the control. This is clearly evident in a spike at C_{14} in *B. licheniformis* But5A1B and at size range of C_{15} and C_{27} in both *B. licheniformis* But5A1A and Wam9A3. Furthermore, there are measurable volumes of C_{18} alkanes in all three *B. licheniformis* samples which are completely absent from the control. This may be due to the bacterial synthesis of secondary metabolites which fall in these size ranges and which remained unidentified by antiSMASH as they are yet to be characterized within the species *Bacillus licheniformis*.

Alternatively, bacteria have been shown to synthesize both alkanes and alkenes which they use as a chemo-attractants or to fend off insects, pathogens or water loss (Kang and Nielsen, 2017; Timmis, 2010). These hydrocarbon compounds are produced at low levels and differ in structure from those found in petroleum fuels (Kang and Nielsen, 2017). However, GC-MS analysis does not have the specificity to differentiate between them. The biosynthesis of alkane hydrocarbons within the *Bacillus* spp. has been mentioned in just a few studies. A study on *Bacillus megaterium* YFM3.25 identified two alkanes, nonane (C_9H_{20}) and hexadecane ($C_{16}H_{34}$) which are toxic to nematodes (Huang *et al*., 2010).

A study conducted by Jones (1969) using gas-liquid chromatography, showed that the bacterium *Bacillus* spp. NCIB 10404 produced alkane hydrocarbons ranging between C_{14}-C_{34} with distinctive peaks formed at C_{27}-C_{29} – this is congruent with the outcome observed amongst all three strains at the C_{27} peak. This heptacosane alkane ($C_{27}H_{56}$) was also identified in the bacterial strain *B. licheniformis* VS16 where it was found to be a prominent constituent of the biosurfactant that it synthesized (Giri, *et al*., 2017). Therefore, it is possible that the heptacosane compound, produced by *B. licheniformis* But5A1A and Wam9A3 (**Figure 9**), is a constituent of an uncharacterized biosurfactant within the species. This would align well with the results of the biosurfactant assays where *B. licheniformis* But5A1A and Wam9A3 yielded excessive and moderate levels of biosurfactant, respectively.

Additionally, the *Bacillus* spp. NCIB 10404 was shown to sometimes, depending on the media, yield a minor peak in the C_{18}-C_{22} alkane range (Jones, 1969). Again, this is congruent with the results from the GC-MS analysis where the three strains, especially *B. licheniformis* But5A1B, were observed to produce C_{18} alkanes. There are currently no existing articles to explain the presence of this peak, however, this again may be an uncharacterized secondary metabolite which may contribute to why this strain is able to degrade motor oil so well.

3.4. Conclusion

Here we have undertaken genome sequencing of three *B. licheniformis* strains with the purpose of identifying the molecular basis underlying hydrocarbon degradation. Although no clear hydrocarbon-degrading pathways were established, gene orthologs of the *alkH*-encoded aldehyde dehydrogenase and *alkK*-encoded fatty acyl-CoA ligase/synthetase were discovered; these enzymes play an important role in the alkane degradation pathway found in *P. putida* Gpo1. In addition, four possible alkane monooxygenases, a FMNH2-dependent alkanesulfonate, a nitronate monooxygenase, an alpha/beta hydrolase and a catechol-2,3-dioxygenase were also discovered encoded within the three genomes; these enzymes have also been linked to either aliphatic or aromatic hydrocarbon degradation in other bacteria. It cannot, however, be excluded that hydrocarbon degradation may be linked to other genetic elements or enzymes encoded on the genome which have to date not been characterized. Additional analyses such as differential gene expression in the presence of hydrocarbons and knock-out mutagenesis may shed further light on the mechanism underlying hydrocarbon degradation.

One stand-out factor on the genomes of the studied *B. licheniformis* strains is the gene cluster coding for the biosurfactant lichenysin, a secondary metabolite secreted by members of the species (Nerurkar, 2010). This biosurfactant enhances the emulsification of the motor oil thus allowing the bacteria to obtain and degrade the motor oil more readily. Because there are several structural variations of this lichenysin molecule (Nerurkar, 2010), further testing would need to be done to accurately classify this lichenysin-type. However, its presence correlates well with the observed oil displacement activities of these bacteria in plate-based assays.

GC-MS analysis showed a general trend in the degradation of C_9-C_{16} alkanes amongst the three *B. licheniformis* strains. However, these strains also appear to be producing alkanes in the C_{18} and C_{27} range, which may represent uncharacterized secondary metabolites whose functions

are yet to be confirmed. Because GC-MS cannot distinguish between the bacterial alkanes and the alkanes in the oil, the alkanes synthesized by the bacterial strains may actually be overshadowing any possible long-chain alkane degradation abilities they have. Overall, the GC-MS results help to strengthen the liquid assay results which once again suggests *B. licheniformis* But5A1A to be the strongest hydrocarbon degrader and *B. licheniformis* Wam9A3 the weakest, amongst the three strains.

Prior to obtaining their pure cultures, *B. licheniformis* But5A1A and But5A1B demonstrated a great synergy in degrading motor oil. This synergy may be explained through the GC-MS analysis results which are indicative of their specificities for the degradation of different alkane chain lengths – *B. licheniformis* But5A1A degraded alkanes of the C_9-C_{14} range to a better degree while *B. licheniformis* But5A1B appears to be better at degrading the C_{15} and C_{16}. This information alludes to the use of a consortium-like system to enhance hydrocarbon-degradation. The use of bacterial consortia has been found to be more advantageous than the use of pure cultures as they enhance both oil degradation and the rate at which it is degraded (Basu *et al*., 2014; García-Alcántara *et al*., 2016). This is because, comparatively, pure bacterial cultures generally display longer growth cycles, and each strain has a specific hydrocarbon-degradation rate which ends up limiting their overall performance as a hydrocarbon-degrading system (García-Alcántara *et al*., 2016). Future work should focus on the design of a large-scale biodegradation system such as a bioreactor-based hydrocarbon-contaminant remediation system which incorporates a consortium of the strains identified in this study.

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Summary

Crude oil is undoubtedly an important resource as roughly a third of the world's total energy is derived from it. Unfortunately, the accidental spillage of oil into the environment, which occurs during the acquisition and transport of this resource, negatively impacts on the environment. As a result, multiple remediation processes have been developed. One approach that has received substantial attention is the use of bacteria in the bioremediation of petroleum hydrocarbons. In this study, we elucidated the hydrocarbon degradation capacities of aerobic thermophilic bacteria as these bacteria display great promise in the bioremediation of petroleum hydrocarbons. This can be attributed to the thermostable enzymes they encode which allow them to have greater reaction kinetics than their mesophilic counterparts.

From a collection of 168 aerobic thermophilic bacterial strains, we have identified many thermophilic bacteria which showed the capacity for oil degradation; this was achieved through a series of liquid-based and plate-based assays. Three candidates, namely *Bacillus licheniformis* But5A1A, But5A1B and Wam9A3, were selected for further study. Sequencing of the complete genomes allowed us to identify a number of candidate genes coding for enzymes with potential roles in the hydrocarbon degradation observed in the screening assays. Further work should focus on the knock-out mutagenesis and functional characterization of these candidate genes.

Furthermore, we identified a genetic locus on the genomes of all three strains coding for a biosurfactant, lichenysin. This biosurfactant has a purported role in increasing the bioavailability of hydrocarbons and may improve the degradation capacities of the *B. licheniformis* strains. Similarly, functional characterization should be undertaken to definitively prove a role for the biosurfactant in hydrocarbon degradation. GC-MS analysis showed that the *B. licheniformis* strains degrade alkane hydrocarbons in the range of $C_{9\text{-}14,16}$ confirming the observed effects in the screening assays. Of note is that these strains also appear to synthesize alkanes, C_{18} and C_{27} in size. These alkanes may represent uncharacterized secondary metabolites which should be further explored experimentally.

Overall, although their hydrocarbon degradation pathways are yet to be elucidated, we have found that aerobic thermophilic bacteria belonging to the species *Bacillus licheniformis* are

potential candidates in the bacterial bioremediation of oil pollutants. This is one step closer to addressing the problems presented by oil pollution in the environment. Future work should concern the characterization of the hydrocarbon pathways used by these aerobic thermophilic *B. licheniformis* strains; this can be achieved through RNA sequencing techniques. Once this has been elucidated, the implementation of these strains in an *ex situ* strategy should be considered as the capacity of these thermophiles for *in situ* bioremediation is restricted by their temperature optima. These strains may be developed into a consortium for bioreactor-based bioremediation of oil contaminants. This type of implementation is advantageous as the parameters relating to both oil contaminant and bioremediating bacteria can be controlled and managed.

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