Investigating the degradation of phenols by aerobic thermophilic bacteria.

by

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Declaration

I declare that this dissertation is my own, unaided work. 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)

17th day of November 2020 at 291 Jenner street, Rembrandt Park, Gauteng.

Preface

Phenol and its derivatives are toxic aromatic hydrocarbons frequently found to contaminate industrial and agricultural wastewaters. Bacterial whole-cell biodegradation is a frequently employed technique for phenol removal as it is environmentally friendly, cost effective and allows for the complete mineralisation of phenol. A promising avenue of research is the use of aerobic thermophilic bacteria for phenol bioremediation. Advantages of high temperature bioremediation strategies include increased phenol solubility at higher temperatures as well faster biomass production and phenol degradation rates in thermophilic bacteria. This study aimed to identify and characterise aerobic thermophilic bacteria which are able to survive in the presence of phenol and actively degrade high concentrations of phenol.

Chapter 1 presents a review of the pertinent literature pertaining to phenols, their environmental effects, mechanisms for their degradation and the pathways utilised by microbial phenol degraders. In Chapter 2 an extensive culture of thermophilic bacteria was screened for the capacity for resistance to phenol toxicity and phenol degradation. A number of taxa tolerant to high concentrations (up to 1,200 mg/l) of phenol were identified. Subsequently, in Chapter 3 the most proficient phenol degrading strain, *Bacillus smithii* Row 2A12.3A was further characterized through genome sequencing and comparative genomic analysis. The molecular determinants underlying its capacity for phenol degradation were determined *in silico*.

Dedication

To my devoted husband, for always believing in me even when I did not believe in myself.

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CHAPTER TWO: SCREENING OF A LARGE COLLECTION OF THERMOPHILIC AEROBIC BACILLI FOR PHENOL TOLERANCE AND BIODEGRADATION

CHAPTER THREE: PHENOL DEGRADATION THROUGH THE *META*-CLEAVAGE PATHWAY IN *BACILLUS SMITHII* NOVEL STRAIN ROW2A12.3A

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Nomenclature

4-AAP	4-aminoantipyrine
5CHMSD	5-carboxymethyl-2-hydroxymuconate-6-semialdehyde dehydrogenase
AAI	Amino acid identity
ANI	Average nucleotide identity
COG	Conserved orthologous group
CPE	Cloud point extraction
dDDH	Digital DNA-DNA Hybridization
DSM	Deutsche sammlung von microorganismen
DSMZ	Deutsche sammlung von microorganismen und zellkulturen
Fdx	Ferredoxin
GC/MS	Gas chromatography-mass spectrometry
GDBP	Genome blast distance pipeline
gDNA	Genomic DNA
GGDC	Genome to genome distance calculator
HMSD	2-hydroxymuconate-6-semialdehyde dehydrogenase
HMSH	2-hydroxymuconate-6-semialdehyde hydrolase
hpi	Hours post inoculation
HPLC	High-performance liquid chromatography
IclR	Isocitrate lyase regulator
LB	Luria Bertani
MAFFT	Multiple Alignment Fast Fourier Transform
MFS	Major Facilitator Superfamily
MSM	Minimal salt media
PH	Phenol hydroxylase
PHASTER	PHAge search tool
RAST	Rapid annotations using subsystems technology
RDP	Ribosomal database project
TCA	Tricarboxylic acid cycle
TPL	Tyrosine phenol lyase
TYGS	Type (strain) genome server

CHAPTER ONE

BIOREMEDIATION OF PHENOL AND ITS DERIVATIVES – A LITERATURE STUDY

1.1 Introduction

Phenol and its derivatives are important in the synthesis of numerous products applied in multiple facets of life, including domestic, industrial and agricultural practices (Gianfreda, *et al.* 2006). Phenols may be naturally or synthetically derived and their continuous use ultimately leads to the contamination of the air, soil and water (Al-Khalid & El-Naas, 2012). While phenols frequently contaminate the air and soil, the most pressing concern is generally their presence and distribution in waterbodies (Al-Khalid & El-Naas, 2012; Christen, *et al.* 2012; Senthilvelan, *et al.* 2014). Many phenols, particularly synthetic ones, are highly toxic and their presence needs to be limited. This has resulted in extensive investigation into phenol removal and remediation strategies (Christen, *et al.* 2012). These strategies can be physicochemical, biological or a combination of both in order to ensure efficient removal of phenol from solution or the degradation of phenol via mineralisation (El-Abbassi, *et al.* 2009). In particular, bioremediation strategies are gaining traction as numerous microorganisms have been identified with an inherent capacity for the degradation of phenols (Nešvera, *et al.* 2015). Numerous studies have been conducted into the use of different remediation strategies, with traditional whole cell mineralisation of phenols taking preference due to the lower cost and relative simplicity of establishing a water treatment system (Basha, *et al.* 2010; Hasan & Jabeen, 2015; Pradeep, *et al.* 2015; Villegas, *et al.* 2016). Here we have discussed pertinent literature relating to phenol and phenolic compounds as important environmental pollutants as well as the latest trends in terms of removal of these pollutants, with particular emphasis on microbial bioremediation strategies, which form the central topic of this thesis.

1.2 Phenol and phenolic compounds

Phenol or hydroxybenzene is a monoaromatic hydrocarbon, which is comprised of a hydroxyl (-OH) group that is directly bound to an aromatic (benzene) ring (Nguyen, *et al.* 2003; Dai & Mumper, 2010). Given its structure, phenol is classed as the simplest member of aromatic organic alcohols (Nguyen, *et al.* 2003; Weber & Weber, 2010). Phenol is characterised as a colourless crystalline solid with a distinct odour (Basha, *et al.* 2010). The physical properties of phenol (C_6H_5OH)

include a molecular weight of 94.11 g/mol, a relatively low melting point of 41-43°C, a boiling point of 181.8°C and a pKa (acid dissociation constant) of 9.89-9.95 making it weakly acidic (Nguyen, *et al.* 2003; Weber & Weber, 2010; Kynadi & Suchithra, 2017). Phenol is completely soluble in alcohols, ethers, acids, ketones and, halogenated and aromatic hydrocarbons (Basha, *et al.* 2010; Weber & Weber, 2010; Mohammadi, *et al.* 2015). Phenol solubility in water is dependent on temperature, being partially miscible in water below 68.4°C and completely miscible in temperatures exceeding this (Nguyen, *et al.* 2003; Weber & Weber, 2010). Interactions with water are also shown to substantially reduce the melting and solidification temperatures of phenol (Nguyen, *et al.* 2003).

Regarding chemical interactions phenol is unique in that the presence of an aromatic ring and a bound hydroxyl group allow it to partake in either electrophilic or nucleophilic reactions (Weber & Weber, 2010). The distribution of the unshared electron pair of the hydroxyl group across the ring structure creates an excess of electrons in the *para*- and *ortho*- positions (Weber & Weber, 2010). This is important as it determines the outcome of electrophilic reactions, the position of the chemical group, the resulting chemistries and the subsequent end use of these phenolic derivatives. It is also important to note that phenol is highly susceptible to oxidation, which has notable carry over effects for producing phenols and for the degradation of phenolic pollutants (Basha, *et al.* 2010; Prasse, *et al.* 2018).

1.2.1 Sources of phenols

Phenols occur in the environment naturally as a result of plant metabolic processes or the decay of organic matter (Michlowicz & Duda, 2007; Stalikas, 2007; Fuchs, *et al.* 2011; Kynadi & Suchithra, 2017). The production of phenols in plants has been selected for throughout the evolution of vascular plants, as it plays numerous important roles in plant development, structure and defence (Stalikas, 2007; Dai & Mumper, 2010; Lattanzio, 2013). Plants produce a wide range of secondary metabolites referred to as phytochemicals (Stalikas, 2007). Phenolic compounds are the largest group of phytochemicals produced, with over 8,000 different plant phenols having been identified (Stalikas, 2007; Dai & Mumper, 2010). These phenols are produced through two major pathways, the shikimate/ phenylpropanoid

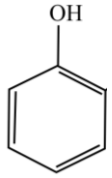
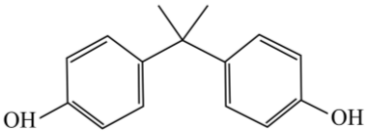
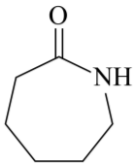
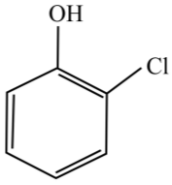
pathway and the polyketide acetate/ malonate pathway (Lattanzio, 2013). The phenols produced can be broadly divided into monomeric, polymeric and polyphenols (Stalikas, 2007; Lattanzio, 2013). Simple monomeric phenols are ubiquitous in plants and include bactericidal compounds, while polymeric and polyphenols have more complex structures and include tannins, lignin and flavonoids (Dai & Mumper, 2010; Fuchs, *et al.* 2011). Despite possessing the pathways to produce numerous phenols, plants cannot effectively remove high concentrations of phenol (Ucisik & Trapp, 2006; Fuchs, *et al.* 2011). This is not necessarily problematic as the concentrations of plant phenols released into the environment do not exhibit toxic effects (Dai & Mumper, 2010; Kynadi & Suchithra, 2017). It is only as a result of anthropogenic activities that the release of phenols – either plant derived or synthetic – has highly negative effects on the environment, due to the high concentrations of phenols released (Kynadi & Suchithra, 2017).

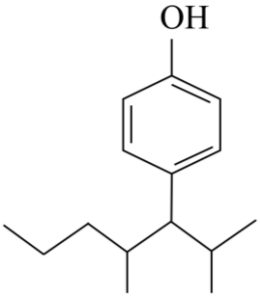
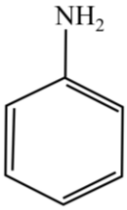
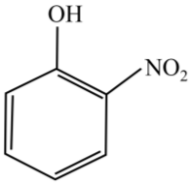
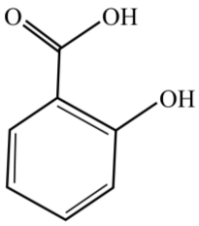
Phenol and phenolic derivatives are widely employed chemicals in numerous agricultural and industrial practices (Gianfreda, *et al.* 2006). Currently the global phenol market value is recorded as being approximately 20 billion US dollars and increasing (IMARC Group, 2019). When phenol mass production first began it was primarily derived from coal tar (Michlowicz & Duda, 2007; Weber & Weber, 2010). The main route of phenol production presently, however, is the cumene (isopropylbenzene – an aromatic hydrocarbon with an aliphatic substitution) to phenol method (Schmidt, 2005; Zakoshansky, 2007; Weber & Weber, 2010). In short, this process consists of the oxidation of cumene to cumene hydroperoxide followed by its cleavage through mineral acid catalysis to phenol and acetone (Schmidt, 2005; Zakoshansky, 2007; Weber & Weber, 2010). Whilst other methods such as sourcing phenol from coal tar are operational, they account for less than 5% of global production (Weber & Weber, 2010).

In commercial applications phenols frequently form intermediates in the production of numerous end products employed in industrial, agricultural and domestic applications (Table 1.1) (Basha, *et al.* 2010; Weber & Weber, 2010; Krastanov, *et al.* 2013; Nešvera, *et al.* 2015; Kynadi & Suchithra, 2017). In terms of supply and usage, Bisphenol A, phenolic resins and caprolactam are in order the most widely

produced and consumed phenols (Michlowicz & Duda, 2007; Weber & Weber, 2010). Their global economic, industrial, agricultural and medical importance makes the supply and use of phenols indispensable. However, a problem emerges in that phenol and various phenol derivatives are highly toxic both to humans and the environment even at low concentrations, making their removal imperative (El-Naas, *et al.* 2009; Al-Khalid & El-Naas, 2012; Rafiei, *et al.* 2014; Kynadi & Suchithra, 2017).

Table 1.1: The structures of phenols and the industries in which they are frequently employed.

Compound & Diagram		Industry - End product	References
Phenol 		Coal processing, oil refineries, steel and smelting industries and a frequent product of petrochemical industries	Gianfreda, *et al.* 2006; Nešvera, *et al.* 2015
Phenol derivatives	Bisphenol A 	Resins/ Plastics – Epoxy resins (fibre glass) and Polycarbonate resins Paint - finishes	Basha, *et al.* 2010; Weber & Weber, 2010; Villegas, *et al.* 2016; Kynadi & Suchithra, 2017
	Caprolactam 	Textiles and plastics – nylon and polyamide resins/plastics for example in the manufacture of fish nets, carpets and clothing	Basha, *et al.* 2010; Krastanov, *et al.* 2013
	Chloro-phenols  2-chlorophenol	Textiles- preservation Pharmaceuticals- antiseptics, disinfectant, bactericides Timber protection – varnish and fungicides Agriculture- pesticides Pulp and paper - bleaching	Alvarez, 2008; Field & Sierra, 2008; Basha, *et al.* 2010; Al-Khalid & El-Naas, 2012; Hasan & Jabeen, 2015;

			Kynadi & Suchithra, 2017
	Alkylphenols  nonylphenol	Resins/ plastics – phenolic resins such as Bakelite Chemical – detergents, surfactants and emulsifiers Agriculture – pesticides	Basha, *et al.* 2010; Weber & Weber, 2010; Nešvera, *et al.* 2015
	Phenylamines  Aniline	Textile – dyes and pigments Pharmaceutical - cosmetics Agriculture – herbicide intermediates	Basha, *et al.* 2010; Mohammadi, *et al.* 2015; Pradeep, *et al.* 2015;
	Nitrophenols  2-Nitrophenol	Textile – dyes Agriculture – pesticides Munitions – explosives	Nešvera, *et al.* 2015
	Salicylic acid 	Pharmaceutical – the core component of aspirin and other similar drugs	Kynadi & Suchithra, 2017

1.2.2 Phenolic pollution and toxicity

Phenols are widespread contaminants of the environment that contaminate soil, air and water (both surface and subsurface), the latter being the focus of this literature review (Al-Khalid & El-Naas, 2012). The chemical nature of phenol makes it a particularly problematic pollutant as its low solubility, high density and stability

make it a long-term and recalcitrant pollutant (Michlowicz & Duda, 2007; Al-Khalid & El-Naas, 2012; Kynadi & Suchithra, 2017). Both phenol and associated derivatives have been shown to exhibit toxic effects on the environment, animals, aquatic life and humans (Michlowicz & Duda, 2007; Basha, *et al.* 2010; Al-Khalid & El-Naas, 2012; Christen, *et al.* 2012; Krastanov, *et al.* 2013; Asadgol, *et al.* 2014; Hasan & Jabeen, 2015; Mohammadi, *et al.* 2015; Nešvera, *et al.* 2015; Pradeep, *et al.* 2015; Liu, *et al.* 2016; Villegas, *et al.* 2016; Kynadi & Suchithra, 2017).

Phenol has different toxicity levels in different organisms. For fish, the range is generally cited as being between 5-25 mg/l phenol, depending on fish maturity (Kumar *et al.* 2005; Al-Khalid & El-Naas, 2012; Villegas, *et al.* 2016). Different animal models have different capacities for phenol metabolism, the toxic oral dose for humans is said to be 25 mg with the lethal oral dose being cited as 1,000 mg (Al-Khalid & El-Naas, 2012; Kynadi & Suchithra, 2017). Phenol exposure can be chronic or acute and leads to systemic poisoning through the denaturation of proteins, inhibition of cell synthesis and promotion of cell death (Michlowicz & Duda, 2007; Basha, *et al.* 2010; Mohammadi, *et al.* 2015; Pradeep, *et al.* 2015; Villegas, *et al.* 2016). As such, it has drastic effects on numerous metabolic processes. These include those in the dermal-, central nervous-, respiratory-, cardiovascular-, gastrointestinal-, renal-, ocular- and reproductive systems (Michlowicz & Duda, 2007; Basha, *et al.* 2010; Mohammadi, *et al.* 2015; Pradeep, *et al.* 2015; Villegas, *et al.* 2016). Whilst reproductive effects have not been demonstrated in humans, animal models show exposure to phenol to adversely affect foetal development, leading to reduced growth and developmental abnormalities (Basha, *et al.* 2010; Pradeep, *et al.* 2015). Similarly, there is a substantial amount of evidence indicating that exposure to phenol plays a role in carcinogenesis and mutagenesis (Krastanov, *et al.* 2013; Nešvera, *et al.* 2015; Liu, *et al.* 2016). While health concerns are generally the key focus of phenolic pollutants, it is also important to note that large concentrations of phenol can reduce soil fertility and lead to the wilting and death of plants – as shown in willow trees exposed to 250-1,000 mg/l phenol (Ucisik & Trapp, 2006; Senthilvelan, *et al.* 2014).

Taken together these factors have led to the classification of phenols as priority pollutants along with strict regulation of the release of phenols into the environment (Basha, *et al.* 2010; Al-Khalid & El-Naas, 2012; Christen, *et al.* 2012; Asadgol, *et al.* 2014; Liu, *et al.* 2016; Villegas, *et al.* 2016; Kynadi & Suchithra, 2017). Regulations for phenolic content in potable water and wastewater differ depending on the governing authority. Drinking water regulations include the WHO recommendation of not exceeding 1 µg/l and the EU Council directive's upper limit of 0.5 µg/l (Al-Khalid & El-Naas, 2012). Wastewater emission values are more variable, according to EU regulations wastewater should not exceed a phenolic content of 0.5 mg/l, while the UAE has lowered this to 0.1 mg/l in industrial and agricultural effluents (Al-Khalid & El-Naas, 2012; Christen, *et al.* 2012; Senthilvelan, *et al.* 2014). Given that industrial and agricultural effluents can range between 0.1 mg/l to 6,800-10,000 mg/l phenol in highly polluted environments, the development and use of efficient removal and remediation strategies are vital (Christen, *et al.* 2012; Krastanov, *et al.* 2013; Kynadi & Suchithra, 2017).

1.3 Remediation/ Removal strategies

The removal or remediation of phenol and phenolic contaminated environments is essential, and several technologies have been developed for this purpose (Al-Khalid & El-Naas, 2012; Villegas, *et al.* 2016; Kynadi & Suchithra 2017). These technologies can be broadly divided into physicochemical and biological treatment technologies (El-Abbassi, *et al.* 2009). Physicochemical treatment technologies allow for either the removal or the recovery of phenols depending on the system employed (Mohammadi, *et al.* 2015). Physicochemical treatment technologies include extractions, adsorption processes, oxidation processes and others to be expanded further below (Mohammadi, *et al.* 2015; Villegas, *et al.* 2016). Biological treatments involve the degradation of phenols by microorganisms or their enzymes (Wilberg, *et al.* 2002; Kumar *et al.* 2005; Gianfreda, *et al.* 2006; Pradeep, *et al.* 2015). Irrespective of whether a physicochemical or biological technology is used, several important factors need to be taken into consideration including temperature, pH and phenol concentration (initial and final) for the water being treated (Esfandyari, *et al.* 2015; Mohammadi, *et al.* 2015; Villegas, *et al.* 2016).

1.4 Physicochemical remediation

A broad range of distinct physicochemical technologies have been developed for the removal of phenolic pollutants. These can be broadly grouped as thermal decomposition, distillation, adsorption, extraction, membrane processes, oxidation processes, electrochemical processes as well as hybrid systems which may combine these processes (Soto, *et al.* 2011; Al-Khalid & El-Naas, 2012; Asadgol, *et al.* 2014; Senthilvelan, *et al.* 2014; Zain, *et al.* 2014; Hasan & Jabeen, 2015; Mohammadi, *et al.* 2015; Villegas, *et al.* 2016; Kynadi & Suchithra, 2017; Alshabib & Onaizi, 2019). These methods are continuously being altered and redeveloped in order to comply with environmental regulations that emphasise the near complete removal of phenol from wastewater (Zain, *et al.* 2014; Mohammadi, *et al.* 2015; Alshabib & Onaizi, 2019). Distillation methods include steam, steam (thermal) plasma jet and solar distillation approaches, but their application has been largely limited to agricultural waste treatment (Mohammadi, *et al.* 2015; Villegas, *et al.* 2016). Adsorption methods include the traditional and expensive activated carbon adsorption approach, as well as more advanced methods which make use of mineral adsorbents, resin adsorbents, industrial and agricultural waste products and bio-sorbents for a more cost effective and environmentally friendly approach (Soto, *et al.* 2011; Zain, *et al.* 2014). Extraction methods include liquid-liquid (solvent) extractions, solid-phase extractions and cloud point extractions (CPE), with CPE being considered the most sustainable (Zain, *et al.* 2014; Villegas, *et al.* 2016).

Membrane processes encompass a large collection of technologies, including membrane distillation, photocatalysis, nanofiltration, reverse osmosis, pervaporation and membrane extractions (Mohammadi, *et al.* 2015; Villegas, *et al.* 2016). These systems are highly effective and sustainable, however, they are hindered primarily by membrane fouling, which occurs as wastewater often contains particles which interfere with these processes (Villegas, *et al.* 2016). Oxidation processes are varied with the inclusion of chemical, electrochemical, Fenton, ultraviolet/hydrogen peroxide, ozonation, wet-air and catalytic wet-air oxidation treatments (Fajador, *et al.* 2014; Esfandyari, *et al.* 2015; Mohammadi, *et al.* 2015; Villegas, *et al.* 2016). The most prevalent problem associated with oxidation treatments is the generation of secondary pollutants (Prasse, *et al.* 2018).

Electrochemical processes have gained popularity due to the increased efficacy of phenol removal and are frequently employed as hybrid systems that combine electrochemical oxidation, flocculation and coagulation, using several different metals as anode materials (Fajador, *et al.* 2014; Esfandyari, *et al.* 2015).

Despite advancements in physicochemical methods there are still several major drawbacks with these approaches, including high cost, complexity, lower efficiency and most importantly the production of toxic secondary pollutants which can be difficult to detect and remove using standard protocols (Al-Khalid & El-Naas, 2012; Krastanov, *et al.* 2013; Asadgol, *et al.* 2014; Senthilvelan, *et al.* 2014; Hasan & Jabeen, 2015; Liu, *et al.* 2016; Kynadi & Suchithra, 2017; Prasse, *et al.* 2018). Given these disadvantages, biological methods tend to be favoured for phenol containing wastewater treatment (Krastanov, *et al.* 2013; Hasan & Jabeen, 2015).

1.5 Biological treatment

Various species of algae, archaea, bacteria and fungi have been identified as capable of degrading phenols with differing efficacies, however, bacterial species remain the most widely investigated and employed (Scragg, 2006; Lu, *et al.* 2009; Basha, *et al.* 2010; Al-Khalid & El-Naas, 2012; Hasan & Jabeen, 2015; Pradeep, *et al.* 2015). Biological phenol degradation may be divided into two main sections, whole cell microbial remediation and enzymatic remediation (Xu & Yang, 2013; Rafiei, *et al.* 2014; Villegas, *et al.* 2016). While whole cell catalysis is generally employed for the complete degradation (through mineralisation) of phenols, enzymatic treatment is most frequently employed for the polymerisation of phenols allowing for recovery (Pradeep, *et al.* 2015). Aerobic and anaerobic microbial whole cell treatment may effectively remove phenol, as microorganisms capable of resisting phenol toxicity may catalyse the complete mineralisation of phenols through intrinsic metabolic pathways, in order to supply carbon and energy to the cell (Fang, *et al.* 2006; Al-Khalid & El-Naas, 2012; Pradeep, *et al.* 2015; Liu, *et al.* 2016; Kynadi & Suchithra, 2017).

1.5.1 Microbial bioremediation

Microorganisms have diverse metabolic capabilities and numerous microorganisms have an inherent capacity for the degradation of polyaromatic hydrocarbons, such as phenol (Fuchs, *et al.* 2011). Microbial bioremediation is one of the most efficacious and widely used methods for the removal of phenols due to the comparatively low cost, ease of design and the generation of simple, non-toxic end products (Al-Khalid & El-Naas, 2012; Villegas, *et al.* 2016). Microorganisms capable of phenol biodegradation may be isolated from contaminated environments or through screening of available strains (Krastanov, *et al.* 2013; Liu, *et al.* 2016). The ability to metabolise phenol is relatively common, especially in bacteria, due to the wide distribution of plant phenols (Omokoko, *et al.* 2008). There are numerous identified whole cell catalysts that may be employed for the degradation of phenols (Table 1.2). The intrinsic ability for phenol removal, substrate concentrations and physicochemical conditions (pH, temperature, oxygen availability, etc.) all play a substantial role in the successful use of the different microorganisms (Kumar *et al.* 2005; Al-Khalid & El-Naas, 2012; Krastanov, *et al.* 2013; Jalayeri, *et al.* 2013; Pradeep, *et al.* 2015; Liu, *et al.* 2016; Kynadi & Suchithra, 2017).

Table 1.2: Various microorganisms identified as being capable of phenol degradation.

	Phylum	**Organism**	**Reference**
Archaea	Crenarchaeota	*Sulfolobus solfataricus*	Christen, *et al.* 2012
Bacteria	Actinobacteria	*Arthrobacter citreus*	Karigar, *et al.* 2006
		Arthrobacter chlorophenolicus	Unell, *et al.* 2008
		Corynebacterium sp. DJ1	Ho, *et al.* 2009
		Nocardia hydrocarbonoxydans	Dabhade, *et al.* 2009
		Nocardiodes sp. NSP41	Cho, *et al.* 2000
		Rhodococcus erythropolis	Margesin *et al.* 2005
		Rhodococcus pyridinivorans	Al-Defiery & Reddy 2018
		Sphingomonas sp. FG03	Liu, *et al.* 2009
	Cyanobacteria	*Spirulina maxima*	Lee, *et al.* 2015
	Firmicutes	*Bacillus brevis*	Bajaj, *et al.* 2009
		Bacillus cereus	Chandra, *et al.* 2011
		Paenibacillus thiaminolyticus	Chandra, *et al.* 2011

	Phylum	Organism	Reference
Bacteria	Proteobacteria	*Acinetobacter tandoii*	Van Dexter, *et al.* 2019
		Acinetobacter calcoaceticus	Liu, *et al.* 2009; Yamaga, *et al.* 2010
		Alcaligenes faecalis	Essam, *et al.* 2010; Jiang, *et al.* 2007
		Burkholderia sp. XTB-5	Chen, *et al.* 2017
		*Burkholderia (*previously *Pseudomonas) psuedomallei*	Afzal, *et al.* 2007
		Citrobacter farmeri	Ren, *et al.* 2014
		Klebsiella oxytoca	Shawabkeh, *et al.* 2007
		Pseudomonas aeroginosa	Afzal, *et al.* 2007
		Pseudomonas fluorescens	Mahiudddin & Faakhruddin, 2012
		Pseudomonas putida	Li, *et al.* 2010; El-Naas, *et al.* 2009
		Ralstonia eutropha	Nickzad, *et al.* 2012
		Ralstonia pickettii	Al-Zuhair, *et al.* 2012
Eukaryotes (Fungi)	Ascomycota	*Aspergillus fumigatus*	Santos & Linardi, 2004
		Candida albicans	Tsai, *et al.* 2005
		Tropicalis spp.	Zhou, *et al.* 2011
		Fusarium flocciferum	Santos & Linardi, 2004
		Graphium sp. FIB4	Santos & Linardi, 2004
	Basidiomycota	*Cryptococcus terreus*	Krallish *et al.* 2006
		Phanerochaete chrysosporium	Lu, *et al.* 2009
		Rhodotorula creatinivora	Krallish *et al.* 2006
		Trichosporon cutaneum	Alexievaa, *et al.* 2004
		Trichosporon dulcitum	Margesin *et al.* 2005
		Trichosporon montevideense	Liu, *et al.* 2011
Eukaryotes (Microalgae)	Chlorophyta	*Chlorella vulgaris*	Scragg, 2006
		Chlorella sp. VT-1	Scragg, 2006
	Ochrophyta	*Ochromonas danica*	Scragg, 2006

Microbial degradation is centred on the concept that microorganisms can use phenolic compounds as a carbon and energy source leading to its complete mineralisation (Fuchs, *et al.* 2011; Pradeep, *et al.* 2015; Liu, *et al.* 2016; Kynadi & Suchithra, 2017). While complete mineralisation is the end-goal, it is important to note that phenols with different substitutions will pass through peripheral pathways which result in the generation of differently substituted metabolic intermediates (Field & Sierra-Alvarez, 2008; Fuchs, *et al.* 2011). Furthermore, many phenolic pollutants are highly toxic, the result of which is microbial inhibition with increasing concentrations of phenols (Kumar *et al.* 2005; Pradeep, *et al.* 2015; Villegas, *et al.* 2016). For these reasons, the kinetic model for the degradation of

phenol is most frequently based on Haldane's inhibitory growth kinetic model and the Monod model (Kumaran & Paruchuri, 1997; Li, *et al.* 2010; Bakhshi, *et al.* 2011; Lee, *et al.* 2011). This may, however, change according to the operating parameters of a given system (Feitkenhauer, *et al.* 2003b).

The extent of phenolic degradation and toxicity is dependent on the phenol substitutions, the microorganism and the physicochemical conditions in question (Alexander & Lustigman, 1966; Krastanov, *et al.* 2013). In terms of phenol substitutions, it has been found that carboxy- and hydroxyl- substitutions may act to increase the degradability of aromatic pollutants, whereas amino-, methoxy-, nitro- and sulfone substitutions reduce the capacity for degradation (Alexander & Lustigman, 1966). Bioremediation strategies rely heavily on the optimisation of conditions, specifically oxygen requirements and operating temperatures, for the removal of phenols (Krastanov, *et al.* 2013).

While an intrinsic capacity for phenolic degradation is essential, there are numerous ways to enhance bioremediation strategies and systems. Field scale applications have indicated that bioprospecting for native phenol degrading microorganisms results in a more stable system, wherein the microorganisms are able to persist (Liu, *et al.* 2016). The use of mixed microbial cultures is also thought to be of benefit as the presence of several microorganisms allows for the coupling of reactions and an increased likelihood of the complete mineralisation of phenols (Ju & Zhang, 2014). Additionally, genetically modified organisms have been designed to accentuate phenol degrading capacities, this includes the introduction of promoters, enhancers or additional gene copies of the necessary proteins (Zìdková, *et al.* 2013; Nešvera, *et al.* 2015). Immobilisation of microorganisms has been shown to increase system stability through protection from high phenol concentrations and washout as well as permitting reuse of immobilised microorganisms (Rafiei, *et al.* 2014). Immobilisation may be achieved in several ways, including adsorption, cell-coating, encapsulation, entrapment and film-attachment, ultimately all of these techniques provide protection and stability making them valuable in industrial scale remediation technologies (Al-Khalid & El-Naas, 2012).

Phenol degrading organisms occur in all the domains of life, however, bacteria are the most frequently used microorganisms in phenol bioremediation strategies (Loh & Chua, 2002; Cao & Loh, 2008; El-Naas, *et al.* 2009; Li, *et al.* 2010; Wang, *et al.* 2011; Al-Khalid & El-Naas, 2012; Mahiudddin & Faakhruddin, 2012; Hasan & Jabeen, 2015). While fungi may have advantages over bacteria, such as greater tolerance to environmental shock, scalability and generally capable of coping with higher concentrations of xenobiotics, bacteria hold numerous advantages with respect to industrial application (Gouma, *et al.* 2014). Bacterial taxa are used in phenolic wastewater treatment as they have faster growth and metabolic rates, with strains capable of surviving in high phenol concentrations (>1500 mg/l), which is important at field-scale operations with high flowrates (Banerjee & Ghoshal, 2010; Al-Khalid & El-Naas, 2012; Krastanov, *et al.* 2013; Gouma, *et al.* 2014; Kynadi & Suchithra, 2017). Bacteria have been used extensively in municipal and industrial wastewater treatment, with optimisation focusing on enhancing the efficacy, reaction rates and stability of these systems (Abeynayaka & Visvanathan, 2011; Hoyos-Hernandez, *et al.* 2014; Chantho, *et al.* 2016).

1.5.2 Enzymatic bioremediation systems

Enzymatic treatment of phenolic contaminated aqueous waste is an attractive option in bioremediation, as it presents several advantages such as high specificity, increased reaction rates and no acclimatisation periods (Dec & Bollag, 1990; Mukherjee, *et al.* 2013; Pradeep, *et al.* 2015; Agarwal, *et al.* 2016; Arca-Ramos, *et al.* 2018). Enzymatic treatment employs enzymes, isolated from a range of different organisms, whose activity triggers the polymerization of phenols and its subsequent precipitation (Xu & Yang, 2013). The success of either bioremediation technology is largely related to the physicochemical properties of the phenols being treated, i.e. the substitutions and structures (Krastanov, *et al.* 2013; Pradeep, *et al.* 2015; Singh *et al.* 2015).

The key enzymes used for the remediation of phenols are the oxidoreductases. There are several oxidoreductases employed for the bioremediation of phenol polluted wastewater, most notably peroxidases and polyphenol oxidases (Mukherjee, *et al.* 2013; Pradeep, *et al.* 2015; Arca-Ramos, *et al.* 2018). The

reaction mechanism of oxidoreductases relies greatly on high redox potentials for phenol polymerisation (Nguyen, *et al.* 2014; Pradeep, *et al.* 2015). Numerous peroxidases have been identified and investigated for phenol remediation including soybean, radish, horse radish, turnip, manganese, lignin, and chloro- peroxidases (Burton, *et al.* 1998; Ryan, *et al.* 2007; Loncar, *et al.* 2011; Pradeep, *et al.* 2015; Arca-Ramos, *et al.* 2018; Husain, 2019). The next most frequently investigated group is the polyphenol oxidases, specifically laccases and tyrosinases. The main benefit of polyphenol oxidases is that, unlike peroxidases, they are activated by oxygen as opposed to hydrogen peroxide (Agarwal, *et al.* 2016; Arca-Ramos, *et al.* 2018; Husain, 2019). Laccases have been isolated from a number of fungal and plant species and their mass production is already conducted due to the ease of isolation, extraction and the subsequent application of these enzymes in numerous industries (Nguyen, *et al.* 2014; Pradeep, *et al.* 2015; Arca-Ramos, *et al.* 2018). The potential of tyrosinases, in phenol degradation has also been relatively well studied. Both tyrosinases and laccases are superior to peroxidases as they do not require hydrogen peroxide (Agarwal, *et al.* 2016). Other enzymes that have been identified for the purpose of treating phenol contaminated wastewater include phenol hydroxylases, phenol oxidases, catechol dioxygenases, cytochrome P450 and sulfotransferases from a wide range of microbial sources (Pradeep, *et al.* 2015; Agarwal, *et al.* 2016; Azizi, *et al.* 2017; Tandjaoui, *et al.* 2019; Ely *et al.* 2020).

The mechanism of action differs for each type of enzyme; however, the central process relies on the radical-catalysed oxidation of the phenolic compounds. The oxidised phenolic compounds are capable of forming polymers which may be removed from solution (Claus, 2004). In peroxidases, for example, the reaction begins with the oxidation of the enzyme by hydrogen peroxide which produces the active form of the enzyme (E1). Once activated the peroxidase accepts the phenolic compound into its active site and triggers its oxidation, generating a radical species of the phenolic compound. The enzyme (E2) is now ready to accept another phenolic compound for oxidation. The enzyme will then return to its native state and the free radical phenols will polymerise or interact with other creating more free radical species, which ultimately polymerise and precipitate out (Dec & Bollag, 1990; Huixian & Taylor, 1994; Claus, 2004; Desentis-Mendoza, *et al.* 2006).

For an enzyme to be considered a good choice of catalyst, it needs to have a high affinity for phenolic compounds, be stable outside of its natural environment and not require cofactors that are expensive to produce (Arca-Ramos, *et al.* 2018). It is also preferential for these enzymes to be diffusible and transported extracellularly for reduced cost and ease of production (Arca-Ramos, *et al.* 2018). In addition to this a number of other factors must be taken into consideration, including additional substrate requirements, mediators, operating parameters and immobilisation (Hou, *et al.* 2014; Nguyen, *et al.* 2015; Agarwal, *et al.* 2016; Becker, *et al.* 2016; Arca-Ramos, *et al.* 2018; Jun, *et al.* 2019; Varga, *et al.* 2019). Whilst enzymatic systems tend not to be resource intensive, additional substrates apart from the phenolic contaminated wastewater are required for effective biocatalysis, these may include hydrogen peroxide, manganese or oxygen, among others (Agarwal, *et al.* 2016; Arca-Ramos, *et al.* 2018). Additives may also be included which help protect the enzymes from deactivation (Agarwal, *et al.* 2016; Arca-Ramos, *et al.* 2018). Another important factor in enzymatic remediation of phenols is the use of mediators. The addition of mediators to the enzymatic system may increase the range of phenolic compounds on which the enzyme can act, the amount of enzyme required as well as the rate at which it acts (Agarwal, *et al.* 2016; Arca-Ramos, *et al.* 2018; Varga, *et al.* 2019). An example of this would be the addition of Triton X-100, which was shown to decrease the required amount of soybean peroxidase for 95% phenol removal by 10-13 fold as it prevents enzyme adsorption to the phenol polymers produced (Steevensz, *et al.* 2014). The use of mediators, such as syringaldehyde, may present their own challenges however, as they may result in the generation of toxic by-products (Nguyen, *et al.* 2015; Becker, *et al.* 2016; Varga, *et al.* 2019).

Operating parameters, such as temperature, pH and agitation, greatly affect the efficiency of an enzymatic system (Kurniawati, *et al.* 2008; Asadgol, *et al.* 2014; Jiang, *et al.* 2014). Temperature and pH are well established in influencing enzyme activity, which may be a result of the increased number of molecule collisions allowing for more rapid reaction rates as temperature increases or the changes in polarity of the side chains of the enzyme with changes in pH (Kurniawati, *et al.* 2008; Jiang, *et al.* 2014; Asadgol, *et al.* 2014; Arca-Ramos, *et al.* 2018). Similarly,

increasing the agitation and subsequent aeration of a system may increase the reaction rate in oxygen requiring systems (e.g. laccases and tyrosinases) (Arca-Ramos, *et al.* 2018). However, balance is essential. Running a system outside of its ideal conditions may increase the rate of a reaction but ultimately decrease the stability of the system (Kurniawati, *et al.* 2008; Jiang, *et al.* 2014). For example, horse radish peroxidase reaches maximal catalytic activity at 45℃, however the stability of the enzyme decreases greatly, and denaturation begins, at temperatures exceeding 40℃ (Jiang, *et al.* 2014). In order to counter complications that may occur during operation, enzymatic systems often make use of immobilised enzymes (Gasser, *et al.* 2014; Hou, *et al.* 2014; Jun, *et al.* 2019). Immobilisation of enzymes generally increases stability, efficacy, reusability and retention, thereby mitigating several of the disadvantages of enzyme systems and reducing the costs in the long run (Fernandez-Lafuente, *et al.* 2000; Gasser, *et al.* 2014; Hou, *et al.* 2014; Liu, *et al.* 2016; Jun, *et al.* 2019). The more recent approach is to include the use of nanoparticles (Hou, *et al.* 2014; Liu, *et al.* 2016; Jun, *et al.* 2019). For example, the immobilisation of a laccase onto silica nanoparticles increased the stability of this enzyme over a wider pH and temperature range as well as improving the scalability of the system (Hou, *et al.* 2014; Jun, *et al.* 2019). Similarly, Liu, *et al.* (2016) created magnetic enzyme aggregates using magnetic nanoparticles and tyrosinase enzymes which were covalently attached to graphene oxide. This drastically improved the efficiency, strength and stability of the system (Liu, *et al.* 2016).

Due to the mixed nature of wastewater and the variety of phenol substitutions, it is postulated that a combination of enzymatic and physicochemical remediation techniques may represent the best approach to phenol remediation (Azizi, *et al.* 2017; Tandjaoui, *et al.* 2019; Ely *et al.* 2020). For example, the combined use of immobilised (alginate) peroxidase and Fenton processes resulted in a substantial increase in phenol removal, increasing removal efficiency to 99.3% (Azizi, *et al.* 2017; Ely *et al.* 2020). Although enzyme systems or hybrid systems present an alternative to traditional whole cell bioremediation techniques, they are not without their own problems.

When compared to whole cell remediation techniques, enzyme systems have several advantages, namely an increase in turnover times due to an increase in reaction velocity (stemming from the enzyme specificity), no acclimatisation requirements or lag phase and reduced waste retention times (Mukherjee, *et al.* 2013; Pradeep, *et al.* 2015; Agarwal, *et al.* 2016; Arca-Ramos, *et al.* 2018). No toxic effects are exerted by the phenols on the enzymes which allows for activation over a broader range of phenolic concentrations (Dec & Bollag, 1990; Mukherjee, *et al.* 2013; Agarwal, *et al.* 2016; Arca-Ramos, *et al.* 2018). These systems furthermore tend to be capable of resisting wider changes in pH and temperature, allowing for more efficient removal of phenolic polymers (Gasser, *et al.* 2014).

While enzyme systems offer several benefits at present their cost is equivalent to that of physicochemical processes such as ozonation (Gasser, *et al.* 2014). The generation of toxic by-products can be circumvented depending on the type of mediator used or combining the process with a physicochemical process, but this often negatively affects either the efficacy or the cost of the system (Gasser, *et al.* 2014; Azizi, *et al.* 2017; Ely *et al.* 2020). Similarly, if using a peroxidase-based system there is the additional complication of introducing hydrogen peroxide to the system (Agarwal, *et al.* 2016). The other frequently cited problem is scalability; while many of these systems have been tested at lab-scale they seldom progress to field testing (Stadlmair, *et al.* 2018). For these reasons the traditional whole cell catalysis approach is more widely employed and successful as it is fully scalable, cost effective and is designed in such a way that the phenolic compounds of interest are completely mineralised.

1.6 Bacterial bioremediation

A number of taxa belonging to the phyla *Proteobacteria*, *Actinobacteria* and *Firmicutes* have been identified for phenol degradation, an indication of a greater inherent capacity for the degradation of aromatic hydrocarbons like phenol (Table 1.2). Members of the phylum *Proteobacteria* have been the subject of the most phenol bioremediation investigations, and include taxa belonging to the genera *Acinetobacter, Burkholderia, Pseudomonas, Ralstonia* and *Sphingomonas* (Nešvera, *et al.* 2015). *Pseudomonas putida* is one of the most prominent examples

of phenol degrading bacteria and has been the subject of numerous studies due to its high degradative efficacy (Al-Khalid & El-Naas, 2012). The efficacy of any bacterial strain, however, is directly related to the physicochemical parameters of the system, particularly regarding the presence of oxygen and the temperature of operation (Christen, *et al.* 2012; Krastanov, *et al.* 2013).

1.6.1 Aerobic and anaerobic bacterial phenol degradation

Phenol degradation has been observed in both aerobic and anaerobic bacterial taxa and have distinct metabolic pathways for the biodegradation of phenols (Pradeep, *et al.* 2015). These pathways are further divided into peripheral and central pathways. The peripheral pathways produce the intermediates catechol, hydroquinone or hydroxyquinol in aerobic pathways or benzoyl-Coenzyme A in anaerobic pathways (Nešvera, *et al.* 2015). The central pathways take several different routes that lead to the production of TCA intermediates which permits complete mineralisation (Figure 1.1) (Basha, *et al.* 2010).

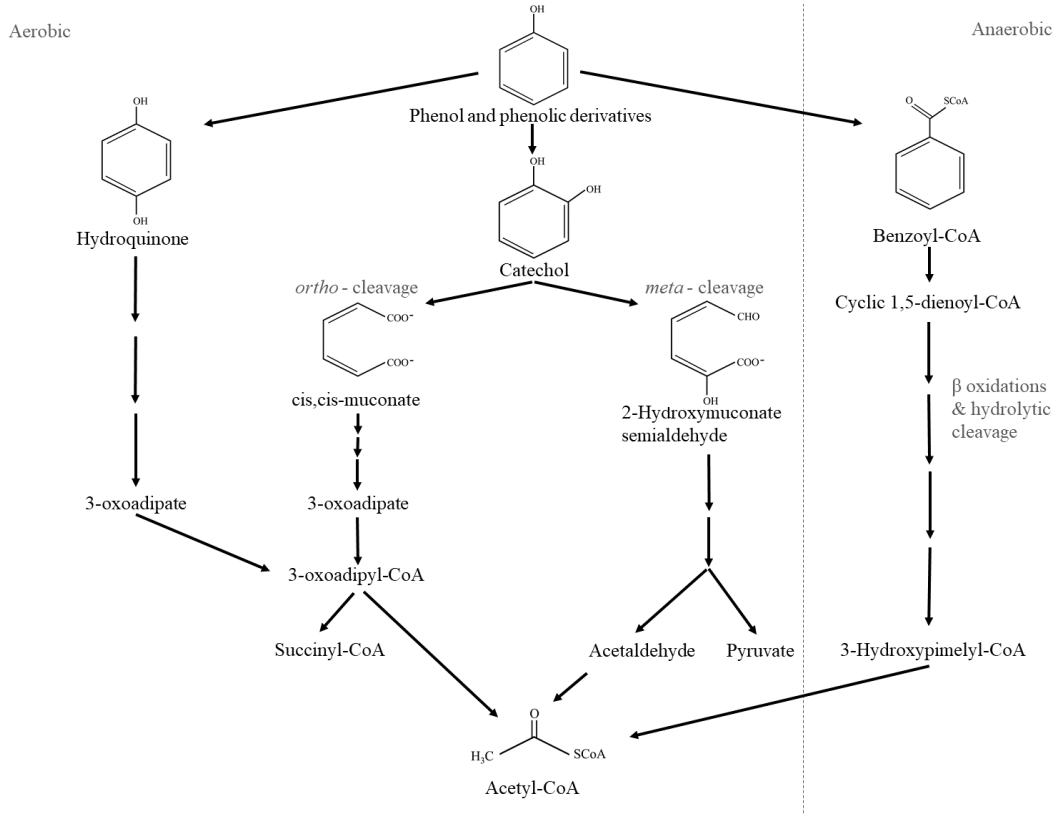


Figure 1.1: Schematic showing the major intermediates in aerobic and anaerobic bacterial degradation of phenols.

The aerobic breakdown of phenol and phenolic derivatives in bacteria is most frequently initialised by phenol hydroxylases (PHs) (EC 1.14.13.7) (Hasan & Jabeen, 2015). These enzymes catalyse the conversion of phenols to catechol or hydroquinone through the hydroxylation of the ring structure (Kynadi & Suchithra, 2017). Three main categories of PHs have emerged (Nešvera, *et al.* 2015). Flavin dependent single component PHs are primarily involved in the hydroxylation of chlorophenols and long chain alkylphenols to chlorocatechols and hydroquinone derivatives, respectively (Ledger, *et al.* 2006; Porter, *et al.* 2012). Two component PHs have both monooxygenase and flavin reductase activity and rely on NADH and FAD being available (Saa, *et al.* 2010). They have been identified primarily in Gram-positive *Actinobacteria* and thermophilic bacilli (Nešvera, *et al.* 2015). The six-subunit multicomponent PHs are the most common form of PH and are found primarily in Gram-negative *Proteobacteria* (Nešvera, *et al.* 2015). While PHs are the most common enzymes in initialising phenol catabolism there are other enzymes such as nitrophenol reductases and benzoquinone reductases, whose action is generally limited to specifically substituted phenolic compounds (Belchik & Xun, 2008; Yin, *et al.* 2010).

Following the formation of the central intermediates there are several pathways available for the remaining catabolism of phenols. Ring cleavage of catechols is catalysed by a catechol dioxygenase and may involve either intradiol (*ortho*-) or extradiol (*meta*-) cleavage (Loh & Chua, 2002; Cao & Loh, 2008; Kwon & Yeom, 2009: Banerjee & Ghoshal, 2010). The *meta*- or *ortho*- ring cleavage of catechol (and its derivatives) represents the first step in the central pathway (for catechol) that culminates in the generation of TCA intermediates (Lee, *et al.* 2011; Hasan & Jabeen, 2015; Nešvera, *et al.* 2015; Kynadi & Suchithra, 2017). The *ortho*- cleavage pathway results in the generation of succinyl-CoA and acetyl-CoA, whereas *meta*-cleavage generates pyruvate and acetaldehyde which is then converted to acetyl-CoA (Omokoko, *et al.* 2008; Basha, *et al.* 2010; Krastanov, *et al.* 2013; Hasan & Jabeen, 2015; Kynadi & Suchithra, 2017). These two pathways can co-exist and function in the same bacterial strain (Omokoko, *et al.* 2008). Hydroquinone undergoes ring cleavage via a hydroquinone dioxygenase, the hydroquinone branch of degradation ends in the formation of 3-oxoadipate which then follows the same

path as that generated as intermediate of the *ortho*- cleavage branch (Basha, *et al.* 2010; Nešvera, *et al.* 2015). Through feeding acetyl-CoA into the TCA cycle phenolic compounds can be completely mineralised into CO_2 and H_2O during aerobic phenol oxidation (Banerjee & Ghoshal, 2010).

Anaerobic catabolism of phenols requires the four-step reaction converting phenols into benzoyl-CoA (Kynadi & Suchithra, 2017). The first step requires a phosphorylation, followed by a carboxylation reaction, then the conversion to a thioester which is finally dehydroxylated allowing for the generation of benzoyl-CoA (Basha, *et al.* 2010; Fuchs, *et al.* 2011; Nešvera, *et al.* 2015; Kynadi & Suchithra, 2017). The initiation of the central pathway in anaerobic degradation relies on the reduction of benzoyl-CoA to 1,5-dienoyl-CoA (Nešvera, *et al.* 2015). This reaction is followed by a sequence of metabolic reactions including beta oxidations, ring cleavage through hydrolysis and a decarboxylation reaction (Figure 1.1) (Basha, *et al.* 2010; Nešvera, *et al.* 2015; Kynadi & Suchithra, 2017). The resultant product can then be used for acetogenic methanogenesis and thus, the phenols are completely mineralised to CO_2 and CH_4 (Fang, *et al.* 2006; Basha, *et al.* 2010; Hoyos-Hernandez, *et al.* 2014; Kynadi & Suchithra, 2017). Not all anaerobic bacteria capable of initialising phenol catabolism are capable of producing methane. This often results in mixed microbial cultures being used for the complete mineralisation of phenols (Ju & Zhang, 2014).

There is some debate as to whether the application of aerobic or anaerobic bacteria in phenol bioremediation is preferable as there are benefits to both. Aerobic bacteria generally exhibit faster growth rates and allow for complete mineralisation of phenols (Al-Khalid & El-Naas, 2012). Anaerobic processes do not require aeration and produce less sludge when applied in bioreactors (Fang, *et al.* 2006). Furthermore, the issues of complete mineralisation can be counteracted by using mixed microbial cultures (Senthilvelan, *et al.* 2014). The choice depends on the end-goal, if the purpose is rapid, high efficiency phenol removal from wastewater then aerobic remediation may be preferable (Senthilvelan, *et al.* 2014; Pradeep, *et al.* 2015). However, if the goal is to combine phenol bioremediation with the generation of biogas (e.g. methane) and phenol free digestates (e.g. fertilisers), then anaerobic processes may be preferred (Zhang, *et al.* 2013; Hoyos-Hernandez, *et al.*

2014; Chantho, *et al.* 2016). However, aerobic degradation can be accomplished using a single strain for complete mineralisation and allows for more rapid biomass generation and a decrease in shock loading times (Al-Khalid & El-Naas, 2012; Krastanov, *et al.* 2013). Thus, aerobic operation lacks some of the metabolic and operational complexities that may appear in anaerobic phenolic biodegradation, making it the preferable option (Krastanov, *et al.* 2013; Ju & Zhang, 2014).

1.6.2 Mesophilic and thermophilic phenol biodegradation

The effect of temperature on the operational stability and success of a bacterial bioremediation system is substantive, as their activity is directly influenced by changes in temperature (Kurniawati, *et al.* 2008; Asadgol, *et al.* 2014). Bacteria which tolerate a wide range of temperatures, including psychrotolerant (<10°C), mesophilic (10°C - 30°C) and thermophilic (optimum 55°C - 65°C) bacteria, have been shown to be capable of phenol degradation (Loh & Chua, 2002; Feitkenhauer, *et al.* 2003a; Margesin *et al.* 2005; Cao & Loh, 2008; Basha, *et al.* 2010; Lee, *et al.* 2018). A number of psychrotolerant phenol degrading bacteria have been identified, including *Rhodococcus* and *Arthrobacter* spp., but this activity is more common in psychrophilic basidiomycetous yeasts (Margesin, *et al.* 2007; Lee, *et al.* 2018). The use of mesophilic bacteria for phenol degradation is the most widely investigated and as a result numerous strains with this capacity have been identified (Nešvera, *et al.* 2015). A number of thermophilic phenol-degrading taxa, although not as frequently investigated, have been identified, with representative taxa from the genera *Bacillus, Geobacillus and Thermus* (Duffner, *et al.* 2000; Feitkenhauer, *et al.* 2003a; Abeynayaka & Visvanathan, 2011; Hoyos-Hernandez, *et al.* 2014; Hussein, *et al.* 2015; Chantho, *et al.* 2016). The use of thermophiles rather than their mesophilic counterparts in phenol bioremediation strategies presents several advantages.

Industrial and agricultural wastewater effluents are often at high temperatures (Christen, *et al.* 2012; Chantho, *et al.* 2016). The use of thermophilic phenol degraders thus precludes the requirement for costly and time-consuming effluent cooling prior to degradation (Feitkenhauer, *et al.* 2003b; Fang, *et al.* 2006; Chantho, *et al.* 2016). High temperature operation furthermore has the advantage of increased

phenol solubility, decreased viscosity and greater diffusion rates of phenol, thereby increasing the bioavailability of phenolic compounds in a given system (Feitkenhauer, *et al.* 2001; Feitkenhauer, *et al.* 2003a). This combined with an increase in reaction and biomass generation rates allows for the more rapid removal of phenols, particularly under aerobic conditions (Chantho, *et al.* 2016). Increased, rapid biomass generation is particularly important during the initial phases of the reaction as it allows the cells to recover from loading shock more timeously and allows for rapid cell replacement in continuous systems that suffer from cell washout (Feitkenhauer, *et al.* 2003b). Moreover, aerobic thermophilic *Bacillaceae* have been shown to operate over a range of pH, temperatures and degrade a broad range of phenolic substrates making them ideal candidates for investigation and potential application in phenol biodegradation (Chantho, *et al.* 2016).

1.7 Conclusions

Phenols are common environmental pollutants, derived from both natural and anthropogenic sources. The toxic nature phenols has led to continued development of physicochemical and bioremediation strategies with greater efficacy in the removal of these contaminants. However, numerous problems remain including cost, efficacy and the incomplete breakdown or removal of phenols leading to the generation of toxic by-products. These problems can often be mitigated using whole cell biocatalysis approaches, which allow for the complete mineralisation of phenols. Numerous bacterial taxa have been identified with the capacity for phenol mineralisation from several environments. While mesophilic bacteria are the most frequently employed and investigated, aerobic thermophilic bacteria represent valuable candidates in phenol biodegradation. The use of aerobic thermophiles permits the rapid removal of phenols due to increases in the bioavailability of phenols, reaction rates and biomass generation.

CHAPTER TWO

SCREENING OF A LARGE COLLECTION OF THERMOPHILIC AEROBIC BACILLI FOR PHENOL TOLERANCE AND BIODEGRADATION

2.1 Introduction

Phenol and its derivatives, defined by the presence of a phenol (hydroxybenzene) ring, are produced in high quantities through both natural and anthropogenic means (Weelink, *et al.* 2010). The broad application of phenols in agriculture and industry makes them widely distributed pollutants that contaminate the water, soil and air (Gupta, *et al.* 2015; Bera, *et al.* 2017; Kynadi & Suchithra, 2017). While many phenols are innocuous, several phenols exhibit genotoxic, mutagenic and carcinogenic effects in both prokaryotic and eukaryotic organisms, even when present at low concentrations (Ghosal, *et al.* 2016; Bera, *et al.* 2017). The toxic nature, high density, low dilution rate and recalcitrant nature of phenols has led to their classification as priority pollutants (Krastanov, *et al.* 2013; Bera, *et al.* 2017).

Wastewater effluents have been shown to contain high concentrations of phenols, with concentrations as high as 5.00 g/l, 6.80 g/l and 10.00 g/l cited in various industrial effluents (Bera, *et al.* 2017; Kynadi & Suchithra, 2017; Ren, *et al.* 2017). In accordance with government regulations, companies are required to reduce these concentrations to 0.1 mg/l - 0.5 mg/l in wastewater (Al-Khalid & El-Naas, 2012; Christen, *et al.* 2012; Senthilvelan, *et al.* 2014). Accordingly, there is substantial research into the removal of phenols from wastewater. One of the primary methods for phenol removal is the use of whole-cell biological systems as they have proven more practical and effective than existing physicochemical and enzymatic techniques (Prasad & Rao, 2013; Ren, *et al.* 2017), primarily because they are comparatively cost effective, environmentally friendly, sustainable and allow for the complete mineralisation of phenols (Li, *et al.* 2019).

Despite being key producers of phenols, plants are not capable of using them as a growth substrate and similarly their usage in animals is limited (Fuchs, *et al.* 2011). By contrast, a broad range of bacteria and fungi are able to degrade phenols, which they couple to energy production (Fuchs, *et al.* 2011). Bacterial taxa are preferred for biodegradation due to their more rapid rates of bioaccumulation and biodegradation (Stoilova, *et al.* 2007; Ren, *et al.* 2017). Both aerobic and anaerobic bacterial taxa are capable of phenol degradation. These bacteria use distinct metabolic pathways for phenol degradation, yielding distinct end-products (Fuchs,

et al. 2011). Aerobic bacterial biodegradation is preferred as it is more rapid with higher turnover rates, rapid biomass regeneration and allows for the complete mineralisation of phenols (Gao, *et al.* 2011). Numerous mesophilic aerobic bacterial degraders have been identified and characterised, particularly those belonging to the genera *Pseudomonas, Bacillus, Rhodococcus* and *Acinetobacter* (Liu, *et al.* 2016; Bera, *et al.* 2017; Kurzbaum, *et al.* 2017; Li, *et al.* 2019).

Another key aspect for whole cell systems for phenol biodegradation relates to the capacity of microorganisms to resist the toxic effects associated with these compounds (Krastanov, *et al.* 2013). Phenol acts as an inhibitory substrate at relatively low concentrations, with 100 mg/l considered to be bacteriostatic. Thus, the identification of phenol biodegrading bacteria, capable of resisting the toxic effects of phenol at higher concentrations is pertinent (Bajaj, *et al.* 2008; Shourian, *et al.* 2009; Christen, *et al.* 2012; Baboshin & Golovleva, 2012; Krastanov, *et al.* 2013). Numerous bacterial taxa have been identified that can resist considerably higher concentrations of phenol, such as *Acinetobacter johnsonni* D1 (1410 mg/l), *A. calcoaceticus* (1700 mg/l) and *Oceanimonas* sp. (1500 mg/l) (Heilbuth, *et al.* 2015; Liu, *et al.* 2016; Tan, *et al.* 2017; Su, *et al.* 2019).

Thermophilic bacteria are of extensive interest in industrial, commercial and environmental biotechnological applications, including bioremediation, biofuel production and composting (Urbieta, *et al*. 2015). The applicability of thermophiles in biodegradation is a result of the accelerated reaction rates that can be attributed to the presence of thermostable enzymes. A further benefit of the high temperature biotechnological operation is the elimination of cooling requirements and the concomitant reduction of contamination associated with operation at moderate temperatures (Abeynayaka & Visvanathan, 2011). Furthermore, thermophilic bacteria are generally more metabolically diverse and resistant to harmful conditions (Prasad & Rao, 2013). Thus, thermophilic bacteria may be suitable candidates for whole cell phenol biodegradation systems. Here we have evaluated an extensive collection of thermophilic aerobic bacteria for their capacity to survive in the presence of high concentrations of phenol and the ability to utilise phenol as sole carbon source with the end goal of identifying a novel efficient thermophilic phenol degrader.

2.2 Methods and materials

2.2.1 Bacterial strains and culture media

A culture collection comprised of 130 aerobic thermophilic bacterial strains is maintained by the Prokaryotic Genomics and Extremophile Biotechnology laboratory at the School of Molecular & Cell Biology. These strains were collected by Professor Don Cowan over a period of ~20 years from a range of high temperature environments (e.g. volcanoes and hot springs) worldwide and have been identified as aerobic gram positives. These strains are maintained as glycerol stocks and stored at – 80 ºC. The strains were cultured and routinely maintained on Luria-Bertani (LB) agar plates [10 g/l casein hydrolysate, 5 g/l yeast extract, 5 g/l NaCl, 15 g/l bacteriological agar, 995 ml distilled water (dH_2O) and 1.25 ml 10% (w/v) NaOH; autoclaved for 20 min at 121°C] and incubated at 56 ºC.

For the biodegradation assays, the aerobic thermophilic strains were first acclimatised to the presence of phenol in LB broth [10 g/l casein hydrolysate, 5 g/l yeast extract, 5 g/l NaCl, 995 ml distilled water (dH_2O) and 1.25 ml 10% (w/v) NaOH; autoclaved for 20 min at 121°C] supplemented with 100 mg/l filter-sterilised phenol once the broth had cooled to 60°C following the autoclave cycle. This acclimatisation period also allowed for the identification of strains unable to resist phenol toxicity at 100 mg/l and their subsequent elimination from further screening.

Phenol utilisation experiments were carried out in a minimal salt medium (MSM) based on the recipe for medium 458 from the Deutsche Sammlung von Microorganismen und Zellkulturen (DSMZ) combined with trace element solution SL-4 from DSMZ medium 14. The base medium comprised of 0.5 g/l K_2HPO_4, 1 g/l NH_4Cl, 0.02 g/l $MgSO_4 \cdot 7H_2O$, 0.2 g/l yeast extract, 0.1 g/l casein hydrolysate, 959 ml/l dH_2O adjusted to pH 7.4 and then autoclaved for 20 min at 121°C. Once cooled to 60°C 1 ml of trace-element solution [0.20 g/l $FeSO_4 \cdot 7H_2O$, 0.10 g/l $ZnSO_4 \cdot 7H_2O$, 0.03 g/l $MnCl_2 \cdot 4H_2O$, 0.30 g/l H_3BO_3, 0.20 g/l $CoCl_2 \cdot 6H_2O$, 0.01 g/l $CuCl_2 \cdot 2H_2O$, 0.02 g/l $NiCl_2 \cdot 6H_2O$, 0.03 g/l $Na_2MoO_4 \cdot 2H_2O$ dissolved in 1,000 ml sterile dH_2O] was added.

In order to establish whether phenol was used as a sole carbon source the MSM and trace element solution was supplemented with a phenol stock solution or a glucose stock solution to bring the final solution to an equivalent of 400 mg/l or 750 mg/l, respectively. Both phenol and glucose were used to determine that any growth inhibition that resulted reflected an inability to use phenol, as opposed to an inability to survive in the MSM media. Following the carbon source usage experiment, the resistance to increasing concentrations was done using phenol concentrations equivalent of 400 mg/l, 800 mg/l, 1200 mg/l and 1600 mg/l of phenol in the MSM media. In order to achieve this a filter-sterilised phenol stock solution of 40 g/l was prepared. This was then diluted in 40 ml of sterile dH_2O and then combined with the 960 ml of MSM to give a final phenol concentration of 400 mg/l, 800 mg/l, 1200 mg/l and 1600 mg/l (10 ml phenol solution/ 30 ml water, 20 ml phenol solution/ 20 ml water, 30 ml phenol solution/ 10 ml water or 40 ml phenol solution, respectively), according to the required concentration for each experimental procedure. A filter-sterilised glucose stock solution of 1.875 g/100 ml was prepared, 40 ml of this glucose solution was added to the 960 ml of MSM and trace element solution to yield a final concentration of 750 mg/l glucose.

2.2.2 Acclimatisation and screening for potential phenol degrading strains

The entire culture collection was initially screened in 10 ml volumes in sterilised 25 ml MacConkey bottles. Single colonies of each strain were inoculated into both 10 ml LB broth and phenol (100 mg/l) supplemented LB broth and incubated at 56°C under shaking conditions (140rpm) for 24 hours. The LB broth was used to ensure that the strains were still viable and capable of growth whereas the phenol supplemented copies were used to produce phenol acclimatised starter cultures for further testing. Those strains that showed growth in the 100 mg/l phenol-supplemented LB broth were used to inoculate, in duplicate, 10 ml of phenol (400 mg/l) containing MSM and a single bottle of glucose-supplemented MSM (carbon source control). For the sake of consistency an estimated cell quantity was established for the initial acclimatisation cultures using spectrophotometry (OD_{600}) and each subsequent bottle was inoculated with an equivalent of 0.5 absorbance (OD_{600}) following centrifugation at 4,000 x *g* for 10 minutes, the removal of the LB broth containing supernatant and resuspension in the MSM media. A set of controls

was prepared in a similar fashion but not inoculated, to ensure no contamination occurred. The experimental cultures were incubated at 56°C on a rotary shaker (140 rpm) over a period of 72 hours. At 24-hour intervals 1 ml of each experimental culture was extracted and an absorbance (OD_{600}) measurement was taken to establish bacterial growth. Isolate preference for either glucose or phenol as a carbon source was inferred based on absorbance (OD_{600}) measurements.

2.2.3 Molecular identification of phenol tolerant aerobic thermophilic bacteria

Strains that showed consistent growth in 400 mg/l phenol supplemented MSM, were identified by selective amplification and sequencing of a 16S rDNA fragment. Genomic DNA was extracted by suspending freshly grown colonies in 200 µl of ddH_2O, homogenisation through vortexing for 30 seconds and incubation at 100°C for 10 minutes. Cellular debris was pelleted by centrifuging for one minute at 16,300 x *g*. The supernatant for each strain was then used as the template for 16S rDNA amplification by PCR. PCR reagents were prepared using the MyTaq™ DNA Polymerase Kit (Bioline, USA). Each reaction system contained 5µl 5X MyTaq reaction buffer, 0.5 µl MyTaq DNA polymerase, 2µl DNA template, 0.5 µl of 8F forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') and 0.5 µl of 1492R reverse primer (5'-GGTTACCTTGTTACGACTT-3') made up to 25µl with 16.5µl ddH_2O. PCR amplification was done using a MyCycler Thermal Cycler (BioRad, USA) using the program: initial denaturation at 95°C for five minutes; 30x (denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and elongation at 72°C for 90 seconds), followed by a final elongation at 72°C for 10 minutes.

The 16S rDNA PCR products were sequenced by Inqaba Biotec (South Africa) using the Sanger sequencing method. In order to gain species identifications, a homology search was done using the Ribosomal database project (RDP release 11) *sequence match* tool (Cole, *et al.* 2014). The cut-off values for 16S identification based on sequence similarity were set at 98.7% for species identity and 95% for genus identity (Beye, *et al.* 2018). A maximum-likelihood phylogeny was established using the 16S rDNA sequences of the selected strains as well as the type strains of species representing the closest match for each of the query sequences.

The 16S rRNA gene sequence of *Clostridium butyricum* DSM 10702 (AJ458420) was used as the outgroup. The type strains of the type species used were *Bacillus subtilis* DSM 10 (MK256302.1), *G. stearothermophilus* NCDO 1768 (X60640.1), *Anoxybacillus pushchinoiensis* K1 (AJ010478.1), *Staphylococcus aureus* DSM 20231 (MN652637.1) and *Aeribacillus pallidus* DSM 3670 (Z26930.1). The type strain for the closest RDP matches were also included in the phylogeny as such *A. gonensis* G2 (AY122325.1), *A. mongoliensis* T4 (EF654664.1), *B. licheniformis* ATCC 14580 (NR_074923.1), *B. smithii* DSM 4216 (X60643.1), *G. thermodenitrificans* DSM 465 (Z26928.1), *G. thermoleovorans* ATCC 43513 (M77488.1), *G. vulcani* 3S-1 (AJ293805.1), *S. equorum* DSM 20674 (Z26895.1) were used. Alignment of the 16S rDNA sequences was achieved through the Multiple Alignment using Fast Fourier Transform (MAFFT version 7) server with default settings (Katoh & Standley, 2013). The aligned sequences were used to construct a maximum-likelihood phylogeny using the ATGC: PhyML web server (version 3.0) using the best evolutionary model determined using the Smart Model Selection tool and bootstrap support (n=1,000 replicates) (Guindon, *et al.* 2010).

2.2.4 Quantitative analysis of phenol tolerance of the aerobic thermophilic bacteria

Those strains that showed consistent growth with phenol (400 mg/l) as the sole carbon source were selected for further investigation. A liquid starter culture was grown in phenol (100 mg/l)-supplemented LB broth and incubated at 56°C under shaking conditions (140rpm) for 24 hours. An absorbance (OD_{600}) equivalent of 0.5 was used to inoculate, in triplicate, 15 ml of phenol supplemented MSM. The phenol concentration was increased in a step wise fashion, from 400 mg/l to 1600 mg/l in 400 mg/l intervals. Strains were progressively removed from the investigation depending on their inability to grow at increased phenol concentrations. All strains were cultivated at 56°C for 72 hours as described above. At 24-hour intervals, 1 ml of each experimental culture was extracted and an absorbance (OD_{600}) measurement was taken (in triplicate) to determine the extent of growth. A set of controls was prepared in an identical fashion for each stepwise increase of phenol, for every experimental batch conducted, with the exclusion of the inoculum.

2.2.5 Quantitative analysis of phenol degradation

The strains that showed the best growth in the presence of phenol were cultivated in MSM supplemented with 400 mg/l of phenol for 72 hours and evaluated using the direct 4-aminoantipyrine (4-AAP) colorimetric assay (APHA, 2017) to determine the extent of phenol degradation. The 4-AAP assay works on the principal that under alkaline conditions 4-AAP will react with phenol in the presence of potassium ferricyanide ($K_3[Fe(CN)_6]$) to form a coloured primary amine (Tandjaoui, *et al.* 2019). A starter culture of each bacterial strain was produced as described previously and the equivalent of 0.5 absorbance (OD_{600}) was used to inoculate 15 ml of MSM with 400 mg/l phenol, five replicates were produced for each strain. The strains were incubated at 56°C for 72 hours on a rotary shaker (140 pm). At 24-hour intervals 1 ml of each experimental culture was extracted for absorbance (OD_{600}) measurements for growth estimation and a volume of 1 ml was used to perform the 4-AAP colorimetric assay.

The direct 4-AAP colorimetric assay was conducted according to the Direct photometric method (section 5530 D) of the Standard Methods for the Examination of Water and Wastewater with the use of an ammonium chloride (NH_4Cl) buffer solution as opposed to a phosphate buffer solution (Fayyad, *et al.* 1989; Ni, *et al.* 2010; APHA, 2017). In brief, a stock solution of 4-AAP (2% w/v; 0.2g/ 10 ml dH_2O) and $K_3[Fe(CN)_6]$ (8% w/v; 0.8g/10 ml dH_2O) was prepared daily. The ammonium chloride – ammonia (NH_4Cl- NH_3) buffer solution was prepared by dissolving 5.3g of NH_4Cl in 900 ml of distilled water and adjusted to a pH of 8.5 by the dropwise addition of NH_4OH (ammonium hydroxide) and then diluted to 1,000 ml. 1 ml of each sample was centrifuged at 7,000 x *g* for two minutes to separate the bacterial cells from the phenol containing MSM. The supernatant was removed and diluted 1:1,000, to the detectable range of 0-1,000,000 µg/l phenol. Following this, 10 ml of each diluted sample was combined with 200 µl NH_4Cl-NH_3 buffer and mixed by vortexing for 30 seconds. Following this, 200 µl of 4-AAP solution and 200 µl of $K_3[Fe(CN)_6]$ solution were added in turn and the colour was allowed to develop for fifteen minutes before spectrophotometric readings were taken at 500 nm. A standard curve was created in the same manner with quadruple concentrations of 0-, 50-, 100-, 200-, 500-, 800- and 1,000 µg/l phenol.

Phenol concentration was determined in accordance with the established standard curve.

2.3 Results and discussion

2.3.1 Acclimatisation and screening for potential phenol degrading strains

A total of 130 aerobic thermophilic bacterial strains were reconstituted from glycerol stocks. Of these only 116 grew in LB broth. These strains were subsequently subjected to phenol acclimatisation. Phenol acclimatisation is often a precursor to the development of any stable phenol degradation setup. The presence of lower concentrations of phenol during the acclimatisation period is adequate to activate the genes associated with its metabolism (Al-Khalid & El-Naas, 2012). The activation of the genes associated with phenol degradation decreases the lag phase and permits accelerated growth in the presence of phenol (Díaz, *et al.* 2013). The resulting increase in phenol metabolism prevents the toxic accumulation of phenol (Ladino-Orjuela, *et al.* 2016). A total of 100 strains (86.2% of strains that grew in LB broth) grew effectively during the acclimatisation step with 100 mg/l phenol. When exposed to minimal media with 400 mg/l phenol as sole carbon source, only twenty-two of the acclimatised strains showed effective and consistent growth. These strains showed two growth patterns, with 77% (17/22) and 23% (5/22) of the strains reaching their maximum growth (absorbance OD_{600}) 24- or 48-hours post-inoculation (hpi), respectively. Those strains whose growth peaked at 24 hpi all showed a notable decrease in absorbance (OD_{600}) from 24 to 48 hours. Between 48 and 72 hpi bacterial growth (absorbance OD_{600}) either plateaued or continued to decrease. Of the five strains whose growth reached a maximum at 48 hpi, three strains showed a steady increase in absorbance (OD_{600}) between 0 and 48 hpi, two strains saw over 100% increase in absorbance (OD_{600}) between 24 and 48 hpi. The strains with the best consistent growth in the presence of 400 mg/l phenol were (in order of average magnitude) Row 2A12.1.1, Row 2A12.3A and G18 A8.1 (Figure 2.1).

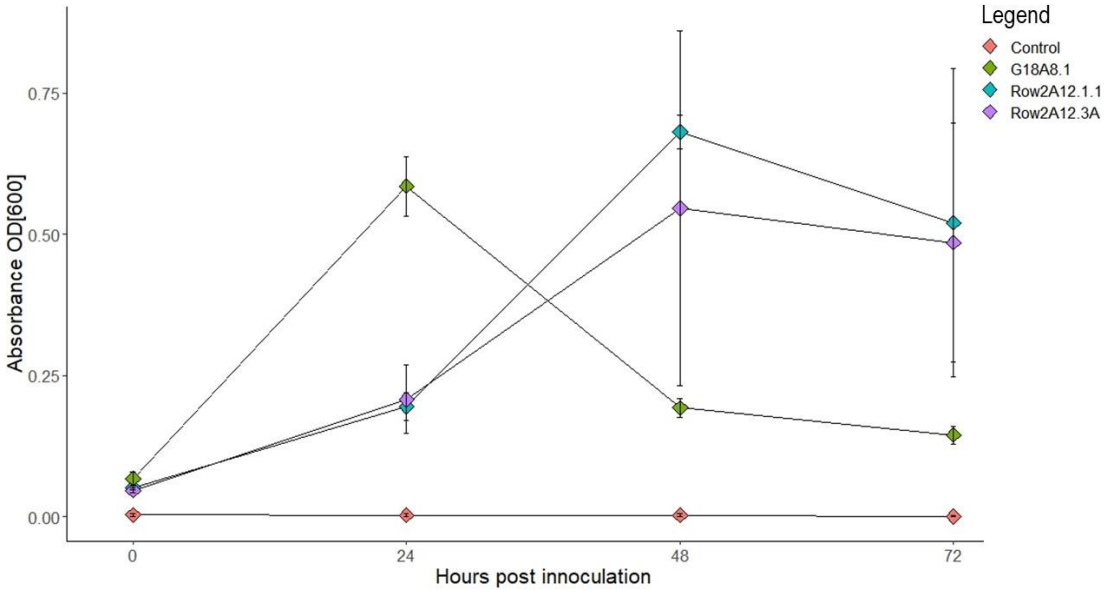


Figure 2.1: Bacterial growth curves (OD_{600}) of the three strains demonstrating the greatest consistent growth over 72 hours in MSM supplemented with 400 mg/l phenol. Error bars represent the growth range seen within the triplicates.

The differences in growth may be influenced by numerous factors as bacterial biodegradation of phenol is sensitive to several environmental influences, including but not limited to acclimatisation, pH, temperature, sources of carbon, nitrogen and other minerals, length of incubation and the degree of aeration (Basha, *et al.* 2010; Chakraborty, *et al.* 2010; Pradeep, *et al.* 2015;). The medium used was at near neutral pH (± 7.0), but optimal pH for growth varies widely for different strains. Additionally, the pH of media has been shown to change over the course of degradation (Shourian, *et al.* 2009; Chakraborty, *et al.* 2010).

Temperature is known to play an important role in the rate of bacterial growth and metabolism (El-Naas, *et al.* 2009). In this study, all experiments were undertaken aerobically at 56 ˚C to be inclusive of moderate thermophiles. Ultimately, however, the various strains may present better growth at alternate temperatures. *Geobacillus (Bacillus) stearothermophilus* for example is capable of growth between 50-60˚C, but a study showed its optimal temperature for the degradation of phenols to be 50˚C (Omokoko, *et al.* 2008; Al-Khalid & El-Naas, 2012).

The growth of each of the twenty-two strains in MSM supplemented with phenol 400 mg/l phenol or 750 mg/l glucose was compared. Overall, 45% (10/22) of these strains showed greater growth in the glucose supplemented MSM, 32% (7/22) showed more substantial growth in the phenol supplemented MSM, while 23% showed roughly equivalent growth rates in both media (Table 2.1). As there are not enough replicates to allow for statistical analysis it is not possible to accurately say whether the strains show preference for either carbon source. To date, only a few studies have reported bacteria with a distinct preference for phenol as a carbon source over glucose. These include *Pseudomonas putida* CSV86 and *Geobacillus thermoleovorans* A2 (Feitkenhauer, *et al.* 2001; Basu, *et al.* 2006; Basha, *et al.* 2010).

Table 2.1: Growth of the thermophilic aerobic bacteria in MSM supplemented with phenol (400 mg/l), with duplicate average, or glucose (750 mg/l) for 72 hours.

Sample	Carbon source	Absorbance (OD_{600}) over 72 hours			
		0 hpi	24 hpi	48 hpi	72 hpi
Control	Glucose	0.001	0	0.001	0.002
	Phenol	0	0	0	0.002
** Fur 6A6.1	Glucose	0.041	0.099	0.113	0.131
	Phenol	0.0345	0.5065	0.3915	0.423
Fur 6A8	Glucose	0.055	0.229	0.224	0.201
	Phenol	0.041	0.2315	0.167	0.1945
G18 A8.1	Glucose	0.058	0.093	0.181	0.278
	Phenol	0.0385	0.1775	0.085	0.0635
* Gey 1A1	Glucose	0.088	0.324	0.439	0.438
	Phenol	0.0545	0.1495	0.1005	0.0575
* Ham 1A2.2	Glucose	0.034	0.251	0.248	0.208
	Phenol	0.045	0.0855	0.079	0.065
* Ham 3A8	Glucose	0.05	0.153	0.176	0.169
	Phenol	0.0545	0.0905	0.121	0.091
** Ork 2A2.1	Glucose	0.042	0.025	0.022	0.023
	Phenol	0.052	0.1215	0.088	0.0835
** Ork 2A6	Glucose	0.053	0.002	0.013	0.014
	Phenol	0.0365	0.1585	0.095	0.0815
** Ork 3A4.1	Glucose	0.063	0.003	0.007	0.115
	Phenol	0.059	0.209	0.108	0.0725
** Ork 3A7	Glucose	0.039	0.03	0.016	0.032
	Phenol	0.0345	0.25	0.1295	0.1065
** Ork 5A2	Glucose	0.031	0.004	0.002	0.022

	Phenol	0.0585	0.197	0.181	0.1305
* Ork 8A7.1	Glucose	0.066	0.206	0.18	0.183
	Phenol	0.0755	0.149	0.041	0.0405
Prf 1A4	Glucose	0.028	0.064	0.08	0.095
	Phenol	0.0345	0.1095	0.056	0.0395
* Prf 1A6.1	Glucose	0.061	0.147	0.132	0.157
	Phenol	0.056	0.0815	0.0645	0.036
* Prf 1A7	Glucose	0.062	0.146	0.163	0.135
	Phenol	0.0545	0.0805	0.099	0.008
* Rec 2A2.2	Glucose	0.046	0.186	0.222	0.185
	Phenol	0.0515	0.1625	0.0875	0.054
* Row 2A11.1	Glucose	0.108	0.541	0.239	0.077
	Phenol	0.082	0.1145	0.0575	0.0515
* Row 2A11.4B	Glucose	0.111	0.361	0.319	0.261
	Phenol	0.128	0.1595	0.0855	0.0905
** Row 2A12.1.1	Glucose	0.059	0.245	0.449	0.492
	Phenol	0.049	0.1945	0.751	0.4095
Row 2A12.1.2	Glucose	0.093	0.349	0.35	0.376
	Phenol	0.081	0.1785	0.357	0.288
Row 2A12.3A	Glucose	0.091	0.44	0.585	0.589
	Phenol	0.073	0.417	0.689	0.427
* Wam 9A3	Glucose	0.09	0.211	0.179	0.174
	Phenol	0.093	0.15	0.06	0.069

Footnote:
* Presumptive preference for glucose
** Presumptive preference for phenol

2.3.2 Molecular identification of phenol tolerant aerobic thermophilic bacteria

The molecular identification of the twenty-two taxa capable of surviving with phenol as the sole carbon source revealed that all identified taxa are Gram-positives from the order *Caryophanales* (previously *Bacillales*), with twenty-one of the identified strains belonging to the family *Bacillaceae*. Based on the maximum likelyhood phylogeny, 45.5% (10/22) and 32% (7/22) of the strains belong to the genus *Geobacillus* and *Bacillus*, respectively (Figure 2). The largest species clusters identified were that of *B. licheniformis* (strains Fur6A8, Ham1A2.2, Prf1A6.1, Prf1A7, Row2A11.4B) and *G. thermoleovorans* (strains G18 A8.1, Gey1A1, Ork2A6, Ork3A4.1, Ork5A2) with five of the strains of interest. The next largest cluster was that of *G. thermodenitrificans* with three associated strains (Ham3A8, Row2A11.1, Wam9A3). Two of the nine remaining strains of interest clustered

with *A. mongoliensis* (Ork3A7, Ork8A7.1), *B. smithii* (Row2A12.1.1, Row2A12.3A) and *G. stearothermophilus* (Prf1A4, Rec2A2.2), respectively. One of each of the three remaining strains clustered with *A. pallidus* (Fur6A6.1), *A, gonensis* (Ork2A2.1) and *S. equorum* (Row2A12.1.2).

Members of the two genera most frequently observed, have previously been shown to tolerate and degrade phenol at high concentrations. *B. amyloliquefaciens* WJDB-1 immobilised on alginate–chitosan microcapsules were capable of complete degradation of 200 mg/l phenol over a period of 36 hours (Lu, *et al.* 2012). *Bacillus cereus* strains AKG1 and AKG2 have been observed to degrade phenol up to a concentration of 2,000 mg/l when immobilised, with degradation reaching 50% within 26-36 days. Similarly, suspended cells of *G. thermoleovorans* A2 were capable of the degradation of 7,000 mg/l phenol daily in a stir tank reactor with a phenol input of 660 mg/l (Feitkenhauer, *et al.* 2001). *G. stearothermophilus* DSM 6285 was shown to decrease phenol concentrations from approximately 450 mg/l to 80 mg/l phenol within 24 hours (Omokoko, *et al.* 2008).

In contrast to *Bacillus* and *Geobacillus*, information pertaining to phenol tolerance and degradation by the other genera identified, namely *Aeribacillus*, *Anoxybacillus*, *Staphylococcaceae*, is considerably more limited. It is evident that taxa belonging to the genera *Bacillus* and *Geobacillus* have a proclivity toward the degradation of phenol when compared to the other identified genera, as they make up a larger portion of the identified strains from the culture collection employed as well as encompassing more observations of their capacity for phenol degradation in literature.

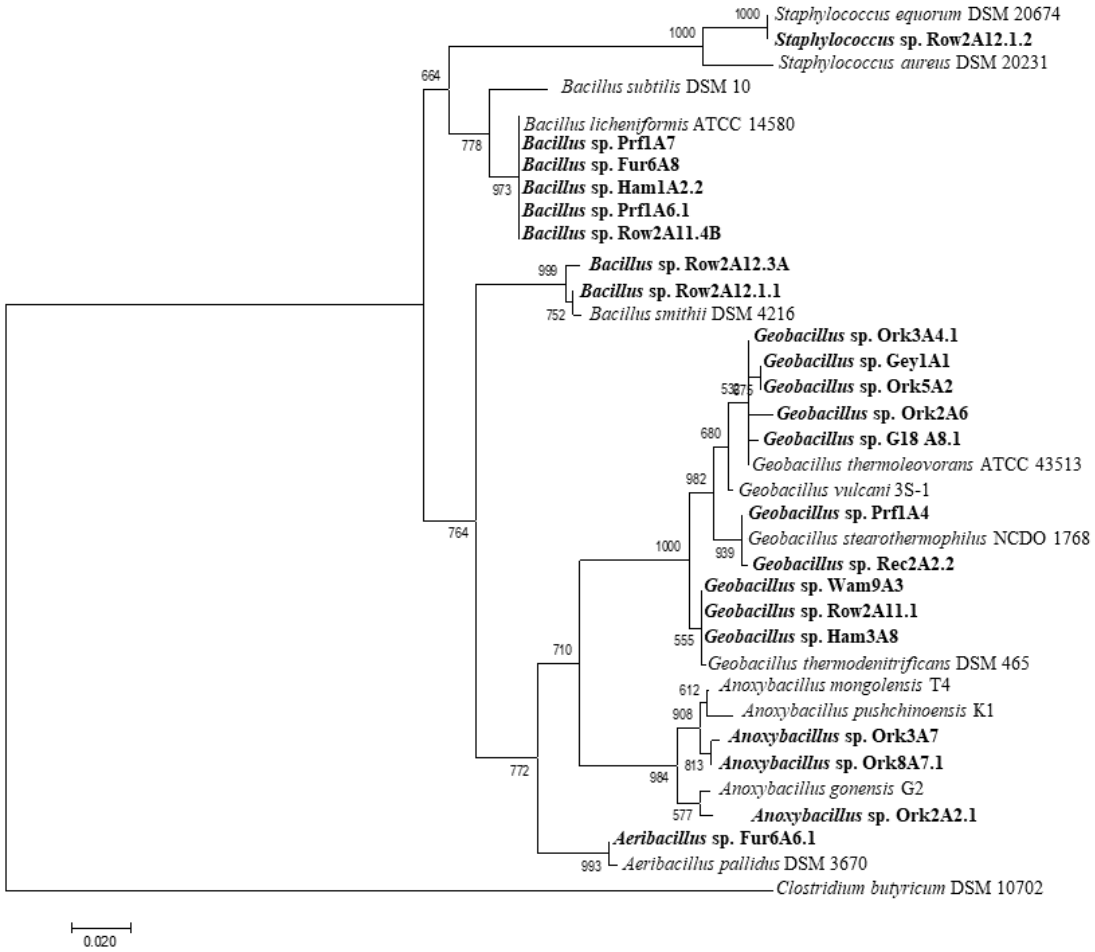


Figure 2.2: 16S rDNA sequence-based phylogeny using the twenty-two strains capable of consistently surviving with 400 mg/l phenol as a sole carbon source. The 0.02 scale bar refers to 2 nucleotide polymorphisms per 100 nucleotides, the common nucleotide length was 515 nucleotides.

2.3.3 Quantitative analysis of phenol tolerance of the aerobic thermophilic bacteria

The twenty-two strains that showed tolerance to phenol at a concentration of 400 mg/l were further evaluated for their ability to tolerate increased phenol concentrations through incremental exposure to 800-, 1200- and 1600 mg/l of phenol as sole carbon source. *Aeribacillus* sp. Fur6A6.1 was removed from screening due to a loss of viability. Thirteen strains showed no growth in the presence of 800 mg/l of phenol 72 hpi, while nine strains demonstrated effective growth at this concentration (Table 2.2; Figure 2.3).

Subsequent exposure to 1200 mg/l of phenol in MSM resulted in the elimination of a further four strains due to the absence of notable growth. The best growth, of the five relevant strains, was observed for *Bacillus* sp. Row2A12.3A which reached a maximal absorbance (OD_{600} = 0.69) at 48 hours hpi (Figure 2.4). The next best growth was observed for *Geobacillus* sp. Ork 5A2 and *Geobacillus* sp. Prf 1A4 after 24 hours, albeit considerably less growth was seen than that observed for *Bacillus* sp. Row2A12.3A (Table 2.3; Figure 2.4). Overall, members of the genus *Geobacillus* demonstrated a greater tolerance to phenol toxicity than the other genera identified. The five thermophilic taxa that grew in the presence of 1,200 mg/l of phenol were subsequently evaluated for growth at 1600 mg/l phenol. However, this concentration proved toxic and inhibited growth completely.

Table 2.2: Growth of the thermophilic aerobic bacteria in MSM supplemented with 800 mg/l phenol for 72 hours.

	Absorbance (OD_{600}) over 72 hours			
	0 hpi	24 hpi	48 hpi	72 hpi
Sample	Avg. Abs (±σ)	Avg. Abs(±σ)	Avg. Abs(±σ)	Avg. Abs(±σ)
Bacillus sp. Fur 6A8	0.061 ±0.007	0.037±0.006	0.024±0.013	0.011±0.003
Geobacillus sp. G18 A8.1	0.067±0.004	0.334±0.122	0.221±0.020	0.195±0.006
Geobacillus sp. Gey 1A1	0.101±0.011	0.238±0.089	0.136±0.032	0.127±0.022
Bacillus sp. Ham 1A2.2	0.078±0.008	0.098±0.004	0.075±0.015	0.054±0.014
Geobacillus sp. Ham 3A8	0.067±0.005	0.103±0.026	0.067±0.029	0.047±0.018
Anoxybacillus sp. Ork 2A2.1	0.074±0.001	0.076±0.029	0.085±0.022	0.078±0.023
Geobacillus sp. Ork 2A6	0.036±0.002	0.295±0.102	0.200±0.043	0.155±0.050
Geobacillus sp. Ork 3A4.1	0.070±0.008	0.232±0.035	0.154±0.036	0.051±0.003
Anoxybacillus sp. Ork 3A7	0.059±0.006	0.047±0.009	0.061±0.002	0.128±0.021
Geobacillus sp. Ork 5A2	0.043±0.004	0.225±0.007	0.190±0.005	0.147±0.010
Anoxybacillus sp. Ork 8A7.1	0.050± 0.010	0.023±0.005	0.039±0.005	0.005±0.003
Geobacillus sp. Prf 1A4	0.048±0.006	0.162±0.039	0.141±0.007	0.049±0.012

Bacillus sp. Prf 1A6.1	0.076± 0.008	0.137±0.018	0.091±0.009	0.065±0.012
Bacillus sp. Prf 1A7	0.093± 0.003	0.134±0.021	0.102±0.005	0.072±0.008
Geobacillus sp. Rec 2A2.2	0.066± 0.021	0.219±0.037	0.125±0.021	0.080±0.003
Geobacillus sp. Row 2A11.1	0.071±0.005	0.130±0.028	0.096±0.018	0.067±0.022
Bacillus sp. Row 2A11.4B	0.073±0.007	0.136±0.015	0.085±0.007	0.066±0.014
Bacillus sp. Row 2A12.1.1	0.030±0.003	0.011±0.002	0.024±0.013	0.013±0.009
Staphylococcus sp. Row 2A12.1.2	0.043±0.008	0.043±0.059	0.024±0.007	0.016±0.009
Bacillus sp. Row 2A12.3A	0.076±0.006	0.153±0.111	0.484±0.060	0.347±0.032
Geobacillus sp. Wam 9A3	0.079±0.009	0.115±0.017	0.075±0.008	0.053±0.010
Controls	0.001±0.001	0.002±0.002	0.001±0.002	0.001±0.001

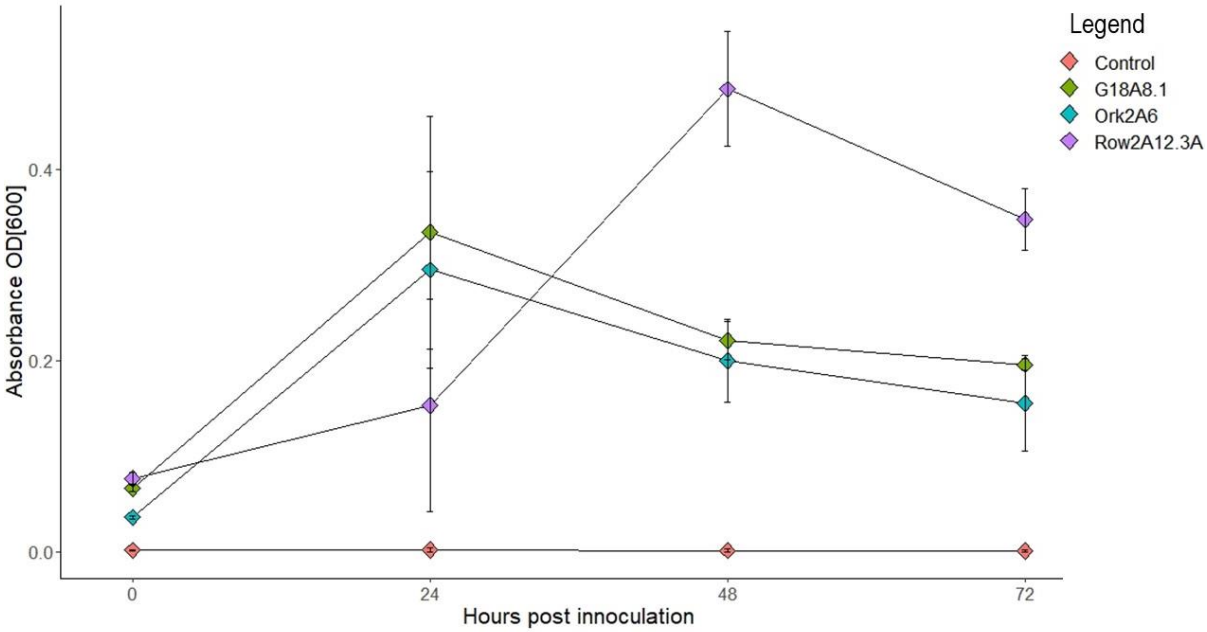


Figure 2.3: Bacterial growth curves (Absorbance OD_{600}) of the three strains demonstrating the greatest growth over 72 hpi in MSM supplemented with 800 mg/l phenol. Error bars represent the growth range seen within the triplicates.

Table 2.3: Growth of the thermophilic aerobic bacteria in MSM supplemented with 1200 mg/l phenol for 72 hours.

	Absorbance (OD600) over 72 hours			
	0 hpi	24 hpi	48 hpi	72 hpi
Sample	Avg. Abs	Avg. Abs	Avg. Abs	Avg. Abs
Geobacillus sp. G18 A8.1	0.033±0.003	0.112±0.168	0.079±0.101	0.064±0.076
Geobacillus sp. Gey 1A1	0.058±0.007	0.034±0.007	0.103±0.030	0.045±0.014
Geobacillus sp. Ork 2A6	0.044±0.004	0.088±0.115	0.054±0.060	0.059±0.069
Geobacillus sp. Ork 3A4.1	0.073±0.014	0.023±0.003	0.040±0.020	0.016±0.006
Geobacillus sp. Ork 5A2	0.035±0.009	0.293±0.017	0.204±0.015	0.186±0.025
Geobacillus sp. Prf 1A4	0.043±0.004	0.189±0.061	0.143±0.025	0.046±0.010
Geobacillus sp. Rec 2A2.2	0.041±0.005	0.135±0.013	0.111±0.020	0.081±0.009
Bacillus sp. Row 2A12.3A	0.031±0.003	0.052±0.026	0.690±0.164	0.418±0.250
Geobacillus sp. Wam 9A3	0.066±0.003	0.081±0.039	0.071±0.016	0.061±0.004
Controls	0.000±0.001	0.001±0.001	0.000±0.000	0.002±0.003

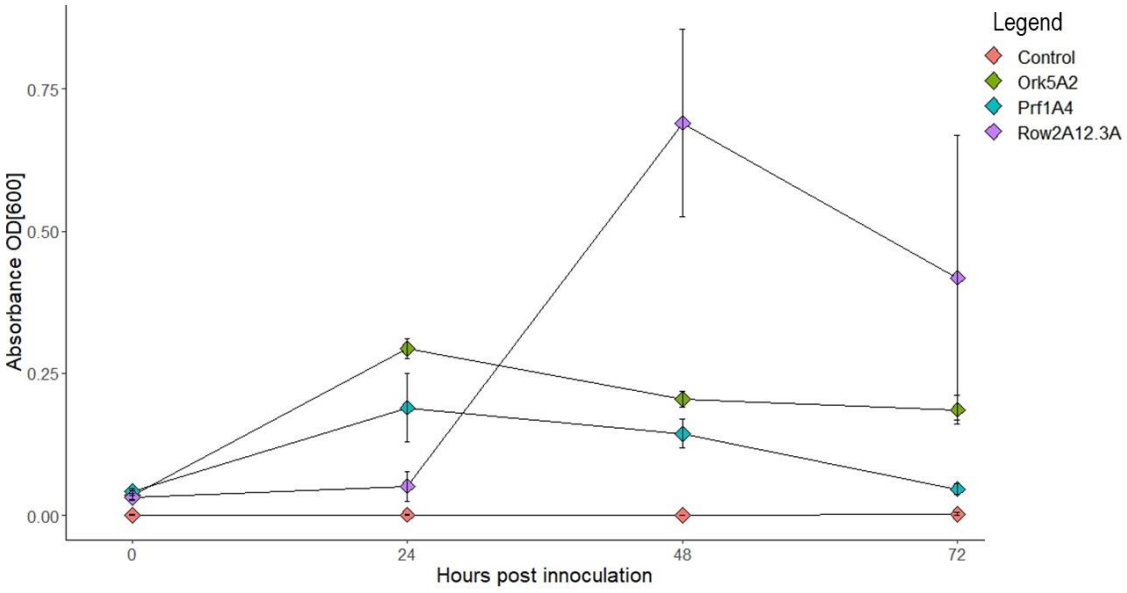


Figure 2.4: Bacterial growth curves (Absorbance OD_{600}) of the three strains demonstrating the greatest growth over 72 hpi in MSM supplemented with 1200 mg/l phenol. Error bars represent the growth range seen within the triplicates.

Bacillus sp. Row2A12.3A demonstrated the highest tolerance to phenol, growing relatively well even at phenol concentrations of 1,200 mg/l. To date, tolerance to higher concentrations than this have been reported in *B. cereus* AKG1 (1,600 mg/l), *B. cereus* AKG2 (1,430 mg/l) and *Bacillus* sp. SAS19 (1,800 mg/l) (Banerjee & Ghoshal, 2010; Banerjee & Ghoshal, 2011; Banerjee & Ghoshal, 2017; Ke, *et al.* 2018). It is important to note that these results were obtained from strains whose conditions for biodegradation had already been optimised (*Bacillus* sp. SAS19 was immobilised on porous carbonaceous gel) or were isolated specifically from environments polluted with high concentrations of phenol (Banerjee & Ghoshal, 2010; Ke, *et al.* 2018). It is likely that with longer and more rigorous acclimatisation and optimisation *Bacillus* sp. Row2A12.3A would display tolerance to even higher concentrations of phenol.

2.3.4 Quantitative analysis of phenol degradation

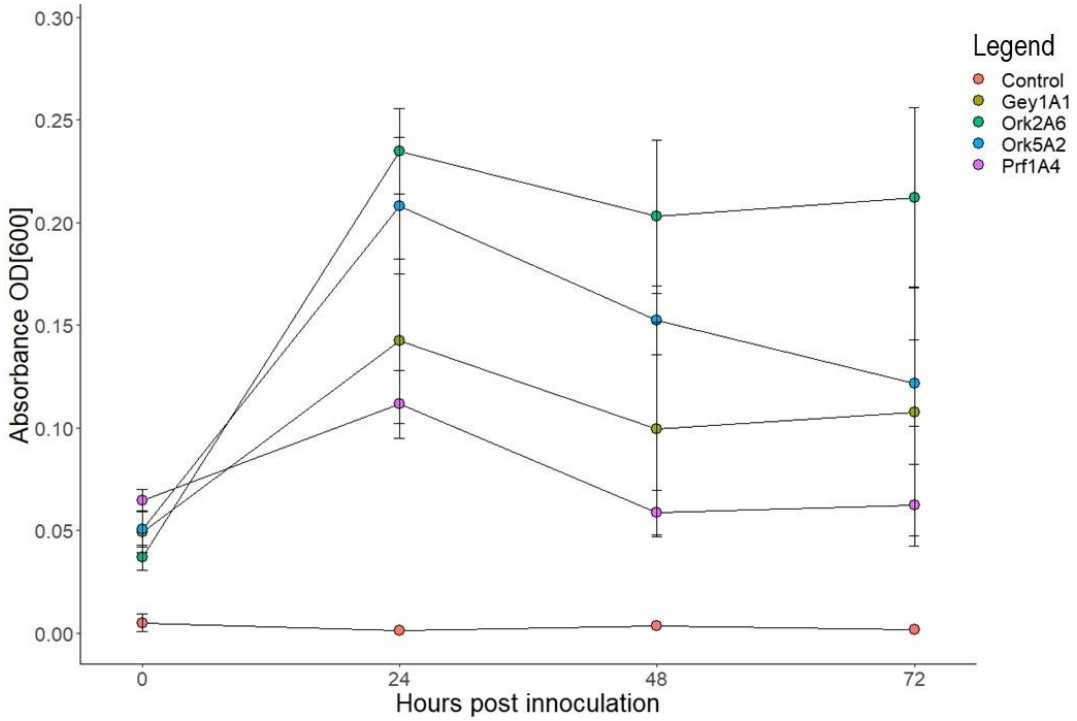
The five aerobic thermophilic bacteria capable of growing at up to 1,200 mg/l were further evaluated for their ability to biodegrade phenol at a concentration of 400 mg/l using the 4-AAP colorimetric assay. The four *Geobacillus* strains, Gey 1A1, Ork 2A6, Ork 5A2 and Prf 1A4 grew to a maximum absorbance 24 hpi, followed by a decline at 48 and 72 hpi (Figure 2.5a). Analysis of the phenol content at the different time-points showed little effective change in the phenol concentration for all four strains (Figure 2.5b). The growth of these four taxa in the presence of 400 mg/l of phenol suggests they are able to tolerate phenol at this concentration but are unable to utilise it as a carbon source. It is likely that C-sources (e.g. from the yeast extract or casein hydrolysate) in the LB broth carried over from the pre-culture medium was utilised in the first 24 hours. After exhaustion of these limited C-sources, these taxa would have gone into decline. Notably, there appeared to be an increase in the phenol concentration compared to the initial concentration (400 mg/l) in the MSM for these strains. A number of bacterial taxa have been shown to be capable of producing phenol via the reversible cleavage of *L*-tyrosine to ammonia, pyruvate and phenol which is catalysed by tyrosine phenol lyase enzymes (TPL, EC 4.1.99.2) (Gosset, 2009; Milic, *et al.* 2011; Zheng, *et al.* 2018). Most studies have focused on *tpl* genes coding for TPL enzymes in Gram-negative taxa such as *Citrobacter freundii, Fusobacterium nucleatum* and *Pantoea agglomerans*

(Gosset, 2009; Milic, *et al.* 2011; Fairhead & Thöny-Meyer, 2012; Zheng, *et al.* 2018). However, there is evidence of similar phenolic producing enzymes in a strain of *G. stearothermophilus*, as determined by the presence of seventeen metabolites associated with the degradation of tyrosine including several phenolic derivatives such as homogentisic acid and cinnamic acid (Afzal, *et al.* 2013).

The fifth strain evaluated, *Bacillus* sp. Row2A12.3A, showed a marked capacity for phenol degradation (Figure 2.6). The phenol concentration decreased from 400 mg/l to 105 mg/l, showing a 74% reduction in phenol concentration by 48 hpi (Figure 2.6b). Based on the exponential phase of phenol degradation, between 0 and 24 hpi, the maximum degradation rate constant for an initial 400 mg/l of phenol is 8.96 mg/l/h. Compared to previously published data this is not particularly high, for example a degradation rate constant of 31.5 mg/l/h was achieved in *Corynebacterium* sp. DJ1 at a phenol concentration of 500 mg/l (Ho, *et al.* 2009). Once again, however, these bacterial cells had previously been optimised for phenol degradation and were used in the form of aerobic granules, resulting in increased stability and toxicity resistance (Ho, *et al.* 2009).

Members of the genus *Bacillus* have been relatively well explored in terms of their capacity for phenol biodegradation. In particular, strains of the mesophilic species *B. subtilis, B. cereus, B. licheniformis* and *B. pumilus* have demonstrated some level of phenol degradation (Liu, *et al.* 2016; Li, *et al.* 2019;). To our knowledge, no strain of *B. smithii*, with which *Bacillus* sp. Row2A12.3A clusters, has previously been demonstrated to degrade phenol, despite evidence that suggests *B. smithii* has the capacity for ring cleavage, as it has been demonstrated to degrade phorbol esters, lignin and cellulose and should thus likewise be able to degrade phenols (Tuo, *et al.* 2012; Chang, *et al.* 2014; Jurado, *et al.* 2014).

A.



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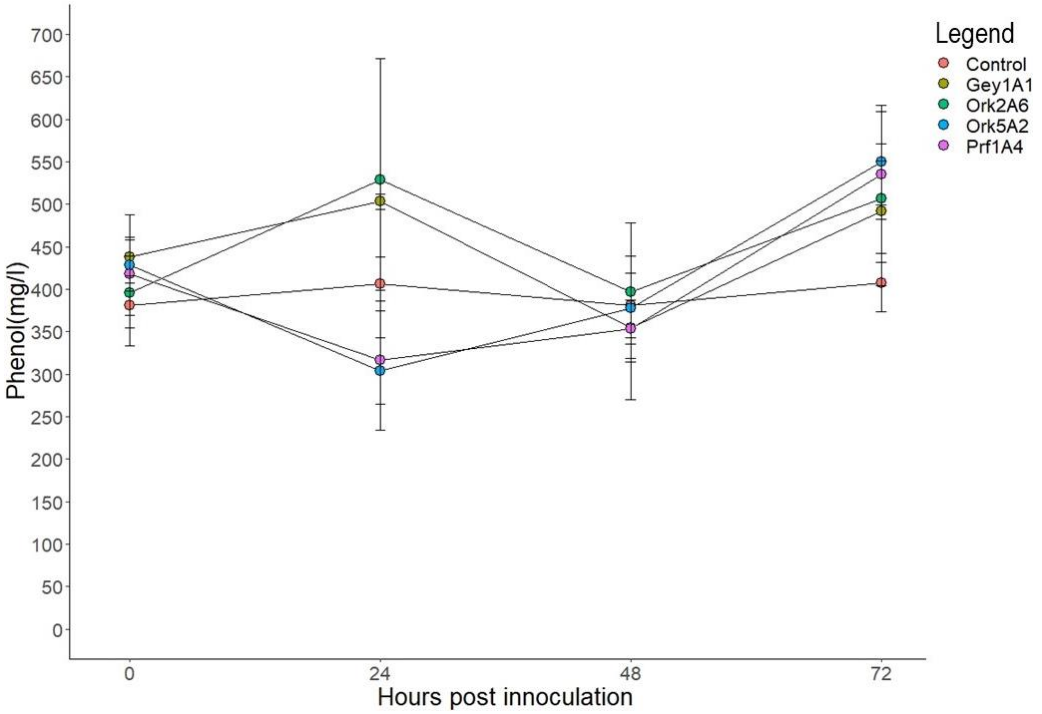
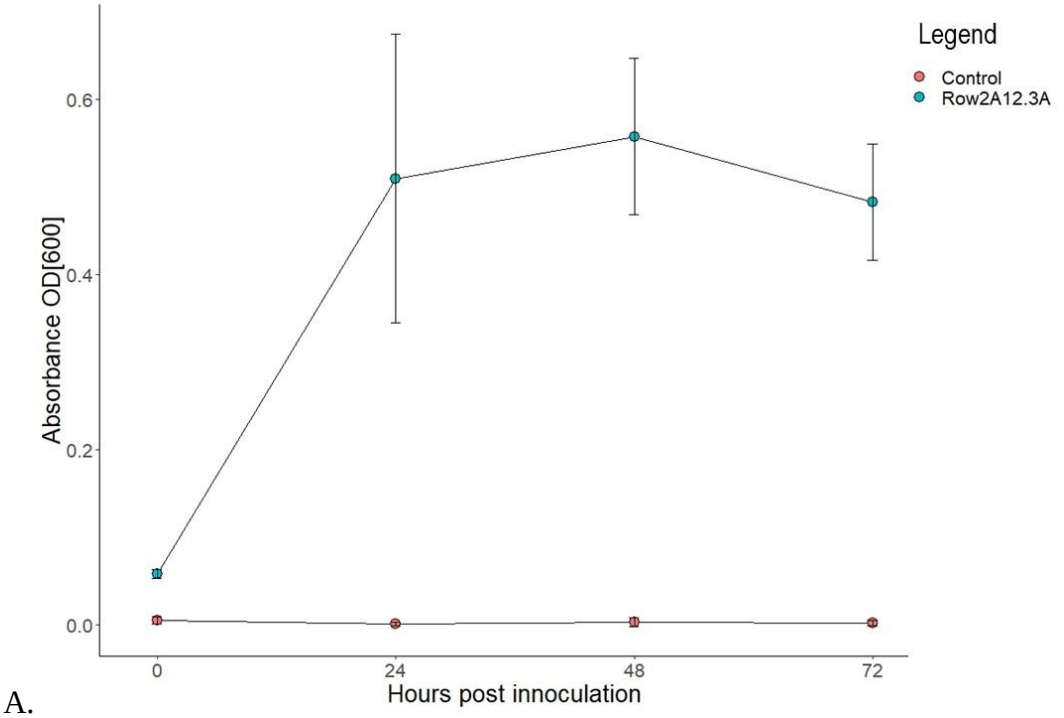
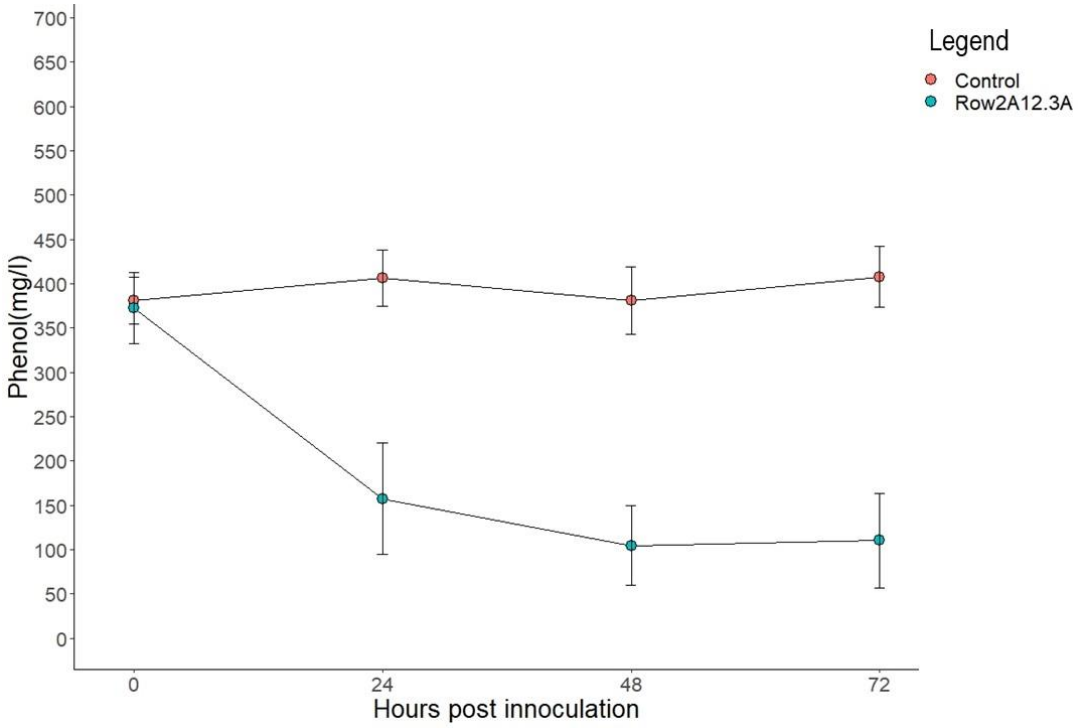


Figure 2.5: A) Bacterial growth curves (OD_{600}) of Gey1A1, Ork2A6, Ork5A2 and Prf1A4 over 72 hours in MSM supplemented with 400 mg/l phenol Error bars represent the growth range within the quintuplets. **B) Degradation profile of phenol (400 mg/l) as estimated by the 4-AAP colorimetric assay in Gey1A1, Ork2A6, Ork5A2 and Prf1A4**. Error bars represent the standard deviation.



A.



B.

Figure 2.6: A) Bacterial growth curve of *Bacillus* sp. Row2A12.3A over 72 hours in MSM supplemented 400 mg/l phenol. Error bars represent the growth range within the quintuplets. **B) Degradation profile of phenol (400 mg/l) as estimated by the 4-AAP colorimetric assay in *Bacillus* sp. Row2A12.3A.** Error bars represent the standard deviation.

2.4 Conclusion

The application of microorganisms in industrial biodegradation strategies requires both consistency in terms of high biomass generation, the continued degradation of the substrate and the ability of the microorganism to withstand high concentrations of the target substrate as well as toxic intermediates and end products. A bioindustrial approach to phenol degradation would likewise need a microorganism that is capable of consistent phenol mineralisation, that is tolerant to high concentrations of phenolic compounds and that can replenish biomass lost during washout. Here we have screened an extensive collection of thermophilic aerobic bacteria that meet all three criteria for application in phenol bioremediation strategies. One strain in particular, *Bacillus* sp. Row2A12.3A may prove valuable in industrial phenol removal. This taxon showed the consistent capacity for growth with phenol as a sole carbon source with comparatively high generation of biomass. Furthermore, *Bacillus* sp. Row2A12.3A exhibited the capacity for effective growth in 1200 mg/l phenol, which is substantially higher than what is found in most phenol-containing waste-streams. Finally, *Bacillus* sp. Row2A12.3A also demonstrated a noteworthy phenol (400 mg/l) reduction of 74% within 48 hpi. Key to the applicability of this strain is its thermophilicity, which allows for increased biomass, reaction rates, toxicity resistance and limited contamination, all of which contribute to more stable and efficient phenol removal systems. To further characterise the phenol tolerance and degradative capacity of this strain, we sequenced its genome to study the molecular basis underlying these biotechnologically relevant phenotypes.

CHAPTER THREE

PHENOL DEGRADATION THROUGH THE *META*-CLEAVAGE PATHWAY IN *BACILLUS SMITHII* ROW2A12.3A

3.1 Introduction

Phenol is a hazardous pollutant whose chemical stability allows for it to persist in the contaminated environment for prolonged periods of time (Min, *et al*. 2014). Phenol generally acts to inhibit growth and induce stress responses in all organisms. However, several bacterial taxa are able to survive exposure to relatively high concentrations of phenol and have the capacity to degrade phenolic compounds (Villegas, *et al*. 2016; Nzila, 2018). This is likely due to the plasticity of their genomes, a result of the rapid genomic replication, the proclivity for gene rearrangements and mutations and the dissemination of genetic material between taxa via mobile genetic elements. These activities promote functional genomic diversity allowing for rapid adaptation and resistance to toxic compounds (Suenaga, *et al*. 2009). Both aerobic and anaerobic bacteria are capable of converting phenol into innocuous compounds through the means of several metabolic pathways (Nešvera, *et al*. 2015). Complete mineralisation of phenol is most rapidly and reliably achieved by aerobic bacteria and as such, they are more favourable for incorporation in bioremediation strategies (Al-Khalid & El-Naas, 2012).

Aerobic bacterial degradation of phenol makes use of oxygen in order to overcome the resonance energy of the phenol ring structure and occurs in three principle steps: 1) ring activation through an initial hydroxylation, 2) ring cleavage by extradiol or intradiol dioxygenase enzymes and 3) the further breakdown of the resultant products into intermediates of the tricarboxylic acid (TCA) cycle, whereupon they are completely mineralised (Fuchs, *et al*. 2011; Kynadi & Suchithra, 2017). As several of the phenol intermediates, such as hydroquinone, represent more toxic compounds than phenol itself, complete degradation is essential (Enguita & Leitão, 2013). The pathways involved in aerobic phenol degradation are separated into upper (peripheral) pathways which involve phenol hydroxylases for ring activation and lower (central) pathways, which are further divided into the *ortho*- or *meta*-catechol oxidation pathways or the hydroquinone/ hydroxyquinol pathway for ring cleavage and mineralisation (Tam, *et al*. 2006; Zhang, *et al*. 2012). The genes coding for the proteins associated with these pathways may be either genome or plasmid bound and often appear in clusters containing both the genes for the peripheral and central pathways for phenol degradation (Nešvera, *et al*. 2015).

Although the basic mechanism for phenol degradation remains the same, there is significant diversity in the pathways employed. Phenol hydroxylases, employed in peripheral pathways, have been identified in three forms; single-, two- and multi-component hydroxylases, with two-component hydroxylases being identified primarily in thermophilic bacilli and Gram-positive *Actinobacteria* while multi-component hydroxylases occur predominantly among *Proteobacteria* (Nešvera, *et al*. 2015; Chen, *et al*. 2018). The central m*eta*- and *ortho*- cleavage pathways are ubiquitous across the broader bacterial taxonomic spectrum (Kynadi & Suchithra, 2017). By contrast, the distribution of the central hydroquinone and hydroxyquinol pathways differs in that Gram-positive bacteria predominantly employ the hydroxyquinol route whereas Gram-negative bacteria may employ the hydroquinone pathway or both pathways simultaneously (Zhang, *et al*. 2012; Enguita & Leitão, 2013).

The genetic and mechanistic diversity exhibited by distinct bacterial taxa affect the both the tolerance to high concentrations of phenol as well as the capacity and efficacy of its degradation. (Krastanov, *et al*. 2013). Hence, careful selection and molecular characterisation of phenol degrading strains and their pathways is pivotal for their incorporation in effective phenol bioremediation strategies. Aerobic thermophilic bacteria are of particular interest in phenol degradation applications due to their increased capacity for resistance to phenol toxicity and favourable growth and reaction rates (Nzila, 2018). Of the thermophilic bacteria identified as capable of phenol degradation 80% belong to the family *Bacillaceae*, with *Geobacillus* sp. and *Parageobacillus* sp. being the most prominent (Nzila, 2018). In Chapter 2, an aerobic thermophilic strain of *Bacillus* sp. Row2A12.3A was identified as being capable of tolerating high concentrations of phenol (1200 mg/l), while a colorimetric assay showed that it effectively degrades phenols, at a rate of 8.96 mg/l/h. As such, the aim of this chapter was to elucidate the molecular basis underlying both the tolerance and biodegradation of phenol exhibited by the strain through full genome sequencing and by means of comparative genomic approaches.

3.2 Methodology

3.2.1 Genome sequencing

Bacillus sp. Row2A12.3A was grown in LB broth for 24 hours at 56°C on a rotary shaker (140 rpm) prior to DNA extraction. Genomic DNA (gDNA) extraction was performed using the ABIOpure total DNA extraction kit (Alliance Bio, U.S.A.) following the recommended protocol for Gram-positive bacteria. In order to maximise the genomic content, gDNA was extracted in five separate reactions, which were pooled in a final volume of elution buffer. The DNA extract A260/A280 and A260/A230 ratios were evaluated using the Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer. An A260/A280 ratio of 1.82, A260/A230 ratio of 1.57 and DNA quantity of 643.10 ng/ µl was considered adequate for full genome sequencing.

The gDNA was sequenced by MrDNA (Texas, USA). Prior to sequencing the DNA concentration of *Bacillus* sp. Row2A12.3A was re-assessed, by MrDNA, using the Qubit® dsDNA HS Assay Kit (Life Technologies) and shown to be 75.20 ng/ µl. Library preparation was performed using 50 ng of gDNA with the Nextera DNA Flex library preparation kit (Illumina) in accordance with the user guide. Library preparation involved simultaneous adapter sequence addition and fragmentation. The adapters were utilised in the addition of a unique index t the sample through a limited (6-cycle) PCR. The final library was evaluated with the Qubit® dsDNA HS Assay Kit (Life Technologies) Agilent 2100 Bioanalyzer (Agilent Technologies). The library had a concentration of 10.50 ng/ µl, with an average fragment size of 701 base pairs. For effective sequencing the library was diluted to 0.6nM. Paired-end (2×150 bp) sequencing was achieved with the NovaSeq 6000 system (Illumina) over 300 cycles.

3.2.2 Genome assembly, annotation and species delineation

Quality control, trimming and initial genome assembly were conducted using the built-in applications in Illumina BaseSpace. Short reads (<130bp), reads of insufficient quality (Q score <28) and Nextera rapid capture adapters were trimmed (trim stringency of 0.9) with FastQ v 2.2.0. FastQC v 1.0.0 was used to evaluate

quality control efficacy (Li & Durbin, 2009). The trimmed and filtered reads were *de novo* assembled using SPAdes v. 3.9.0, with default settings except for the careful option being disabled and the kmer set to automatic selection (Nurk *et al*. 2013). Contigs which were <500bp in size were removed from the initial assembly. Further reference-based assembly was performed using Mauve (v2.4.0) and the multi-draft based scaffolder MeDuSa v. 1.3 (Darling *et al*. 2004; Bosi *et al*. 2015), using the genome of the type strain of *B. smithii* DSM 4126^{T} (NZ_CP012024) as reference genome. Manual gap closure was done by BlastN analysis against the NCBI nucleotide (nt) database and localised BlastN analysis with the NCBI BlastN hits against the unassembled reads to identify homologous fragments, using Bioedit v 7.2.5 (Hall, 1999). Most of the initial gaps represented repeat sequences either in the form of rDNA or transposable elements but were closed in the manual curation phase.

3.2.3 Genome-based taxonomy of *B. smithii* Row2A12.3A

The initial taxonomic placement of *B. smithii* Row2A12.3A on the basis of the partial 16S rDNA sequence was confirmed using the TYpe (strain) Genome Server (TYGS v. 2.12). TYGS determines a distance matrix using the Genome Blast Distance Pipeline (GDBP) approach and then infers a whole-genome based phylogeny using FastME v2.1.6.1 (Lefort *et al*. 2015; Meier-Kolthoff & Göker, 2019). The species-level relatedness of Row2A12.3A to the type strain of *B. smithii*, DSM 4216^{T} and *B. smithii* 7_3_47FAA was confirmed by determining digital DNA:DNA hybridization (dDDH) values using the DSMZ Genome to Genome Distance Calculator (GGDC) web server (Auch *et al*. 2010) and Average Nucleotide Identity (ANI) values using OrthoANI v0.93.1, (Lee *et al*. 2015).

3.2.4 Comparative genomics

For comparative genomic purposes, the complete genome of *B. smithii* DSM 4216^{T} (CP012024.1 and CP012025.1) and high-quality draft genome of *B. smithii* 7_3_47FAA (ACFW000001.1) were obtained from the NCBI genome database. The genome assembly of *B. smithii* 7_3_47FAA was improved (from an initial 175 contigs to a final 14 contigs) by means of reference-based scaffolding and manual gap closure as described above. The genomes (chromosome and plasmid

sequences) were first visually compared using the CGView server (Grant & Stothard, 2008). Both comparator genomes, as well as the complete genome of *B. smithii* Row2A12.3A, were structurally (protein prediction) and functionally annotated on the Rapid Annotation using Subsystems Technologies (RAST) server (Aziz *et al*. 2008). The resultant protein datasets were compared using Orthofinder v.1.1.4 (Emms & Kelly, 2015) to identified core (protein orthologues identified in all three strains), accessory (protein orthologues conserved in two of the three strains) and those proteins which are unique to the individual *B. smithii* genomes. The distinct orthogroup datasets were then functionally classified according to their Non-supervised Orthologous Groups (NOGs v 5.0) using eggnog mapper (Huerta-Cepas, *et al.* 2019).

3.2.5 Identification of genomic determinants for phenol degradation

Aerobic phenol degradation occurs through several pathways, genes for each component of these pathways were selected based on their presence in complete functional phenol degradation pathways as identified in literature. A total of twenty -eight proteins were selected from *G. stearothermophilus* (DQ146476.2), *Thermoflavimicrobium dichotomicum* (FORR01000003.1: 240078..240875) and *B. licheniformis* (AE017333.1: 3817696..3819171) for the *meta*-cleavage pathway (Veith *et al*. 2004; Omokoko, *et al*. 2008; Li, *et al*. 2019), *Acinetobacter pittii* PHEA-2 (NC_016603.1: 1419738.. 1426069) for the ortho-cleavage pathway (Zhan, *et al*. 2012) and *Sphingobium cloacae* for the hydroquinone/ hydroxyquinol pathway (AP017659.1; AP017660.1) (Ootsuka, *et al*. 2016; Ootsuka, *et al.* 2018). The protein coding sequences of relevant genes were retrieved from the NCBI server and compared to the *B. smithii* genomes using local BlastP and tBlastN searches in Bioedit (v7.2.5). Orthology was assigned to BlastP or tBlastN matches if they fulfilled the minimum requirements of a bit score of 50, an E-value of 0.0001 (1E-4) with a > 40% sequence identity (or 30% when the coding sequence is less than 100 amino acids) over 65% of the query sequence (Zhan, *et al*. 2012; Pearson, 2013). As prophage elements are often found in bacterial genome and may interfere with the functioning of metabolic pathways. For this reason, the *B. smithii* genomes were passed through the phage identification and annotation server PHAge Search Tool Enhanced Release (PHASTER) (Zhou, *et al*. 2011; Arndt, *et al*. 2016).

3.3 Results and discussion

3.3.1 General genome characteristics

The paired-end genomic DNA sequencing of *Bacillus* sp. Row2A12.3A yielded ~ 134 Gigabases of data (14,184,954 raw read pairs with average read length of 2×150 bp; genome coverage ~ 260x). A total of 513 contigs were generated following *de novo* assembly, 53 of these contigs were larger than 1,000 bp in size with the largest contig being ~875 kilobases in length. Tentative identification of *Bacillus* sp. Row2A12.3A based on its 16S rDNA sequence placed it as a strain of *Bacillus smithii*. Following reference-based approaches and manual curation the genome of the phenol degrading *Bacillus smithii* Row2A12.3A was assembled to complete status with a circular chromosome of ~3.42 Mb. Two genome sequences of *B. smithii* were available for comparative analysis, *B. smithii* 7_3_47FAA and the type strain *B. smithii* DSM 4216^{T}, which was the only complete genome, with a size of 3.38Mb. The genome of *B. smithii* Row2A12.3A is the largest of the three compared strains (36,431bp and 167,097bp larger than those of DSM 4216 ^{T} and 7_3_47FAA, respectively) (Table 3.1). The genome of *B. smithii* Row2A12.3A also codes for the most proteins with a total of 3,858 distinct proteins (128 and 73 more than are encoded on the genomes of DSM 4216 ^{T} and 7_3_47FAA, respectively). *B. smithii* Row2A12.3A incorporates a small circular plasmid, pRow2A12.3A**,** which is 6,020 bp in size, with a G+C content of 34.9% and codes for eleven proteins. Similarly, a plasmid is incorporated in the genome of *B. smithii* DSM 4216^{T}, pDSM4216 (CP012025.1), but the plasmid of the latter strain is more than twice as large as that observed in Row2A12.3A (Table 3.1). By contrast, no plasmid could be identified in the genome sequence of *B. smithii* 7_3_47FAA. While certain genetic elements related to plasmid replication were identified these were shown to form part of the chromosome due to their distribution and the chromosomal location of their counterpart orthologues in *B. smithii* DSM 4216^{T} (Bosma, *et al*. 2016).

Table 3.1: Summarised genome characteristics for the *B. smithii* strains

Strain	Row2A12.3A	DSM 4216^T	7_3_47FAA
Genome size (bp)	3,417,723	3,381,292	3,250,626
# Contigs	2 (complete)	2 (complete)	14 (high quality draft)
Plasmids	1 (6,020bp)	1 (12,514 bp)	0
Chromosome GC content	40.5%	40.8%	40.7%
Plasmid GC content	34.9%	35.9%	-
# Total CDS	3,858	3,730	3,785
# RNAs	86	127	117

Taxonomic placement of *B. smithii* Row2A12.3A

A phylogeny was constructed on the basis of the GDBP algorithm using TYGS (Meier-Kolthoff *et al*., 2013; Meier-Kolthoff & Göker, 2019). This phylogeny shows Row2A12.3A clustering with the genome of the type strain *B. smithii* DSM 4216^T (Figure 3.1). On the basis of blast distance, they belong to the same species. This was further supported by the dDDH (78%) and ANI values (98.12%) between Row2A12.3A and the type strain. These values are substantively above the species delineation boundaries of >70% and >96% for dDDH and ANI, respectively (Auch *et al*., 2010; Lee, *et al*. 2016). Further dDDH and ANI analysis of the strain 7_3_47FAA, shows that it also belongs to the species *B. smithii* (Table 3.2).

Table 3.2: OrthoANI (upper quadrant) and dDDH (lower quadrant) values between the pair-wise compared *B. smithii* strains.

	ROW2A12.3A	DSM 4216^T	7_3_47FAA
ROW2A12.3A	-	98.12	98.12
DSM 4216^T	78.0	-	98.30
7_3_47FAA	82.7	84.4	-

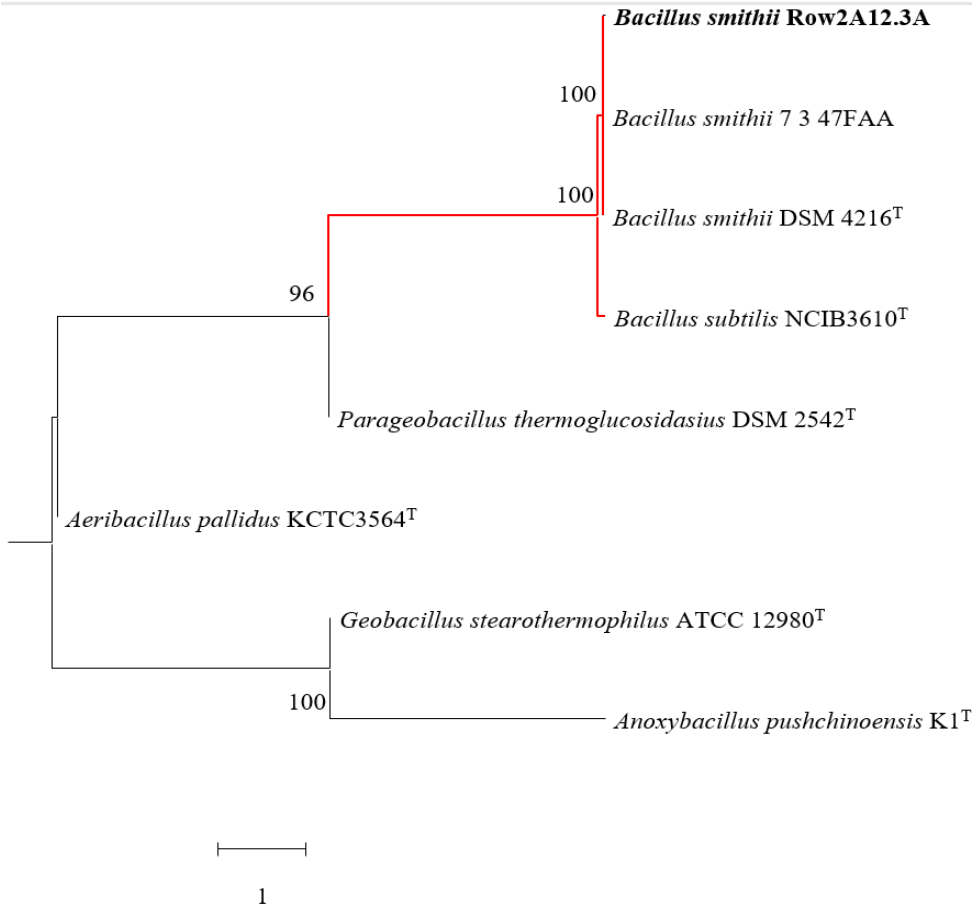


Figure 3.1: Genome Blast Distance phylogeny generated using TYGS (Lefort *et al*. 2015; Meier-Kolthoff & Göker, 2019)

3.3.2 Comparative genomic analysis of *B. smithii* Row2A12.3A, DSM 4216^T and 7_3_47FAA

The complete genome sequence of *B. smithii* Row2A12.3A was first visually compared to those of the other *B. smithii* strains using CGView. Comparison of the chromosome sequences (Figure 3.2) showed extensive sequence conservation among all three genomes. There are, however, several genomic islands which are unique to Row2A12.3A and may be responsible for the larger genome size and greater number of proteins encoded on the genome of this strain. Similarly, the complete plasmid sequences from *B. smithii* Row2A12.3A and DSM 4126^T were visually compared (Figure 3.3 A and B). In contrast to the chromosomal comparison, the plasmids show far less sequence conservation from both the pRow2A12.3A - and pDSM4216-centric perspective (Figure 3.3 A and B).

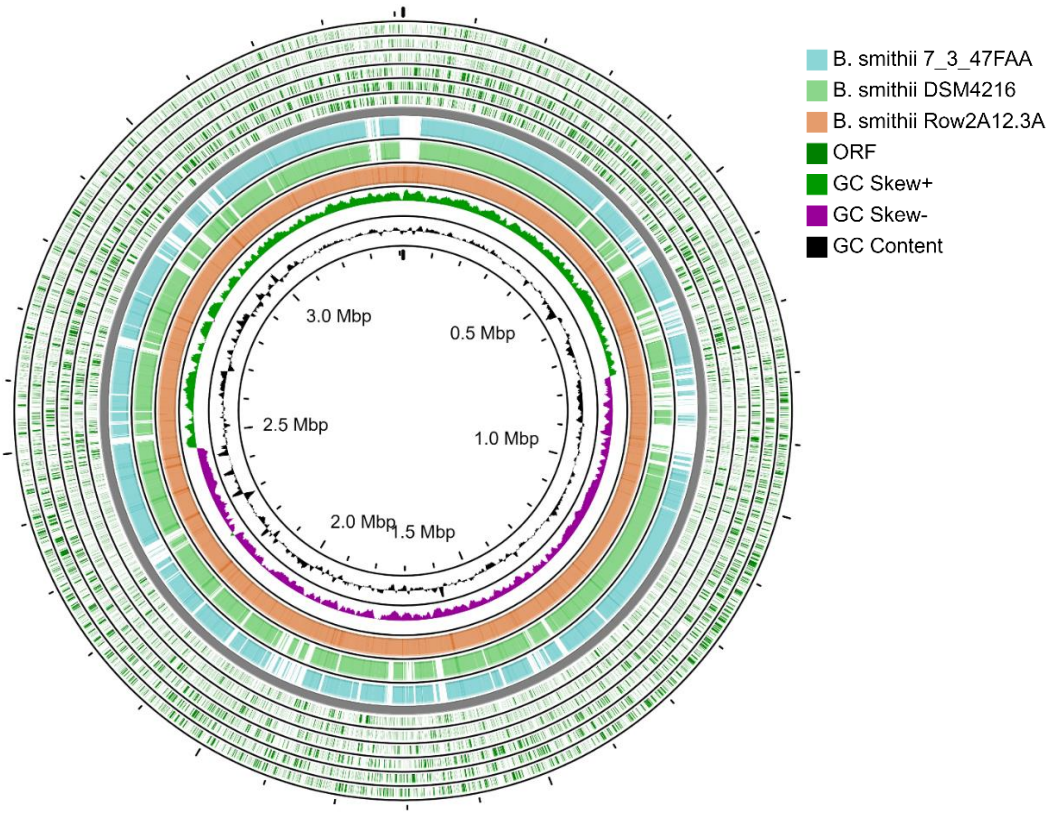
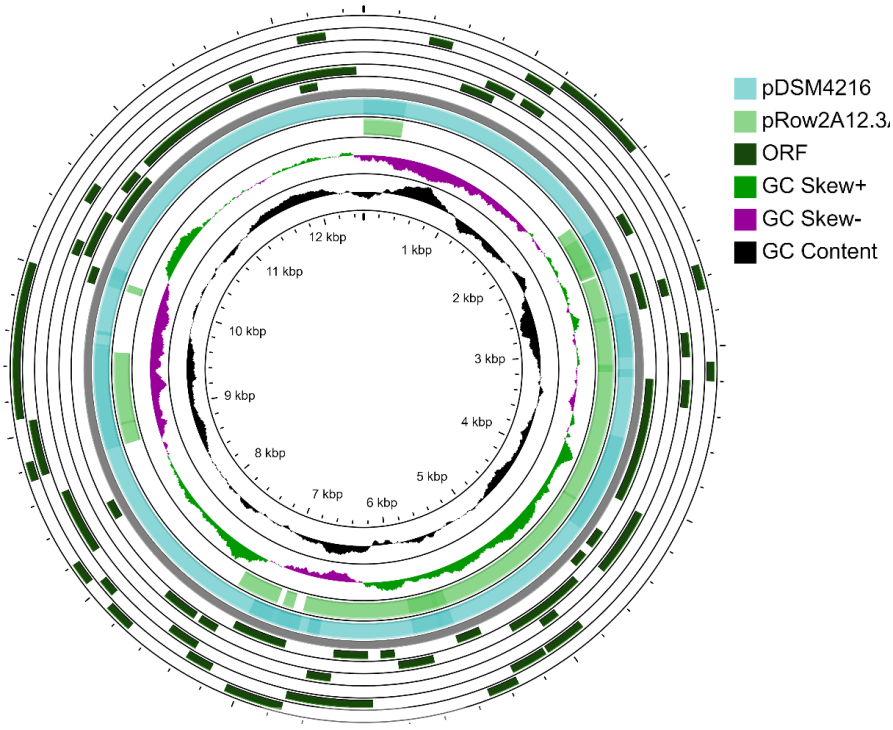
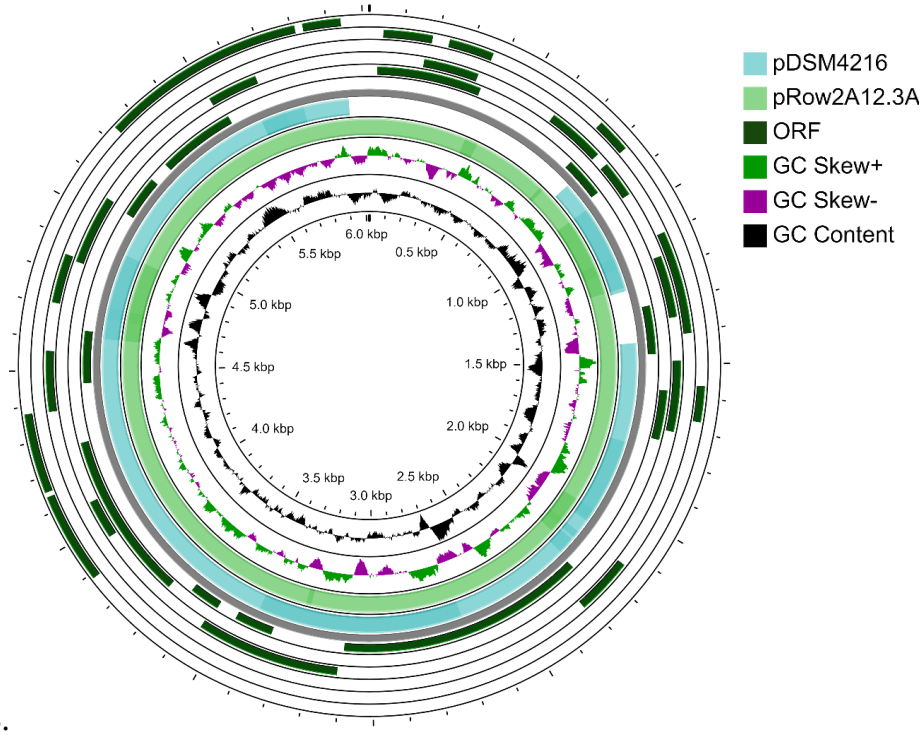


Figure 3.2: Comparative genome map generated using CGView. Starting from the innermost ring outward, Ring 1 represents the reference genome (*B. smithii* Row2A12.3A), Ring 2 the GC content; Ring 3 shows GC skew, Rings 4-6 depict BlastN comparison results of the reference genome against itself, DSM 4216^{T} and 7_3_47FAA, respectively. Rings 7-12 indicate the ORFs greater than 100 codons in the different reading frames. The BlastN results are depicted with partial opacity wherein the darker portions are indicative of several hits in the corresponding portion of the reference sequence *B. smithii* Row2A12.3A.



A.



B.

Figure 3.3: Plasmid map comparing the plasmids of *B. smithii* Row2A12.3A and DSM 4216^{T}. A) pDSM 4216^{T} centric plasmid map. B) pRow2A12.3A centric plasmid map. The contents of each ring are as described in the figure legend for Figure 3.2.

The proteins encoded on the genomes of the three *B. smithii* strains were clustered according to their orthologous groups using Orthofinder (Figure 3.4). This analysis showed that 2,585 proteins are shared by all three strains, with the core proteins contributing 69.2%, 73.4% and 71.2% of the total proteins encoded on the genomes of *B. smithii* Row2A12.3A, DSM 4216^T and 7_3_47FAA, respectively. While the phylogenomic analyses indicated Row2A12.3A and 7_3_47FAA are more closely related, a larger proportion of the accessory fraction of the genome is shared only by *B. smithii* Row2A12.3A and DSM 4216^T. This may be due to the fact that the genome of 7_3_47FAA is not complete. The genome of *B. smithii* 7_3_47FAA coded for the largest number of unique proteins (694; 19.1% of total genome content), followed by Row2A12.3A (690; 18.5%) and DSM 4216^T (426; 12.1% of total genome content). As the accessory genome (unique or shared by only two of the three genomes) comprises of 28.8% of the total protein complement of the three taxa, *B. smithii* can be suggested to display an open pan-genome. Open pan-genomes have been associated with increased intra-species diversity and the acquisition of genetic material that allows functional diversification (Vernikos, *et al*. 2015; Wang, *et al*. 2020). *B. subtilis*, *B. cereus sensu lato* and *Geobacillus* spp. exhibit open pan-genomes and are capable of adapting to a variety of environmental niches (Bazinet, 2017; Brito, *et al*. 2018; Wang, *et al*. 2020). In members of the genus *Geobacillus*, this diversification has been proposed to enhance the bioremediation activities of select taxa within the genus.

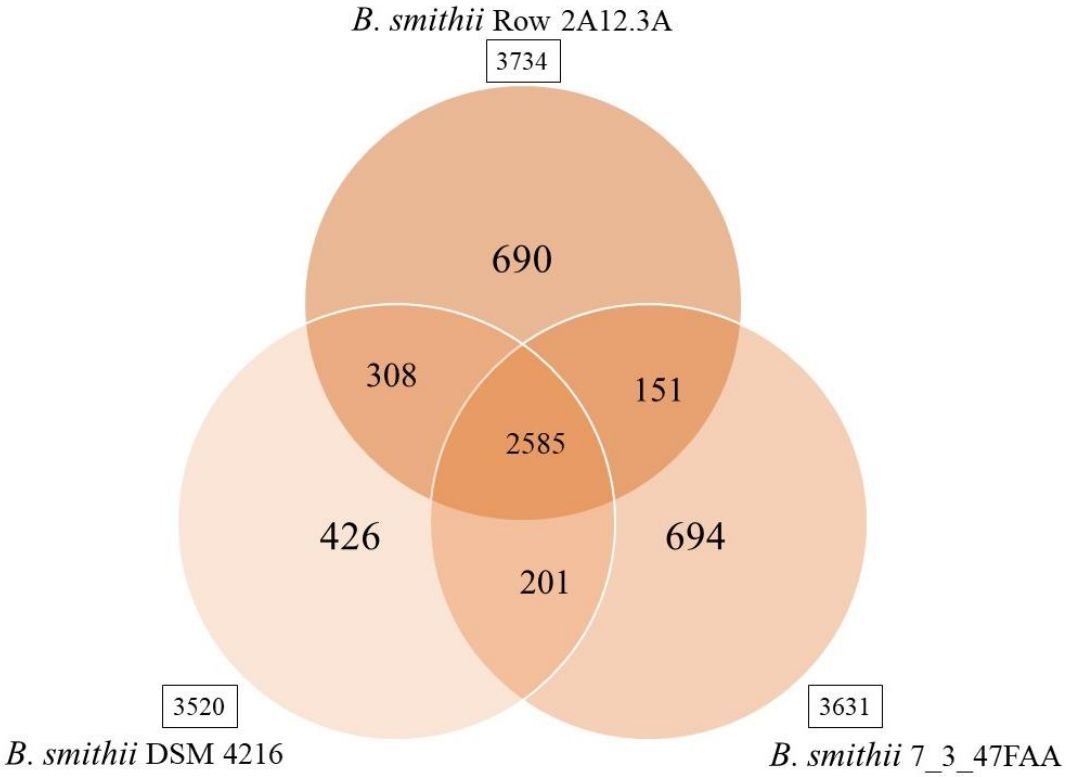


Figure 3.4: Venn diagram showing total number of core and accessory proteins (shared by two strains or unique to a particular strain). Proteins found in multiple copies were included in protein families and treated as a single protein for pan-genome analyses.

The genomes of *B. smithii* strains show evidence of functional diversification

To gain further insight into the potential functional diversification of *B. smithii*, the different genomic fractions (core, accessory and unique) were classified according to Conserved Orthologous Groups (COGs) (Huerta-Cepas, *et al*. 2017) and according to the supra-functional categories Cellular processes and signalling, Information storage and processing and Metabolism (De Maayer, *et al.* 2017).

When considering the core dataset, it is predominated by proteins involved in Metabolism (50.3%) at a ratio of 2:1:1 with Cellular processes and signalling (25.5%) and Information storage and processing (24.2%) (Figure 3.5). A similar trend was observed for most of the accessory and unique genome fractions with the exception of the accessory fractions incorporating *B. smithii* 7_3_47FAA and its unique fraction, where a shift towards proteins involved in Information storage and processing is observed (Figure 3.5). When considering the individual COG

categories of these fractions it can be observed to be pre-dominated by proteins involved in COG category L (DNA replication, recombination and repair). Proteins involved in this function encompass 70.5% of the total proteins unique to *B. smithii* 7_3_47FAA, while they represent the largest fraction of the Row2A12.3A + 7_3_47FAA (22.1%) and DSM 4216^T + 7_3_47FAA (51.0%) accessory fractions (Figure 3.6). The encoded proteins were predominantly identified as transposases. Large numbers of mobile genetic elements, such as transposases, are shown to result in the apparent inactivation of genes and ultimately reductive genome evolution (Darmon & Leach, 2014; De Maayer, *et al*. 2017).

By contrast, when considering the accessory fraction comprising proteins shared by Row2A12.3A and DSM 4216^T only, as well as the proteins unique to Row2A12.3A**,** the relative proportion of proteins in the supra-functional category Metabolism is substantively larger than what is observed in the core protein fraction (6.8% higher in both cases). Considered at the COG category level, this predominance can be ascribed mainly to Carbohydrate transport and metabolism (G) and Energy conversion (C). While carbohydrate metabolism contributes 7.4% of the total core proteins, the proportion of proteins involved in this function are 2.8x and 2.1x fold higher in the Row2A12.3A unique fraction and the Row2A12.3A + DSM 4216 accessory fraction, respectively. This suggests that the latter two strains, and particularly Row2A12.3A, have diversified in terms of the carbohydrates they can utilise. Both fractions encode numerous carbohydrate transporters. The Row2A12.3A + DSM 4216^T accessory fraction encodes proteins involved in the utilisation of mannose, xylose, chitin and N-acetylglucosamine. In addition, the unique fraction for Row2A12.3A encodes proteins associated with the utilisation of benzoate, ribose, gluconate and ketogluconate. The contribution of proteins involved in Energy conversion (C) is particularly higher in the Row2A12.3A + DSM 4216^T accessory fraction (1.7x fold higher relative proportion). Among the accessory fractions pertaining to *B. smithii* Row2A12.3A, whether unique or shared, there was a notable increase of proteins assigned to COG classes T (Signal transduction mechanisms), U (Intracellular trafficking, secretion, and vesicular transport) and Q (Secondary metabolites biosynthesis, transport, and catabolism) which was not reflected in the core protein dataset. Taken together *B. smithii* DSM

4216^{T} and Row2A12.3A encode a higher number of proteins that allow utilisation of a more diverse range of carbohydrates (Li, *et al*., 2019).

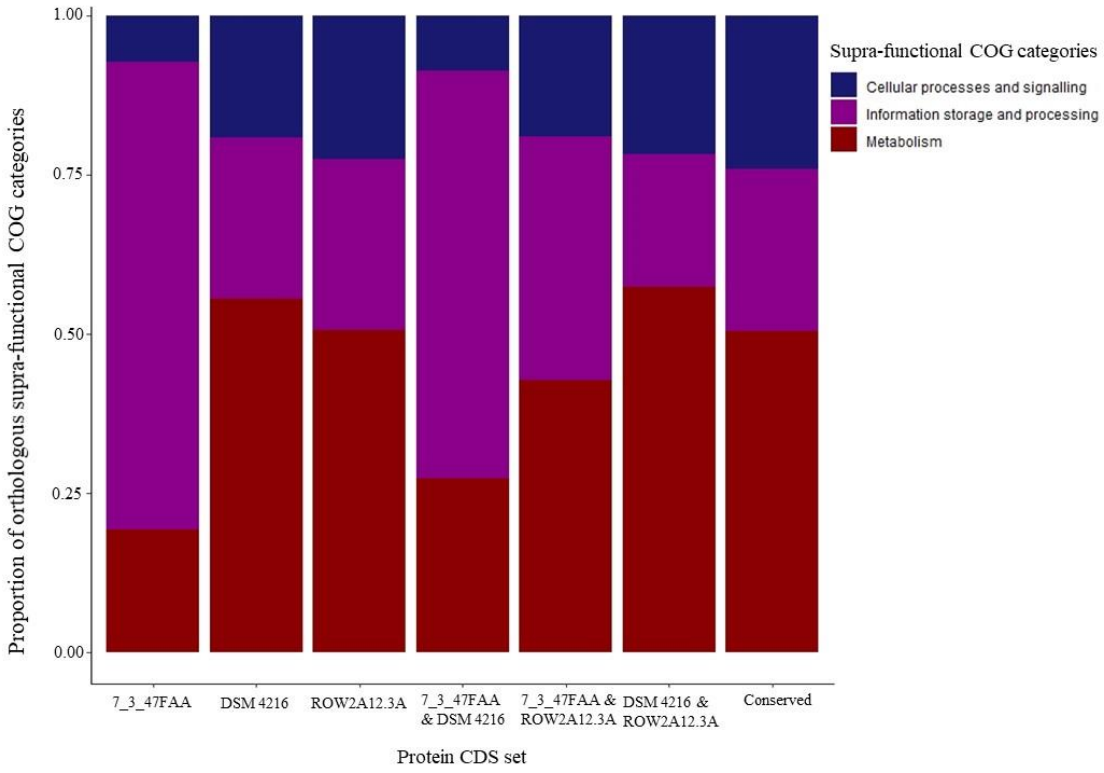


Figure 3.5: Proportions of core and accessory proteins encoded on the genomes of the compared *B. smithii* strains involved in the-functional categories Cellular processes and signalling, Information storage and processing and Metabolism.

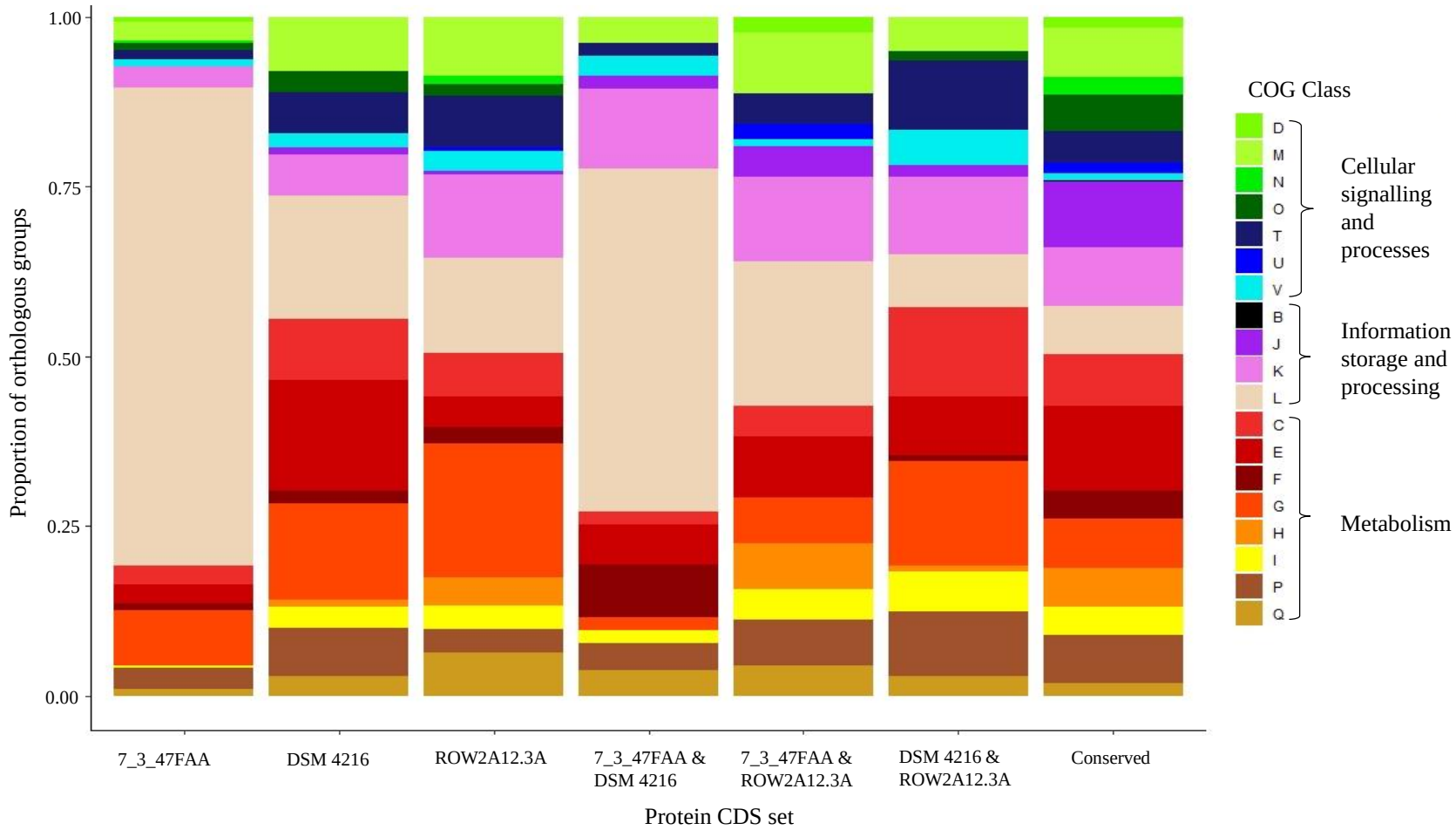


Figure 3.6: COG classification proportions for the conserved core and accessory protein fractions encoded on the genomes of the three *B. smithii* strains.

The genomes of *B. smithii* Row2A12.3A, DSM 4216T and 7_3_47FAA code for the metabolism of a range of aromatic compounds.

The RAST subsystems information for all three *B. smithii* genomes was perused to identify pathways involved in the metabolism of aromatic compounds. The genome of *B. smithii* Row2A12.3A codes for the most proteins (16 proteins) involved in this metabolic capacity, while those of *B. smithii* 7_3_47FAA and DSM 4216^T encode five and eight proteins involved in this function, respectively. These proteins can further be sub-classified as those involved in "peripheral pathways for catabolism", "anaerobic degradation" and "metabolism of central aromatic compounds", although, some proteins may be involved in more than one of these categories. The proteins encoded by all three strains were associated with quinate and p-hydroxybenzoate degradation, the catechol branch of the β-ketoadipate pathway and salicylate and gentisate catabolism. *B. smithii* Row2A12.3A and DSM 4216^T both had three proteins involved in aromatic amin catabolism, including either a 3,4-dihydroxyphenylacetate 2,3-dioxygenase or 4-hydroxyphenylacetate 3-monooxygenase, respectively, and two copies of the 4-hydroxyphenylacetate 3-monooxygenase reductase component. In addition, the genome of Row2A12.3A also encodes three hydroxyaromatic decarboxylases which function anaerobically, three additional proteins involved in gentisate/salicylate catabolism and two copies of the benzoate Major Facilitator Superfamily (MFS) transporter BenK.

Overall Row2A12.3A codes for a larger cohort of aromatic degrading proteins, although none appear specific for phenol. However, several of the compounds targeted for degradation are classed as phenolics – gentisate and salicylate – moreover the structural components of the proteins and the regulators of this catabolic pathway have been shown to influence aromatic toxin degradation (Ghoshal, *et al*. 2016). Phenylacetate, though often derived from phenol condensation, is an acetate ester (Martin & McInerney, 2009). There is evidence that this pathway participates in the catabolism of several other aromatic hydrocarbons, although the range appears limited to those with a phenyl group (Navarro-Llorens, *et al*. 2005; Martin & McInerney, 2009). It cannot be assumed that the proteins encoded would participate in phenol degradation, due to the differences in structure. Nevertheless, the β-ketoadipate pathway does form a

portion of the *ortho*- pathway for phenol degradation (Brzostowicz, *et al*. 2003; Tuan, *et al*. 2011; Sridevi, *et al*. 2012; Zhan, *et al*. 2012). The RAST annotation does not indicate the presence of the entire pathway and as such BlastP and tBlastN searches were performed with gene/protein sequences previously identified to form part of phenol degradation pathways in other bacterial taxa, to identify orthologous pathways encoded on the genome of *B. smithii* Row2A12.3A and the other strains of *B. smithii*.

Genomic identification of the molecular determinants for phenol degradation in *B. smithii*

The *B. smithii* genome sequences and protein datasets were searched by BlastP and tBlastN analysis for orthologues of proteins involved in the *meta*-cleavage (*G. stearothermophilus* DSM 6285; DQ146476 – Omokoko *et al.* 2008, *B. licheniformis* DSM 13; AAU42807.1 – Veith *et al*. 2004 and *T. dichotomicum* DSM 44778; SFJ02046.1 – Li, *et al*. 2019), *ortho*-cleavage (*A. pittii* PHEA-2; NC_016603.1 – Zhan *et al.* 2012) in phenol metabolism as well as the hydroquinone/hydroxyquinol pathway (*S. cloacae* JCM 10874^{T} plasmid 5 and plasmid 6; AP017659.1 and AP017660.1 – Ootsuka, *et al*. 2016). Peripheral pathways related to phenol degradation contain numerous proteins, however, phenol hydroxylases are the most frequently responsible for the conversion of phenol to either catechol, as employed in the *meta*-cleavage and *ortho*-cleavage, or hydroquinone/ hydroxyquinol (Nešvera, *et al*. 2015). The two-component phenol hydroxylase (PheA1 and PheA2) identified in *G. stearothermophilus* was chosen as the representative phenol hydroxylase in this study because two-component phenol hydroxylases are the most frequently identified in thermophilic bacilli (Nešvera, et al. 2015). This taxon is furthermore closely related to *B. smithii* than *A. pittii* and *S. cloacae*.

The hydroquinone/hydroxyquinol pathway involves eight distinct proteins, hydroxyquinol 1,2-dioxygenase (1,2-Hqd), putative AraC-type transcription regulator (HqdR), hydroquinone dioxygenase small subunit (HqdA), hydroquinone dioxygenase large subunit (HqdB), 4-hydroxymuconic semialdehyde dehydrogenase (HqdC), maleylacetate reductase (HqdD), intradiol 1,2-dioxygenase

(HqdE) and putative ferredoxin (HqdF), which are encoded on plasmid 5 and plasmid 6 of *S. cloacae* JCM 10874^{T}. Plasmid 5 codes for 1,2-Hqd, which is the primary enzyme involved in the conversion of hydroxyquinol to maleylacetate. Plasmid 6 incorporates the remainder of the hydroquinone pathway, which is encoded by a total of seven genes in a single 7.9kb locus in the order *hqdRAB-orf1-orf2-hqdCDEF* (Ootsuka, *et al*. 2016). BlastP analysis showed that only one protein shared orthologues in the *B. smithii* strains, namely the 4-hydroxymuconic semialdehyde dehydrogenase HqdC (BAV66980.1), of which three, three and one distinct orthologue(s) were identified in *B. smithii* strains Row2A12.3A**,** DSM 4216^{T} and 7_3_47FAA, respectively. None of the *B. smithii* orthologues shared > 46 % amino acid identity (AAI) with the HqdC protein of *S. cloacae*. It is likely that these orthologues play distinct roles in the *B. smithii* strains as dehydrogenases of other semialdehydes or aldehydes (Perozich, 1999). As such, *B. smithii* can be considered to lack the hydroquinone/hydroxyquinol pathway for phenol degradation.

The *ortho*-cleavage pathway requires six distinct proteins, catechol 1,2-dioxygenase (CatA), muconate cycloisomerase (CatB), muconolactone delta-isomerase (CatC), beta-ketoadipyl CoA thiolase (CatF), 3-oxoadipate CoA-transferase (CatIJ) and 3-oxoadipate enol-lactonase (CatD), which are encoded as an 8 kb gene cluster on the chromosome of *A. pittii* PHEA-2 (Zhan *et al.* 2012). BlastP analysis indicated that two proteins shared orthologues in the *B. smithii* strains, namely the beta-ketoadipyl CoA thiolase CatF (ADY81914.1) and the two subunits that comprise the 3-oxoadipate CoA-transferase CatIJ (ADY81912.1; ADY81913.1). Only one orthologous copy of each 3-oxoadipate CoA-transferase subunit (CatI and CatJ) was identified in each strain. However, multiple copies of the beta-ketoadipyl CoA thiolase (CatF) are present with five, four and three distinct orthologues identified in *B. smithii* strains Row2A12.3A**,** DSM 4216T and 7_3_47FAA, respectively. The *B. smithii* orthologues share amino acid sequence identities of < 44.89% with *catF* of *A. pittii* PHE-A2 (ADY81914.1). The presence of these orthologues is not an indication of the capacity for phenol degradation. Rather, the β-ketoadipate pathway is involved in a range of aromatic hydrocarbon degradation pathways and the *B. smithii* orthologues may thus function in one of

these alternate pathways (Kurbatov, *et al*. 2006; Wells & Ragauskas, 2012). Though the *B. smithii* strains lack the complete, functional protein complements for the *ortho*-cleavage or hydroquinone/hydroxyquinol pathways *B. smithii* Row2A12.3A and DSM 4216^T encode for the entire *meta*-cleavage pathway.

3.3.3 Molecular characterisation of the phenol *meta*-cleavage pathway in *B. smithii* strains

Phenol degradation requires the activity of both the central and peripheral pathways and the activity of phenol hydroxylase (PheA1-2) is the commitment step that permits the conversion of phenol into the central intermediate catechol prior to entry into the *meta*-cleavage pathway (Omokoko *et al.* 2008). Both *B. smithii* Row2A12.3A and DSM 4216^T encode for single orthologues of the two subunits of phenol hydroxylase (PheA1-2). However, *B. smithii* 7_3_47FAA does not encode PheA1-2 and thus is not likely to degrade phenol. The *meta*-cleavage pathway is comprised of two possible branches, incorporating either the enzyme 2-hydroxymuconate semialdehyde hydrolase (HMSH) or 2-hydroxymuconate semialdehyde dehydrogenase (HMSD) (Nešvera, *et al*. 2015). Both branches sequentially convert catechol into acetyl-CoA and pyruvate. The HMSH branch of the pathway requires five distinct proteins in order to function, namely catechol-2,3-dioxygenase (PheB), HMSH, 2-hydroxypenta-2,4-dienoate-hydratase (PheD), 4-hydroxy-2-oxovalerate aldolase (PheE) and acetaldehyde dehydrogenase (PheF). The HMSD branch, also referred to as the 4-oxalocrotonate route, requires seven proteins including PheB, HMSD, 4-oxalocrotonate decarboxylase (PheC), 4-oxalocrotonate tautomerase (PheH), PheD, PheE and PheF (Figure 3.7). Three further proteins have been predicted to be required for overall functioning of the *meta*-cleavage pathway. PheR is a transcriptional regulator which has been shown to activate the *pheA1-2* genes with DNA binding being a result of two helix-turn-helix motifs (Chen, *et al*. 2018). PheX incorporates a predicted lipase active domain and may represent either a carboxylesterase or phospholipase (Omokoko *et al.* 2008). At present, the role this protein plays in phenol degradation remains unknown. Furthermore, a ferredoxin (fdx) is predicted (Omokoko *et al.* 2008).

The protein complement for phenol *meta*-cleavage, which is encoded on the pGGO1 plasmid of *G. stearothermophilus* DSM 6285 (DQ146476 – Omokoko *et al.* 2008) was searched against the *B. smithii* genomes, using local BlastP and tBlastN analyses. The *meta*-cleavage pathway of *G. stearothermophilus* is comprised of eleven protein coding genes, *pheBHA1A2-fdx-pheRDFECX* which are co-localised on the plasmid. The gene cluster identified on the pGGO1 plasmid did not contain the genes encoding HMSH or HMSD, as such representatives of these genes were taken from phenol degrading *B. licheniformis* DSM 13 and *T. dichotomicum* DSM 44778 (Veith *et al*. 2004; Li, *et al*. 2019). BlastP and tBlastN analyses showed that the genomes of all three *B. smithii* strains incorporate genes coding for orthologues of proteins involved in the *meta*-cleavage pathway (Table 3.3) although substantial differences could be observed in both gene content and organization among the three taxa (Figure 3.7).

Table 3.3: BlastP and tBlastN matches of *B. smithii* strains DSM 4216^T, 7_3_47FAA and Row2A12.3A for known phenol degrading proteins

Protein	Common Name	Organism	NCBI Accession	DSM 4216^T			7_3_47FAA			Row2A12.3A			Reference
				CDS	AAI (%)	Location	CDS	AAI (%)	Location	CDS	AAI (%)	Location	
Phenol hydroxylase													
phenol-hydroxylase large subunit	PheA1	*G. stearo-thermophilus* (plasmid)	AAZ76885.1	CDS 1706	82	C:1547172-1548375				CDS 1101	82	C:926970-925768	Omokoko, *et al.* 2008
phenol-hydroxylase small subunit	PheA2		AAZ76886.1	CDS 676	66	C:650917-651396				CDS 2101	66	C:1791861-1791382	
Meta-cleavage													
2-hydroxymuconate semialdehyde dehydrogenase	HMSD	*B. lichen-iformis* DSM 13	AAU42807.1	CDS 871	73	C:818278-819720	CDS 2209	45	S1:1895287-1896771	CDS 1236	45	C:1035893-1037377	Veith, *et al.* 2004
										CDS 1922	75	C: 1641602-1640133	
										CDS 3417	44	C:3010838-3012310	
2-hydroxymuconate semialdehyde hydrolase	HMSH	*T. dichoto-micum* DSM 44778	SFJ02046.1	CDS 1712	72	C:1551699-1552517				CDS 1031	72	C:877598-876780	Li, *et al.* 2019
catechol-2,3-dioxygenase	PheB	*G. stearo-thermophilus* (plasmid) DSM 6285 pGG01	AAZ76884.1	CDS 1705	88	C:1546002-1546916				CDS 1102	88	C:928142-927228	Omokoko, *et al.* 2008
				CDS 678	70	C:652071-652991				CDS 2099	70	C:1790707-1789787	
4-oxalocrotonate decarboxylase	PheC		ACA01540.1	CDS 876	69	C:824169-824960	CDS 2879	69	S1:2471468-2470677	CDS 1918	69	C:1637396-1636605	
2-hydroxypenta-2,4-dienoate-hydratase	PheD		AAZ76889.2	CDS 873	68	C:821443-822228	CDS 2882	68	S1:2474196-2473411	CDS 1921	70	C:1640122-1639337	

4-hydroxy-2-oxovalerate aldolase	PheE		ACA01539.1	CDS 875	67	C:823146-824156	CDS 2880	66	S1:2472791-2471481	CDS 1919	66	C:1638419-1637409	
acetaldehyde dehydrogenase	PheF		ACA01538.1	CDS 874	72	C:822248-823132	CDS 2881	45	S1:2473390-2472812	CDS 1920	71	C:1639317-1638433	
4-oxalocrotonate tautomerase	PheH		ACA01537.1	CDS 877	34	C:824980-825168	CDS 2878	34	S1:2470658-2470470	CDS 1917	34	C:1636586-1636398	
putative regulator	PheR		AAZ76888.1	CDS 1709	73	C:1548872-1550692				CDS 1033	48	C:879876-878611	
carboxylesterase	PheX		ACA01541.1	CDS 1711	54	C:1550956-1551435				CDS 1032	75	C:878343-877654	
ferredoxin	fdx		AAZ76887.1							CDS 1100	53	C:925569-925267	
Ortho- cleavage													
catechol 1,2-dioxygenase	CatA	*A. pittii* PHEA-2	ADY81911.1										Zhan, *et al*. 2011
muconate cycloisomerase	CatB		ADY81909.1										
muconolactone delta-isomerase	CatC		ADY81910.1										
beta-ketoadipyl CoA thiolase	CatF		ADY81914.1	CDS 2038	39	C:1853009-1851822	CDS 816	49	S1:695456-696634	CDS 3476	41	C:3070845-3072023	
				CDS 3010	41	C:2720504-2719326				CDS 668	39	C:557698-558885	
										CDS 985	39	C:835841-834666	
3-oxoadipate CoA-transferase subunit A	CatI		ADY81912.1	CDS 805	48	C:767993-768682	CDS 2948	48	S1:2523223-2522534	CDS 1977	47	C:1687312-1686623	
3-oxoadipate CoA-transferase subunit B	CatJ		ADY81913.1	CDS 806	48	C:768721-769395	CDS 2947	48	S1:2522495-2521821	CDS 1976	48	C:1686584-1685910	

3-oxoadipate enol-lactonase	CatD		ADY81915.1										
Hydroquinone/ hydroxyquinol													
hydroxyquinol 1,2-dioxygenase	1,2-Hqd	*S. cloacae* JCM 10874^{T} plasmids pSCLO_5 and pSCLO_6	BAV66899.1										Ootsuka, *et al*. 2015; Ootsuka, *et al*. 2018
hydroquinone dioxygenase small subunit	HqdA		BAV66976.1										
putative AraC-type transcription regulator	HqdR		BAV66975.1										
hydroquinone dioxygenase large subunit	HqdB		BAV66977.1										
4-hydroxymuconic semialdehyde dehydrogenase	HqdC		BAV66980.1	CDS 1590	46	C:1451974-1450490	CDS 2209	46	S1:1895287-1896771	CDS 1236	43	C:1035893-1037377	
				CDS 3070	43	C:2780409-2778937				CDS 1922	40	C:1641602-1640133	
				CDS 871	40	C:818278-819720				CDS 3417	46	C:3010838-3012310	
maleylacetate reductase	HqdD		BAV66981.1										
intradiol 1,2-dioxygenase	HqdE		BAV66982.1										
putative ferredoxin	HqdF		BAV66983.1										

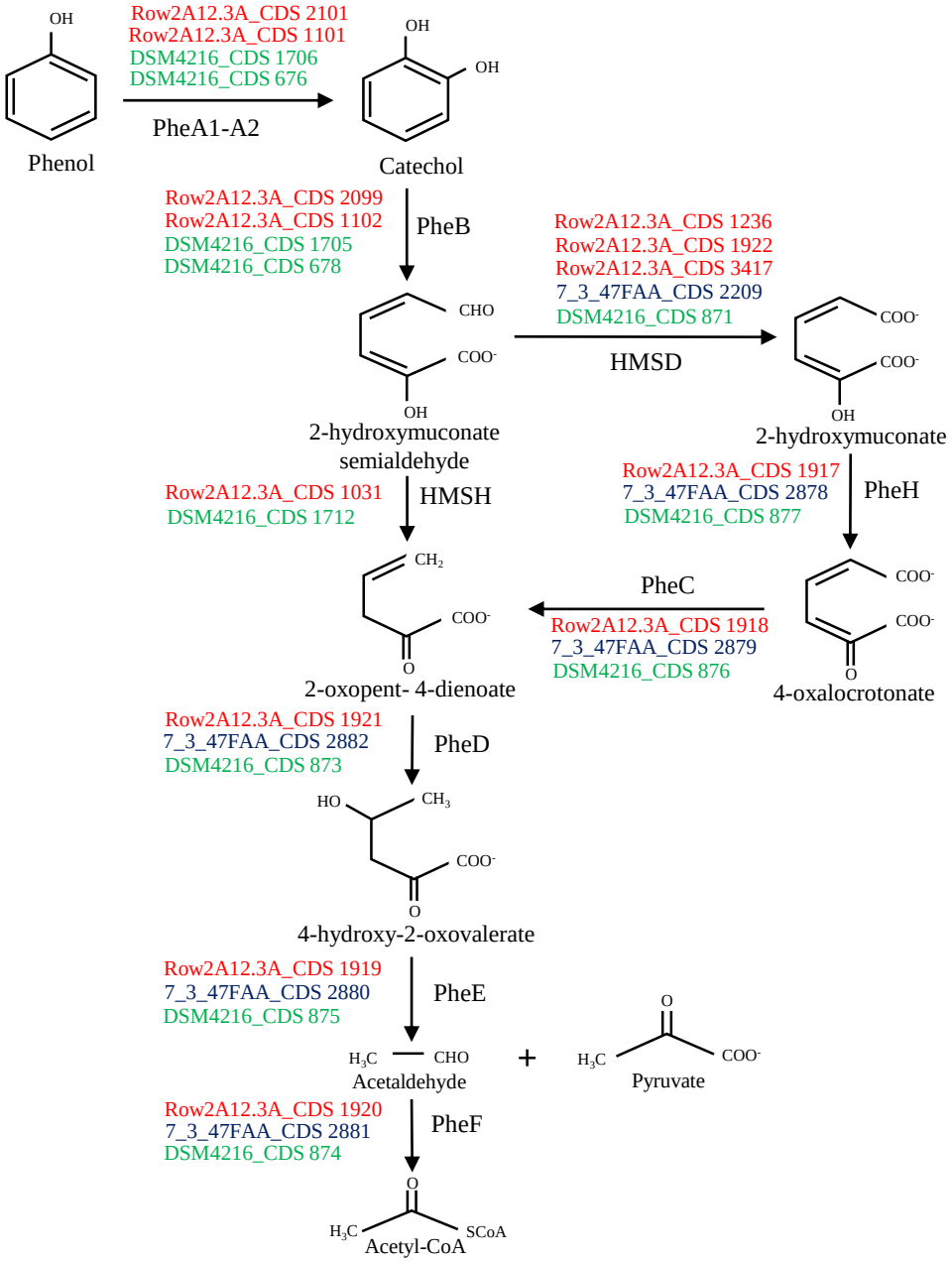


Figure 3.7: Phenol degradative pathway via *meta*-cleavage of catechol. Phenol is first transformed to catechol via the activity of a phenol hydroxylase (PheA1-A2). Catechol then enters the *meta*-cleavage pathway and is metabolised via the sequential activity of catechol-2,3-dioxygenase (PheB); 2-hydroxymuconate semialdehyde dehydrogenase (HMSD); 4-oxalocrotonate tautomerase (PheH); 4-oxalocrotonate decarboxylase (PheC); 2-hydroxymuconate semialdehyde hydrolase (HMSH); 2-oxopent-4-dienoate hydratase (PheD); 4-hydroxy-2-oxovalerate aldolase (PheE); acetaldehyde dehydrogenase (PheF). The corresponding locus tags for the CDSs of each strain are shown alongside each protein.

The genome of *B. smithii* 7_3_47FAA encodes six of the thirteen proteins. These proteins share 60% average amino acid identity (AAI) with their *G. stearothermophilus* DSM 6285 and *B. licheniformis* DSM 13 (HMSD) counterparts. When compared to *B. smithii* strains DSM 4216^T and Row2A12.3A, the corresponding AAI values were 90%. The proteins are all associated with the HMSD branch of the *meta*-cleavage pathway. A single gene cluster*, pheHCEFD*, was found with a gene coding for an HMSD orthologue alongside *pheD* (Figure 3.8). As no orthologues of both phenol hydroxylase components (PheA1 and PheA2) nor the catechol-2,3-dioxygenase (PheB) it can be assumed that this strain lacks the complete pathway required for complete phenol mineralisation. Furthermore, the reading frame of the *hmsD* genes appears to be disrupted, resulting in an *hmsD* pseudo-gene, suggesting that the encoded protein is also non-functional.

The genome of *B. smithii* DSM 4216^T incorporates three distinct gene clusters coding for twelve orthologues of the thirteen *meta*-cleavage proteins. The proteins share an AAI value of 71% with their counterparts in *G. stearothermophilus* pGGO1, *B. licheniformis* DSM 13 (HMSD) and *T. dichotomicum* DSM 44778 (HMSH), while they share 94% average amino acid identity with those orthologues encoded on the genome of *B. smithii* Row2A12.3A was 94%. The gene clusters comprise of *hmsH-pheXRA1B* (6515bp), *pheHCEFD- hmsD* (6890bp) and *pheBA*2 (2074bp) (Figure 3.8). These clusters are separated from each other by ±721kb (cluster 1 to cluster 2) and ±165kb (cluster 2 to cluster 3) nucleotides, respectively. An orthologue for the ferredoxin could not be identified on the genome sequence. However, all other primary proteins for phenol degradation via the *meta*-cleavage pathway are accounted for, and thus this pathway is likely to function in *B. smithii* DSM 4216^T.

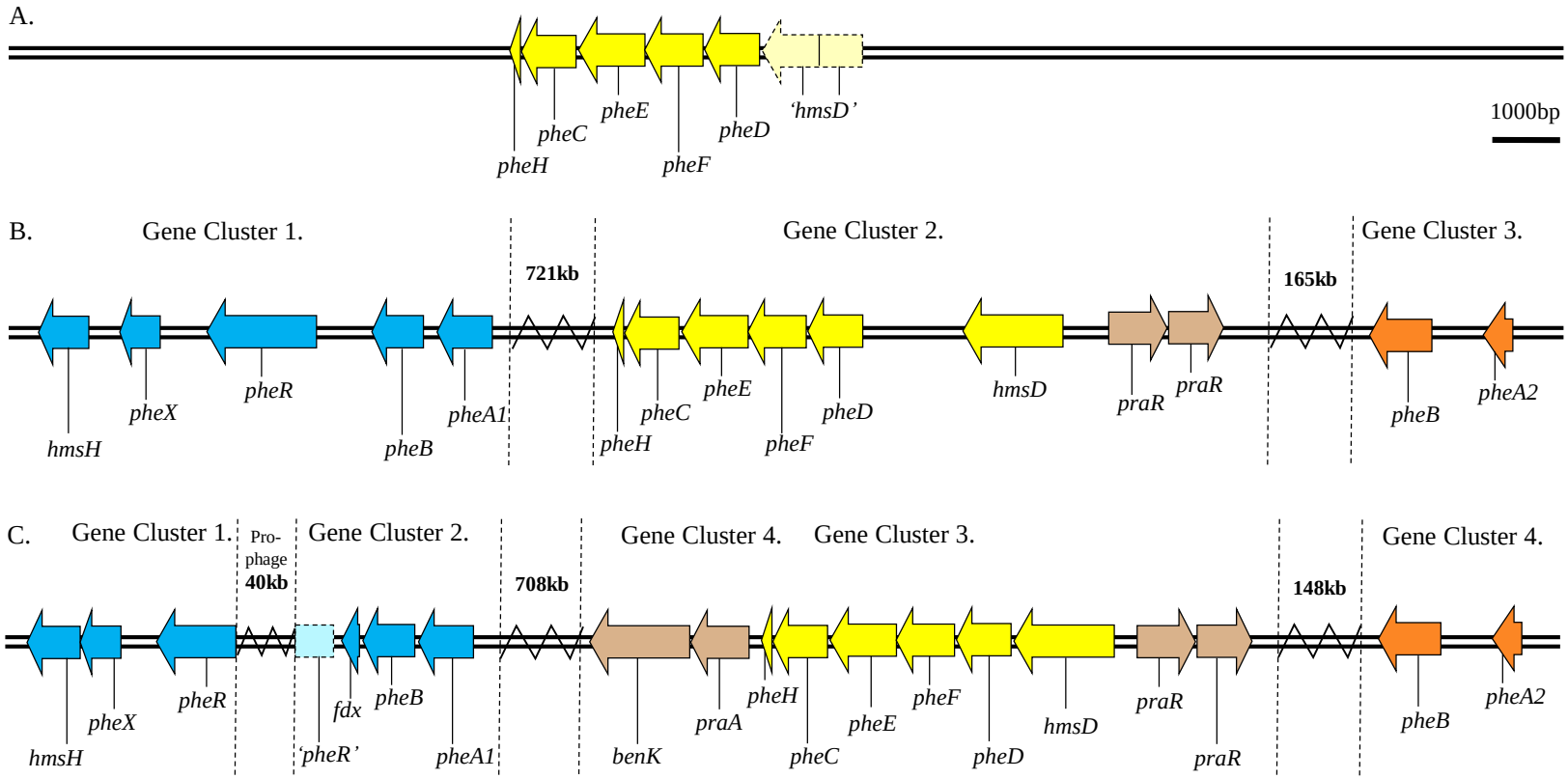


Figure 3.8: Gene cluster(s) encoding the *meta*-cleavage phenol degradation pathway in *B. smithii* 7_3_47FAA (A), DSM 4216^{T} (B) and Row2A12.3A (C). Arrows with partial opacity and dotted boarders are used for '*hmsD'* in *B. smithii* 7_3_47FAA and *'pheR' B. smithii* Row2A12.3A as the orthologous coding sequence is split.

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The genome of the phenol degrading strain *B. smithii* Row2A12.3A encodes orthologues for all thirteen proteins of the *meta*-cleavage pathway. Unlike with *B. smithii* DSM 4216^T, the genes encoding these proteins on the Row2A12.3A genome are contained in four spatially separated gene clusters (Figure 3.8). These four gene clusters are separated from each other by ±45.4kb (cluster 1 to cluster 2) ±708kb (cluster 2 to cluster 3) and ±148kb (cluster 3 to cluster 4) nucleotides, respectively. The total AAI of the orthologous sequences when compared to the established *meta*-cleavage pathway was 64%. The orthologous sequences were dispersed across the genome in the following clusters *hmsH – pheX* (pheR split), (pheR split)-*fdx-pheA1B*, *pheHCEFD- hmsD* (AAI 69%) and *pheBA*2 (AAI 68%). The other *meta*-cleavage pathway gene clusters in *B. smithii* ROW2A12.3A, Cluster 3 (*pheHCEFD- hmsD*) and Cluster 4 (*pheBA2*) strongly resemble Cluster 2 and Cluster 3 in *B. smithii* DSM 4216^T, respectively and the proteins encoded on all the gene clusters also share extensive sequence identity (94%), suggesting they share a common ancestry.

Analysis of the RAST annotation for the proteins indicates that the 45.4 kb region between gene cluster 1 and cluster 2 is a prophage element, which has likely integrated into the complete cluster. Comparison of this region against the PHASTER server identified this prophage element as PHAGE Lister B054 (NC_009913.1) (Zhou, *et al*. 2011; Arndt, *et al*. 2016). This prophage element codes for 59 proteins over a 40.4kb region, 879876 – 925569bp, with a GC content of 40.4%. Analysis of the integration site shows that it has integrated within the reading frame of *pheR*, which codes for the transcriptional regulator PheR. Cluster 1 thus incorporates the genes *hmsH-pheX-pheR* (partial) and is 3096 bp in size. Cluster 2 incorporates *pheR* (partial) – *fdx* – *pheA1B* and thus resembles gene cluster 3 in *B. smithii* DSM 4216^T. However, Cluster 2 in *B. smithii* Row2A12.3A also incorporates a gene coding for an orthologue of Fdx, which is absent from Cluster 1 in the other strain. While DSM 4216^T does show two BlastP hits for *fdx* in the same region as gene cluster 1 and 3 neither the AAI, bit score nor the E-values were adequate for these sequences to be classed as orthologues of *fdx*.

The absence of PheR, an AraC/XylS type regulator, has been shown to result in the internalisation and bioaccumulation of phenol as opposed to its degradation in *Corynebacterium glutamicum* (Chen, *et al*. 2018). The evidence from the 4-AAP degradation assay shows that phenol degradation still occurs despite the disruption of the PheR orthologue. It is also suspected that the putative PheR regulator may act on genes other than the phenol degrading genes and may contribute to differing capacities for the degradation of various phenols as well as resulting in co-metabolism (Min, *et al*. 2014; Nzila, 2018). In addition to the four gene clusters two *hmsD* orthologues were identified elsewhere on the genome of *B. smithii* Row2A12.3A. These share lower AAI values (44 and 45%) than that of the one in Cluster 3 of *B. smithii* Row2A12.3A (Table 3.3). As it possesses orthologues for the entire complement of the *meta*-cleavage pathway it is likely that *B. smithii* Row2A12.3A employs this route for phenol degradation.

The genes flanking the *meta*-cleavage orthologues may provide insight and context for the activation of these gene clusters. The *pheHCEFD-hmsD* gene clusters in *B. smithii* Row2A12.3A and DSM 4216^{T} are flanked upstream by two isocitrate lyase regulator-type (IclR) family transcriptional regulators. These regulators exhibit control over a diverse range of functions, notably carbon metabolism and the degradation of aromatic compounds (Krell, *et al*. 2006). Two orthologues of the IclR type transcriptional regulator PraR (BAH79098.1; *Paenibacillus* sp. JJ-1b; Kasai, *et al*. 2009), involved in regulating protocatechuate metabolism, were identified in each strain displaying AAI values of between 40% and 42% among the *B. smithii* strains. As the cleavage of protocatechuate in *Paenibacillus* sp. JJ-1b employs orthologues of the proteins identified as PheHCEFD- HMSD it is plausible that the PraR orthologues identified in the *B. smithii* strains similarly regulate this portion of the *meta*-cleavage pathway (Kasai, *et al*. 2009).

As phenol degradation genes frequently cluster, it is common for there to be a single regulator for the operon (Omokoko, *et al*. 2008). However, as the gene set may be fragmented across a genome, two possibilities for cluster regulation have been observed. Clusters may be regulated by either individual regulation of each cluster by an adjacent regulator or the regulation of several clusters by a distant regulator, forming a regulon (Cai & Xun. 2002; Nešvera, *et al*. 2015). The transcriptional

regulation of these gene clusters appears to be split with PheR, an ArC/XylS regulator, orthologues regulating the *pheBA1RX-hmsH* gene clusters of *B. smithii* DSM 4216^{T} and Row2A12.3A. The *pheHCEFD- hmsD* gene cluster, found in all three strains is likely regulated by two IclR type regulators orthologous for *praR* involved in protocatechuate degradation. The phenol degrading gene clusters on the genome of *B. smithii* Row2A12.3A are flanked by several other notable genes which were not identified in *B. smithii* DSM 4216^{T} or 7_3_47FAA. These flanking sequences encode orthologues of the protocatechuate degradation pathway and the major facilitator superfamily (MFS) type transporter for benzoate, BenK.

Like many peripheral aromatic carbon degradation pathways, protocatechuate and phenol metabolism coincides with the production of (5-carboxy-)2-hydroxymuconate-6-semialdehyde (5CHMS) which can then be metabolised by (5C) HMSD along the 4-oxalocrotonate route of the *meta*- cleavage pathway for catechol (Kasai, *et al*. 2009; Nešvera, *et al*. 2015). The genome of *B. smithii* Row2A12.3A incorporates genes coding for all the required orthologues for this portion of the *meta*-cleavage pathway as well as an orthologue for protocatechuate 2,3-dioxygenase (PraA) which allows for the conversion of protocatechuate to 5CHMS (Kasai, *et al*. 2009). The PraA orthologue in *B. smithii* Row2A12.3A shares 59% amino acid identity with that of *Paenibacillus* sp. JJ-1b (BAH79099.1; Kasai, *et al*. 2009) and the gene coding for this protein is localised adjacent to the gene coding for PheH. Previous research on *Rhodococcus jostii* RHA1 indicates that alternate phenolics such as biphenyl and polychlorinated biphenyls are metabolised through the protocatechuate route (Gonçalves, *et al*. 2006). Taken together it is likely that *B. smithii* Row2A12.3A will employ this portion of the identified *meta*-cleavage pathway for the degradation of phenol, protocatechuate and other potential phenolics.

MFS transporters are capable of transporting a diverse range of low molecular weight solutes, in bacteria they are primarily used for nutrient up-take and toxin expulsion (Quistgaard, *et al*. 2016). The substrate specificity of MFS transporters is similarly diverse with a single MFS transporter capable of interacting with a range of structurally similar solutes, as is seen in the transportation of aromatic compounds (Paulsen, *et al*. 1998; Michalska, *et al*. 2012). A gene coding for a

predicted MFS was transporter is found adjacent to *praA* and hence Cluster 3 on the genome of *B. smithii* Row2A12.3A. The encoded protein shares 40% AAI with the MFS transporter BenK of *A. pittii* PHEA-2 (ABS81304; Zhan *et al*. 2008) There is evidence that the BenK transporter is orthologous to PcaK and is capable of protocatechuate and 4-hydroxybenzoate transportation (Collier, *et al*. 1997; Kasai, *et al*. 2009). Given the broad substrate specificity, the orthology between benzoate and protocatechuate MFS transporters, the structural similarity of phenol to these compounds and the genomic location of the gene coding for this MFS transporter in *B. smithii* Row2A12.3A, it is possible that this protein plays a role in the transportation of phenol across the cell membrane.

3.4 Conclusion

In this study, the genome of the phenol-degrading *B. smithii* Row2A12.3A was sequenced and fully assembled to a complete circular chromosome and plasmid sequence. The genome was compared to the other two available *B. smithii* genomes, the complete genome of type strain DSM 4216^{T} and the high-quality draft genome *B. smithii* 7_3_47FAA. Comparative genomics indicated that overall, the genomes are highly similar with respect to genome size, GC content and the number of proteins they encode. Several differences between the strains emerged with COG class identification and RAST subsystem annotation of the proteins encoded on the genomes, where the proportions of unique and shared proteins with roles in carbohydrate metabolism were greater for *B. smithii* DSM 4216^{T} and Row2A12.3A than are encoded on the genome *B. smithii* 7_3_47FAA. The SEEDViewer results also indicated a larger number of aromatic degradation associated proteins are encoded on the genome of *B. smithii* Row2A12.3A than those of the other two strains.

tBlastN and BlastP comparisons with protein sequences from known phenol degrading pathways against the genomes, demonstrated that all three *B. smithii* strains encode orthologues of proteins involved in the *meta*- cleavage phenol degradation pathway. While *B. smithii* 7_3_47FAA did not contain the entire complement of proteins required for *meta*-cleavage of phenol both DSM 4216^{T} and Row2A12.3A encoded either twelve or thirteen orthologues, respectively. This

represents the first members of this species for which such tolerance and degradative capacities have been described and the molecular mechanism has been identified.

The genes encoding the orthologues for this pathway in *B. smithii* DSM 4216^{T} and Row2A12.3A do not appear as a single operon but rather as three or four gene clusters, respectively. However, this should show no encumbrance on the activity of this pathway, as fragmental pathways for phenol degradation via *meta*-cleavage have been previously identified (Suenaga, *et al*. 2009; Min, *et al*. 2014). The presence of orthologues for the *meta*-cleavage pathway for phenol degradation is to be expected when considering thermophilic phenol degradation as the *meta*-pathway appears universally preferred. (Mohammadi, *et al*. 2015; Zhou, *et al*. 2016; Nzila, 2018). In addition, the *pheHCEFD- hmsD* gene cluster of *B. smithii* Row2A12.3A is flanked by an orthologue of protocatechuate 2,3-dioxygenase, a key enzyme in protocatechuate degradation, as well as MFS transporter BenK which has similarly been shown to be involved in protocatechuate metabolism. It is thus plausible that *B. smithii* Row2A12.3A has evolved a complexed system for the degradation of a range of aromatic substrates.

Elucidation of the phenol degradation pathway provides insight into the genes and regulatory mechanisms that permit phenol degradation in *B. smithii* Row2A12.3A. The deeper understanding of these factors, as garnered from this study, indicate the best approaches for future system design, gene manipulation and potential co-metabolism of aromatic pollutants.

Summary

Phenols are frequent by-products of a range of agricultural and industrial applications and are recalcitrant environmental pollutants. Hence their environmental removal is an active area of research. Microbial biodegradation is a key avenue of research as microorganisms can completely mineralise phenols. Thermophilic aerobic bacteria represent promising candidates for phenol bioremediation, due to their inherent resistance to toxic compounds and their high reaction rates. Here we have screened an extensive collection of thermophilic aerobic bacteria for their tolerance to high concentrations of and effectively degrade phenols. A number of thermophilic taxa were able to tolerate high concentrations of phenol. One strain in particular, *Bacillus smithii* Row2A12.3A showed the consistent capacity for growth using phenol as a sole carbon source with comparatively high generation of biomass, in phenol concentrations of up to 1,200 mg/l. This phenol concentration is considerably higher than that of most phenol-containing wastewaters. Furthermore, this strain demonstrated a noteworthy phenol (400 mg/l) reduction of 74% within 48 hours.

To further characterise the molecular basis underlying phenol tolerance and degradation, the genome of *B. smithii* Row2A12.3A was sequenced, assembled and compared to those of other *B. smithii* strains for which genomes are available. These analyses indicated the presence of genes coding for the complete *meta*-cleavage pathway for phenol degradation.

The results garnered from this study suggest that *B. smithii* Row2A12.3A for incorporation in industrial phenol bioremediation strategies. However, several avenues of further research are recommended. Firstly, optimisation of operating conditions (e.g. medium composition and pH, temperature) can lead to both better growth and phenol degradation by *B. smithii* Row2A12.3A. Increased reaction rates and phenol tolerance may furthermore be achieved simultaneously through extended periods of acclimatisation with small incremental increases in phenol concentrations and through cell immobilisation or aggregation. Once a well-adapted and suitably acclimatised strain of *B. smithii* Row2A12.3A is generated the next phase would be lab-scale bioreactor testing. Furthermore, while this strain

showed the capacity to tolerate and degrade phenol itself, it is necessary to determine of it is able to tolerate and degrade phenol derivatives such as chloro- and nitro-phenols. Furthermore, it is advised that Gas Chromatography- Mass Spectrometry (GC-MS) or High-Performance Liquid Chromatography (HPLC) analyses be undertaken, to ensure complete mineralisation of phenolic compounds without the production of other undesirable metabolic intermediates.

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