GENOMIC AND TAXONOMIC STUDIES OF AN ANTARCTIC PSYCHROTOLERANT QUORUM QUENCHING BACTERIUM, *Planococcus versutus* L10.15^{T}

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GENOMIC AND TAXONOMIC STUDIES OF AN ANTARCTIC PSYCHROTOLERANT QUORUM QUENCHING BACTERIUM, *Planococcus versutus* L10.15^{T}

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GENOMIC AND TAXONOMIC STUDIES OF A ANTARCTIC PSYCHROTOLERANT QUORUM QUENCHING BACTERIUM, *Planococcus versutus* L10.15^T

ABSTRACT

Quorum quenching (QQ) has been suggested as an alternative to antibiotic treatments for pathogenic infections as it does not affect the viability of the pathogen and imposes minimum selection pressure promoting drug resistance. During an ecological survey for quorum quenching (QQ) bacteria from soil collected in Lagoon Island (Ryder Bay, Adelaide Island, Antarctica), a bacterial strain was isolated and identified as *Planococcus* species, L10.15^T, based on 16S rRNA gene analysis. The genomic, phylogenetic, chemotaxonomic and phenotypic data obtained showed that L10.15^T represents a novel species for which the name *Planococcus versutus* sp. nov. is proposed. Previously, QQ activity of L10.15^T was studied, and it was confirmed to be capable of inactivating synthetic *N*-acyl homoserine lactones (AHLs) with acyl side chain lengths C_4-C_{12}, and active at a temperature as low as 4 °C. In this study, the gene responsible for the QQ activity of *P. versutus* L10.15^T was identified and confirmed for its function in a gene expression study. The cold-active characteristics of the enzyme coded by this gene suggested that it belonged to a novel class of *N*-acyl homoserine lactonase, and we therefore term the gene as 'autoinducer degrading gene from *Planococcus* sp.' (*aidP*). The *aidP* gene was then cloned, and its gene product AidP enzyme, which is a novel class of AHL lactonase from the metallo-ß-lactamase superfamily gene, was characterized. Multiple sequence alignment analysis and amino acid composition suggested that the *aidP* gene encoded a cold-adapted enzyme. Interestingly, the *aidP* gene has only been detected in *Planococcus* spp. that have been isolated from Antarctica. Therefore, branch-site analysis was conducted on several *Planococcus* spp. branches within the phylogenetic tree of homologous gene, showing evidence of episodic positive selection for the *aidP*

gene in cold environments. The gene showed homology to several metallo-β-lactamase proteins obtained from Bacteroidetes but not to any known AHL-degrading enzymes. Liquid chromotography-mass spectrometry analysis confirmed that AidP functions as an AHL-lactonase that hydrolyzes the ester bond of the homoserine lactone ring of AHLs. Furthermore, the effects of covalent and ionic bonding were demonstrated and the results indicate that Zn^{2+} is important for AidP activity *in vivo*. A pectinolytic inhibition assay using Chinese cabbage confirmed that this enzyme is a potential anti-quorum sensing agent. A phylogenomic study was also carried out for members of the family *Planococcaceae*. The results identified a misclassification of the genus *Planomicrobium,* which should be reclassified to *Planococcus.* The core genomic study revealed an essential set of survival genes shared across *Planococcaceae*. A positive selection assessment conducted on the 352 core genes identified 50 genes to be under high selection pressure in cold-adapted *Planococcus* spp. Finally, cold-adapted traits were identified in *Planococcus* spp., revealing information about cold-adaptation strategies in cold habitats.

Keywords: Genomic, taxonomic, Antarctica, bacteria.

KAJIAN GENOMIK DAN TAKSONOMI MENGENAI *Planococcus versutus* L10.15^T, BAKTERIA PSIKROTOLERAN DENGAN KEBOLEHAN QUORUM QUENCHING DARI ANTARTIKA

ABSTRAK

Quorum quenching (QQ) telah terbukti berpotensi sebagai rawatan alternative antibiotik untuk patogen, kerana QQ tidak mempengaruhi daya tahan patogen dan oleh itu mempunyai tekanan pemilihan minimum pada rintangan. Semasa kajian ekologi bakteria quorum quenching (QQ) di Lagoon Island (Ryder Bay, Adelaide Island, Antartika), kami telah mengasingkan spesies *Planococcus* baru, L10.15^T, mengikut analisis gen 'housekeeping' dan gen rRNA 16S. Data genomik, phylogenetic, chemotaxonomic dan phenotypic yang diperoleh menunjukkan bahawa L10.15^T mewakili spesies baru yang mana nama *Planococcus versutus* sp. nov. telah dicadangkan. Dalam kajian ini, kita juga mencirikan aktiviti QQ bagi L10.15^T, yang mampu menyahaktifkan lakton homoserine N-acyl sintetik (AHL) sintetik dengan panjang rantai sisi acil C_4-C_{12}, dan berfungsi pada suhu serendah 4 °C. Gen yang bertanggungjawab untuk aktiviti QQ *P. versutus* L10.15^T telah dikenal pasti dan disahkan untuk fungsinya dalam kajian ekspresi gen. Ciri aktif enzim enzim yang dikodkan oleh gen ini mencadangkan bahawa ia tergolong dalam kelas baru lactonase homoserine N-acyl, dan oleh karenanya kita menggambarkan gen sebagai 'autoinducer degrading gene from *Planococcus* sp.' (*aidP*). Kami telah mengenal pasti gen *aidP dari Planococcus* sp. diklonkan, and enzim telah diasingkan, dan dibuktikan bertindak sebagai AHL-lactonase. Enzim AidP ini, dari kelas baru AHLs lactonase adalah ahli gen superfamily ß-metallo-lactamese. Analisis keselarasan pelbagai urutan dan komposisi asid amino menunjukkan bahawa gen ini mengkodekan enzim yang disesuaikan dengan sejuk. Menariknya, gen *aidP* hanya dikenalpasti dalam *Planococcus* sp. yang terpencil atau dikenal pasti di Antartika. Kami selanjutnya menjalankan analisis tapak cawangan di beberapa *Planococcus* sp. cawangan pada pokok gen dan hasil kami

menunjukkan bukti pemilihan positif episod gen bantuan di persekitaran yang sejuk. Gen ini menunjukkan homologi kepada beberapa protein metallo-β-laktamase dari Bacteroidetes, walaupun gen-gen tersebut tidak dikenali sebagai enzim AHL lactonase yang berfungsi. Analisis kromotografi-cecair spektrometri cecair mengesahkan bahawa AidP berfungsi sebagai AHL-laktonase yang menghidrolisis ikatan ester cincin lakton homoserine AHLs. Tambahan pula, kami menunjukkan kesan ikatan kovalen dan ionik, menunjukkan bahawa Zn^{2+} adalah penting untuk aktiviti AidP dalam vivo. Pengaruh pektinolitik yang menggunakan kubis membuktikan bahawa enzim ini adalah reagen pendengaran anti-kuorum yang berpotensi. Kajian phylogenomic telah dijalankan untuk *Planococcaceae*, keluarga bakteria di mana *P. versutus* L10.15^{T} diklasifikasikan. Hasilnya mengenalpasti genus *Planomicrobium* telah salah dikelaskan, yang mana harus dikelaskan kepada genus *Planococcus*. Kajian genomik teras menunjukkan set penting gen survival untuk *Planococcaceae*, dan penilaian pemilihan positif telah dijalankan 352 gen teras, 50 gen telah dikenal pasti di bawah tekanan pemilihan yang tinggi pada *Planococcus* spp. yang disesuaikan dengan sejuk. Akhirnya, ciri-ciri sejuk yang dikenal pasti di spesies *Planococcus*, mendedahkan strategi penyesuaian sejuk dalam habitat sejuk.

Kata kunci: Genomik, taksonomi, Antartika, bakteria.

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LIST OF SYMBOLS AND ABBREVIATIONS

%T	:	% Transmittance
A	:	Adenine
AT	:	Adenine-thymine
Ala	:	Alanine
≈	:	Almost equal to
AL	:	Aminolipids
Arg	:	Arginine
Asn	:	Asparagine
Asp	:	Aspartic acid
bp	:	Base pair(s)
β	:	Beta
Ca^{2+}	:	Calcium, ion
χ^2	:	Chi-square
Co^{2+}	:	Cobalt (II), ion
Cu^{2+}	:	Copper, ion
Cys	:	Cysteine
C	:	Cytosine
°	:	Degree
°C	:	Degree Celsius
H_2O	:	Dihydrogen oxide
Fe^{2+}	:	Ferrous, ion
Glu	:	Glutamic acid
Gln	:	Glutamine
Gly	:	Glycine

g	:	Gram(s)
≥	:	Greater than or equal to
G	:	Guanine
GC	:	Guanine-cytosine
His	:	Histidine
HCL	:	Hydrochloric acid
IlE	:	Isoleucine
kb	:	Kilobase(s)
kDa	:	Kilodalton(s)
<	:	Less than
Leu	:	Leucine
L	:	Litre
Lys	:	Lysine
Mg^{2+}	:	Magnesium, ion
Mn^{2+}	:	Manganese, ion
m/z	:	Mass-to-charge ratio
Mb	:	Megabase(s)
Met	:	Methionine
m	:	Metre
μ	:	Micro
μL	:	Microlitre
μm	:	Micrometer
μM	:	Micromolar
mL	:	Mililitre
mm	:	Milimetre
mM	:	Milimolar

M	:	Molar
>	:	More than
nL	:	Nanolitre
nm	:	Nanometer
nM	:	Nanomolar
Ni-NTA	:	Nickel-nitrilotriacetic acid
#	:	Number of
PON	:	Paraoxonase
Pa	:	Pascal
%	:	Percent
Phe	:	Phenylalanine
His_6	:	Polyhistidine-tag
′	:	Prime
Pro	:	Proline
M-H	:	Quasimolecule
A260/230	:	Ratio of absorbance at wavelength of 260 nm and 230 nm
A260/280	:	Ratio of absorbance at wavelength of 260 nm and 280 nm
®	:	Registered trademark
N50	:	Sequence length at 50% of the total genome length
Ser	:	Serine
NaCl	:	Sodium choride
NaOH	:	Sodium hydroxide
Na_2SO_4	:	Sodium sulfate
g	:	Standard Gravity (relative centrifugal force)
S	:	Svedberg
Thr	:	Threonine

T	:	Thymine
×	:	Times
™	:	Trademark symbol
Trp	:	Tryptophan
Tyr	:	Tyrosine
Val	:	Valine
v/v	:	Volume per volume
w/v	:	Weight per volume
Zn^{2+}	:	Zinc, ion
MOPS	:	3-(*N*-morpholino)propanesulfonic acid
MES	:	2-(*N*-morpholino)ethanesulfonic acid
AP	:	Alkaline phosphatase
AA	:	Amino acid
APL	:	Aminophospholipid
AAI	:	Average amino acid identity
ANI	:	Average nucleotide identity
BEB	:	Bayes empirical Bayes method
BAS	:	British Antarctic Survey
COG	:	Clusters of orthologous groups
d	:	Day(s)
DNA	:	Deoxyribo nucleic acid
DPG	:	Diphosphatidylglycerol
DTT	:	Dithiothreitol
DDH	:	DNA–DNA hybridization
ESI-MS	:	Electrospray ionization-mass spectorometry
EDTA	:	Ethylenediaminetetraacetic acid

FA	:	Fatty acid
FAME	:	Fatty acid methyl esters
GCs	:	Gene clusters
gDNA	:	Genomic DNA
GEBA	:	Genomic Encyclopedia of Bacteria and Archaea
HMM	:	Hidden Markov model
HGAP	:	Hierarchical genome assembly process
HPLC	:	High-performance liquid chromatography
HGT	:	Horizontal gene transfer
h	:	Hour(s)
IPTG	:	Isopropyl *β*-D-1-thiogalactopyranoside
LUCA	:	Last universal common ancestor
LGT	:	Lateral gene transfer
LRT	:	Likelihood ratio test
LC/MS	:	Liquid chromatography–mass spectrometry
LSPN	:	List of Prokaryotic with Standing in Nomenclature
LBA	:	Luria-Bertani Agar
MAGIS	:	Mapping and Geographic Information Centre of BAS
MA	:	Marine agar
MCL	:	Markov cluster algorithm
ML	:	Maximum likelihood algorithm
MBR	:	Membrane bioreactors
min	:	Minute(s)
MLSA	:	Multilocus sequence analysis
MLST	:	Multilocus sequence typing
AHLs	:	*N*-acyl homoserine lactones

NCBI	:	National Center for Biotechnology Information
CDD	:	NCBI conserved domain database
NJ	:	Neighbor-joining algorithm
NADP	:	Nicotinamide adenine dinucleotide phosphate
NADPH	:	Nicotinamide adenine dinucleotide phosphate hydrogen
sp. nov.	:	Novel species
ORF	:	Open reading frames
OGs	:	Orthologous groups
PacBio	:	Pacific Biosciences
POCP	:	Percentage of conserved protein
PBS	:	Phosphate buffered saline
PE	:	Phosphatidylethanolamine
PG	:	Phosphatidylglycerol
CDS	:	Predicted coding sequences
PGAP	:	Prokaryotic genome annotation pipeline
PFGE	:	Pulsed-field gel electrophoresis
QQ	:	Quorum quenching
QS	:	Quorum sensing
RAPD	:	Randomly amplified polymorphic DNA
RAST	:	Rapid annotation using subsystem technology
RRLC	:	Rapid resolution liquid chromatography
RFLP	:	Restriction fragment length polymorphism
rt	:	Retention time
RNase	:	Ribonuclease
RNA	:	Ribonucleic acid
rRNA	:	Ribosomal RNA

rpm	:	Round per minute
SEM	:	Scanning electron microscope
STEM	:	Scanning transmission electron microscope
SMRT	:	Single-Molecule Real Time
SSU	:	Small ribosomal subunit
SDS-PAGE	:	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
spp.	:	Species (plural)
sp.	:	Species (singular)
TLC	:	Thin layer chromatography
tRNA	:	Transfer RNA
TBT	:	Tributyltin
TCA	:	Tricarboxylic acid
TBE	:	Tris/Borate/EDTA
UPLC	:	Ultra performance liquid chromatography
WGS	:	Whole genome sequencing

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CHAPTER 1: INTRODUCTION

1.1 Biotechnological potential of psychrophilic bacteria

Over recent decades, psychrophilic/psychrotolerant bacteria are gaining attention of researchers because of their cold adaptation traits that may confer biotechnological potential (Hamdan, 2018). Studies on these bacteria was particularly focused on applications of cold-adapted enzymes identified or isolated from these bacteria in various industries, including pharmaceutical, food production, cosmetic, waste processing, environmental bioremediations, agriculture, biosensor, and molecular diagnostics (Cavicchioli *et al*., 2011).

1.2 Strain selection and strain history

In a microbiological survey of Antarctic quorum quenching (QQ) bacteria (See-Too, 2013), numerous bacteria were isolated that are capable of inactivating *N*-acyl homoserine lactones (AHLs), a type of signaling molecule produced by certain Proteobacteria. Among all the QQ bacteria isolated, one isolate in particular was designated as L10.15, and was selected for further study as (1) it formed a distinct branch in 16S rRNA gene phylogenetic tree from the nearest type strains, and (2) it had high QQ activity at 4 °C, the lowest temperature for bacteria to show QQ activity that has yet been identified.

1.3 Significance of the study

Since L10.15 exhibits high QQ activity at low temperature, rigorous studies are required as the gene encoding the QQ enzyme are required as it may represent a novel QQ enzyme family. The QQ enzyme should be purified for further characterization, as the availability of a cold-adapted QQ enzyme would be beneficial to avoid the selection

pressure on emerging of QQ resistant bacteria. In addition, the potential of the QQ enzyme in field application should be investigated through *in vivo* studies.

Strain L10.15 belongs to the genus *Planococcus*, and a BLASTn search against the NCBI non-redundant nucleotide database indicated that it is closely related to other Planococcus species isolated from polar regions. Even though it shared 99% and higher 16S rRNA gene sequence similarity with known type strains of *Planococcus* species in the EzBiocloud database, strain L10.15 formed a distinct branch within cluster of *Planococcus* type strains in the 16S rRNA gene phylogenetic analysis.

Before this study, QQ activity of *Planococcus* species has not been reported. This study thus focused on strain L10.15 that may represent a novel species, and its QQ activity as there is high potential that strain L10.15 produces a cold-adapted QQ enzyme that has biotechnological application.

1.4 Objectives of the study

The objectives of this study are:

(1)To conduct a taxonomic study of strain L10.15 using a polyphasic approach (chemotaxonomic, phenotypic and genotypic studies).

(2)To conduct a phylogenomic study of strain L10.15 using genomic data from members of the family *Planococcaceae* in order to improve understanding of the adaptation of bacteria in cold environments.

(3)To characterize the QQ enzyme produced by strain L10.15, and to examine the potential of the QQ enzyme *in planta*.

CHAPTER 2: LITERATURE REVIEW

2.1 The post-genomic era

The first complete bacterial genome sequence, of a strain of *Haemophilus influenzae*, was released in 1995 (Fleischmann *et al*., 1995), eight years before the release of the complete human genome (Collin *et al*., 2003), and 20 years after Sanger & Coulson (1975) demonstrated their work in DNA shotgun sequencing, a breakthrough technology that sequenced 80 nucleotides in one go. The *H. influenzae* sequencing project took about a year, and within months the same method was applied to *Mycoplasma genitalium* (Fraser *et al*., 1995). Genomic data of other bacteria were then released, spurring comprehensive studies of bacterial genomes, and altering our understanding of these most ancient organisms on Earth.

In only one decade, the development of second-generation sequencing changed the game by producing sizeable numbers of draft genomes with drastic reductions in running time and cost. The third-generation sequencing, Single-Molecule Real Time (SMRT) sequencing, managed to complete bacterial genome in a few hours, and concurrently provide the possible DNA methylation pattern of the genome (Land *et al*., 2015). The latest genome sequencing technology, the fourth-generation, by Oxford Nanopore Technologies, is a real-time sequencing technique with only a pocket DNA sequencer (Pennisi, 2016). Advances in sequencing techniques and bioinformatics generate massive amounts of genomic information on a daily basis. These genomic data are stimulating new insights into the interrelationships of bacterial populations, revealing that bacterial diversity and the size of the bacterial gene pool are higher than previously thought (Binnewies *et al*., 2006).

Two decades ago, it was difficult to conduct a complex study as there were inadequate genome sequences and computational processing power to handle massive amounts of genomic data. Genomic research has now shifted to consortia-based projects (Reuter *et al*., 2015). Large-scale genomic projects have provided vast amounts of open-access genomic information that would be difficult to achieve in an individual lab. The Genomic Encyclopedia of Bacteria and Archaea (GEBA) project has sequenced more than 1000 type strains (Mukherjee *et al*., 2017; Whitman *et al*., 2015), with the aims to extend the coverage of the tree of life and expand our understanding on the functional and evolutionary diversity of microbial life.

Genomic data also allow us to carry out extensive analyses on genome diversity or dynamics in term of gene gain/loss, which require large amounts of genomic data from a particular taxon. Characterizing the dynamics of the pan-genome and full Orthologous Groups (OGs) in a bacterial taxon (Medini *et al*., 2005) accelerates our understanding on bacterial evolution, host adaptation (Palomo *et al*., 2018), and virulence and pathogenesis (Lefébure & Stanhope, 2007; Muzzi *et al*., 2007). Advances in genome sequencing are also driving the discovery of novel natural products (Challis, 2008), providing a resource of novel industrially-important enzymes (See-Too *et al*., 2017a), antimicrobial agents (Cassell & Mekalanos, 2001) and other products.

2.2 The bacterial genome

As a single-celled microorganism, the bacterial genome is much smaller in size than those of eukaryotic organisms. Just like eukaryotic organisms, the bacterial genome consists of DNA, including coding sequences (genes) that are co-transcribed in operons, and other regulatory elements (Rocha, 2008). Based on the autoradiograph of DNA from *Escherichia coli*, the bacterial genome was assumed to have only a single circular

chromosome (Cairns, 1963), sometimes including one or more non-essential circular plasmids (Wake, 1973). We now understand that more than 10% of the bacterial genome is multiparte, consisting of more than one chromosome, and is not circular all the time (diCenzo & Finan, 2017). The multiparte genome may be beneficial in terms of regulation of gene expression, since genes coding enzymes from the same pathway may often co-transcribe in the same operon. The expression level also affected by the gene copy number (Bryant *et al*., 2015; Dryselius *et al*., 2008).

The general genomic features are diverse between species. Sometimes, strains of a bacterial species may have great differences in genomic features as bacteria can easily gain genetic material through horizontal gene transfer (HGT) from other organisms (Arber, 2014). The general genomic features that will be greatly affected in the acquisition of new genetic material are the GC content, codon usage, genome size and gene number. However, some of these features, for example the GC content, are still widely used characters in bacterial taxonomy (Stackebrandt & Liesack, 1993). Generally, the GC content of bacterial genomes ranges from 15~75% (diCenzo & Finan, 2017). Bacteria with broader temperature tolerance ranges have been documented to have higher GC content, possibly due to higher thermal stability of GC base pairs compared to AT (Musto *et al*., 2006).

The bacterial genome has been used to study the minimum number of genes needed for an organism to survive (Koonin, 2000). Deeper understanding of the minimal-gene-set to maintain life is crucial particularly in the generation of strains with desirable traits in synthetic biology (Juhas *et al*., 2012). This information is also important to extend our understanding of the origin of life, by aiding in determining the last universal common ancestor (LUCA) of all living cells (Weiss *et al*., 2018). LUCA not only contains

important information for the evolution of all organisms, but the study of these essential genes could also help us to develop efficient drugs in fighting pathogens (Roemer *et al*., 2003).

2.3 Revisiting the taxonomy boundary of bacteria with genomic data

In the 1960s, DNA–DNA hybridization (DDH) was introduced into prokaryote systematics (Brenner *et al*., 1967; De Ley *et al*., 1966). For more than half a century, using DDH to measure the degree of genomic similarity has been a gold standard that allows us to delineate a novel species from phylogenetically coherent group by a 70% DDH threshold.

The recent developments of DNA sequencing techniques allow us to use the small ribosomal subunit (SSU) gene sequence as a marker to differentiate bacterial species. For more than a quarter of a century, novel bacterial species or higher taxa have been classified/reclassified mainly through comparing the 16S ribosomal RNA (rRNA) gene sequence (Weisburg *et al*., 1991; Woese *et al*., 1990), A good correlation between similarity in 16S rRNA gene sequences of bacteria and DDH was reported. In general, a 70% cutoff in DNA–DNA re-association is the general rule of thumb of bacterial species delineation, which equates to less than 98% 16S rRNA gene sequence similarity (Dighe *et al*., 2004; Keswani & Whitman, 2001).

Multilocus sequence typing (MLST) and multilocus sequence analysis (MLSA) using housekeeping genes that provide higher resolution have occasionally been applied by bacterial taxonomists (Glaeser & Kämpfer, 2015). However, results of MLSA are often supported by DDH data in species delineation. Before journal like the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM), that publishes 90% of

new classifications, sets genomic data as the minimum standard for taxonomic description for prokaryotes, phylogenetic analysis of 16S rRNA sequences was used as the sole molecular data for taxonomic study (Chun *et al*., 2018; Prakash *et al*., 2007). Molecular fingerprinting methods such as Restriction Fragment Length Polymorphism (RFLP), Ribotyping, and Randomly Amplified Polymorphic DNA (RAPD) remain extremely useful and are widely applied in bacterial classification although techniques only allows us to differentiate clonal isolates (Jarocki *et al*., 2016), but remain extremely useful.

Entering the era of genomics, the widespread availability of whole genome sequencing (WGS) has enabled us to obtain complete and high quality draft genomes as the techniques are becoming increasingly affordable. Genome-based taxonomy has led to the progressive discovery of novel bacterial taxa. The application of user-friendly software and online-tools to determine pairwise genome relatedness, such as JSpecies (Richter & Rosselló-Móra, 2009) and OrthoANI (Lee *et al*., 2016) to determine pairwise genome relatedness by calculation of average nucleotide identity (ANI), has been generally recognized by the scientific community (Colston *et al*., 2014; Li *et al*., 2015; Oren & Garrity, 2014). Taxonomists have recognized the higher accuracy of these software, leading to a shift from the outdated DDH method for bacterial species delineation. The use of ANI, with a narrowed boundary of ≈95–96%, has become the new gold standard in offering a relatively stable species definition.

Nonetheless, none of these techniques or analyses gives a complete picture of bacterial evolutionary history, which is important to infer a true relationship in the tree of life. Comprehensive evolutionary analysis of the core-genome can improve our knowledge of bacterial evolution and population structure. On the other hand, comparison of the

biological functions encoded in the pan- and accessory-genome has helped us to understand the biological traits for adaptation of bacteria in their natural environment.

2.4 Phylogenomic Approach in Bacterial Phylogeny

The 16S rRNA gene sequence has been extensively used for establishment of the molecular phylogeny of bacteria for more than four decades (Fox *et al*., 1977). This common housekeeping gene that is present in most bacteria, is functionally important, and it gradually accumulates random sequence changes. Such features permit accurate measurement of the evolutionary relationships within a phylogenetically coherent group. However, this single-gene phylogenetic approach has low resolving power in many bacterial taxa, for example among members of the family *Enterobacteriaceae* (Janda & Abbott, 2007).

With genomic data, the total genomic information allows us to infer the true evolutionary history of any group of organisms using multiple loci of the whole genome, resolving phylogenies more reliable (Gee, 2003; Rokas *et al*., 2003). The genome-scale data include all evolutionary signals in the phylogenetic analysis, improving the signal–noise ratio. Since different genes in a genome may have undergone various degrees of conservation, combined analyses of different genes will improve the resolution and resolve distinct parts of the phylogeny (Felsentein, 1978). Core genes are regarded as conserved and less likely to undergo horizontal transfer as compared to other genes. Therefore, the core-genome has been suggested as a fundamental genomic unit for defining bacterial species (Lan & Reeves, 2001).

Generally, a 'supermatrix' is constructed by concatenating alignments of the core-genome, which includes all the OGs that are present in all members of an evolutionarily

coherent group, obtaining a result with high precision and high statistical support (Philippe *et al*., 2005). This 'promising method' however, has a few major flaws that may affect the accuracy of the results. First, long-branch attraction artefacts may occur if taxa subdividing the long branches are missing (Bergsten, 2005). Second, mis-defined evolutionary models for the analysis may result in maximum support for a mis-clustering clade, especially when the divergence level is high (Jeffroy *et al*., 2006; Phillips *et al*., 2004). To analyze the massive amount of data, selecting an appropriate evolutionary model (Leigh *et al*., 2008; Steel, 2005) and partitioning for multiple-sequence gene alignment in both maximum likelihood and Bayesian inference analysis have been a laborious process (McGuire *et al*., 2007), although this is no longer a substantial issue with improvements in computational power.

Genomic data also allow comparisons of multiple independent data sources including, for example isolation source, specific phenotype, or growth temperature ranges, to assess different hypotheses or to predict future occurrence of specific events, for example an outbreak of a pathogen. The gene order and rare genomic changes (Rokas & Holland, 2000) render a reliable phylogenetic reconstruction, using only gene alignment data (House, 2009; Philippe *et al*., 2005). The reconstruction of gene phylogenies based on evolutionary phenomena provides a more accurate tree (Eisen & Fraser, 2003). The phylogenetic network may also be reconstructed for non-tree-like evolutionary processes including recombination and lateral gene transfer (LGT) (Dagan, 2011). Therefore, all hypotheses can now be tested using the genomic data. Since the phylogenomic approach provides more insight of the evolution of bacteria, this approach should be more widely applied in taxonomic studies.

2.5 Comparative genomics for bacterial evolution study

Studies of comparative genomic composition are widely applied to reveal or predict the adaptive features of bacteria in contrasting environments (Browne *et al*., 2017; Martino *et al*., 2016). Phylogenomic approaches using the core genomic data have been used to gain insights into the history of evolutionary adaptation (Palomo *et al*., 2018; Simon *et al*., 2017). Tettelin *et al*. (2005) initially proposed the pan-genome concept, based on genomic analysis of eight *Streptococcus agalactiae* strains. Improvements in bioinformatics are now allowing the analysis of more strains spanning the whole bacterial family (Simon *et al*., 2017). Genomic studies have generally focused on medically important strains, mostly pathogenic strains. However, studies of the genomics of non-pathogenic strains in extreme environments, such as the marine *Prochlorococcus* sp. and member of *Rhodobacteraceae*, have provided important insights into the role of these bacteria in biogeochemical cycles in marine environments (Simon *et al*., 2017; Sun & Blanchard, 2014).

2.5.1 Cold-adaptation strategies of bacteria

For psychrophilic bacteria, comparison of genomes with mesophilic phylogenetic relatives has revealed aspects of their survival strategies (De Maayer *et al*., 2014). Comparative studies of the genomes of *Alteromonas* sp. (Math *et al*., 2012) and *Halobacterium* sp. (DasSarma *et al*., 2013; DeMaere *et al*., 2013) suggested that the psychrophilic strains of these bacterial species possess ecological fitness in cold environments, with distinct genomic features in comparison with the mesophilic strains. Adaptive features such as cold-associated osmotic and oxidative stress responses, and modulation of membrane fluidity of psychrophilic bacteria, can all be revealed by comparative genomic studies (Aliyu *et al*., 2016). In some studies, comparison among

psychrophilic strains coupled with expression studies can also give insight into ecological fitness (Dsouza *et al*., 2015).

By comparing amino acid (AA) modifications with the matched homologous protein in the genomes of psychrophilic bacteria and their mesophilic counterparts, evidence of cold-adaptation can be revealed (Raymond-Bouchard *et al*., 2018). AA composition greatly affects the flexibility of cold-adapted proteins, even in more mobile parts of the molecule's structure (Saavedra *et al*., 2018). Even though the AA composition of cold-adapted proteins does not differ much in their mesophilic form (Rabus *et al*., 2004; Yang *et al*., 2015), studies have also revealed consistent AA substitution, postulating high association of certain AA composition in cold-adapted proteins (Ayala-del-Río *et al*., 2010; Saunders *et al*., 2003; Zhao *et al*., 2010). With the rapid emergence of new genome sequences, AA composition studies of cold-adapted enzymes offer higher reliability and the potential to increase understanding of how AA composition is associated with cold-adaptation.

2.6 Taxonomic status of the family *Planococcaceae*

Plan.o.coc.ca'ce.ae. N.L. masc. n. *Planococcus*, type genus of the family; L. suff. -aceae, ending denoting family; N.L. fem. pl. n. *Planococcaceae*, the *Planococcus* family.

The family *Planococcaceae* (Krasil'nikov, 1949) belongs to the order Bacillales (Pre´vot, 1953) within the phylum Firmicutes, and at the time of writing includes 14 genera (Table 2.1). The type genus is *Planococcus* (Migula, 1894; emend. Nakagawa *et al.*, 1996; emend. Yoon *et al*., 2010), which was first proposed to accommodate aerobic, Gram-positive, motile, cocci- or rod-shaped bacteria. Phylogenetically the

Planococcaceae is closely related to *Bacillaceae* (Shivaji *et al*., 2014). Other type species of genera from *Planoccoaceae* are summarized in Table 2.1. Members of the *Planococcaceae* have been isolated or detected in various environmental samples from extreme environments, including deep sea sediments, marine solar salterns, glaciers, permafrost, Antarctic deserts, faeces, cyanobacterial mats and sea ice brine (Kim *et al*., 2015; Margolles *et al*., 2012; Pearson & Noller, 2011; Reddy *et al*., 2002). The capability to survive in extreme environments suggests that *Planococcaceae* provide a good model for comparative genomic study which will generate clues for the evolution of adaptation strategies in extreme environments.

The all species living tree project (Yarza *et al*., 2010) reveals that members of *Planococcaceae* form a monophyletic branch based on 16S rRNA gene sequences using the neighbor-joining algorithm (NJ), even though *Jeotgalibacillus* appeared as a separate sister clade and appeared to be closely related to *Listeriaceae* using the maximum likelihood (ML) algorithm (Shivaji *et al*, 2014). The genera *Planococcus* and *Planomicrobium* did not resolve into distinct clades. Some taxa from *Planomicrobium*, which was previously a member of *Planococcus*, clustered with *Planococcus*, contradicting the criterion of that classification of a genus or a species should be monophyletic based on sequence-based phylogenetic analysis (Christensen *et al*., 2007).

Table 2.1: Type species of genera from *Planococcaceae.*

Type species	Isolation source	Reference
Bhargavaea cecembensis	Oceanic ridge system	Manorama *et al*., 2009
Caryophanon latum	Animal feces	Peshkoff, 1939
Chryseomicrobium imtechense	Seawater	Arora *et al*., 2011
Filibacter limicola	Lake sediment	Maiden & Jones, 1985
Jeotgalibacillus alimentarius	Fermented food	Yoon *et al*., 2001a
Kurthia zopfii	Turkey caecum	Trevisan, 1885
Paenisporosarcina quisquiliarum	Soil	Krishnamurthi *et al*., 2009

Table 2.1, continued.

Type species	Isolation source	Reference
Planococcus citreus	Seawater	Migula, 1894
Planomicrobium koreense	Fermented seafood	Yoon *et al*., 2001b
Rummeliibacillus stabekisii	Spacecraft-assembly clean room	Vaishampayan *et al*., 2009
Solibacillus silvestris	Soil	Krishnamurthi *et al*., 2009
Sporosarcina ureae	Soil	Kluyver & van Niel, 1936; emend. Yoon *et al*., 2001c
Ureibacillus thermosphaericus	Landfill air	Fortina *et al*., 2001
Viridibacillus arvi	Soil	Albert *et al*., 2007

2.6.1 Taxonomy of the genera *Planococcus* and *Planomicrobium*

At the time of writing, the List of Prokaryotic with Standing in Nomenclature (LSPN) (http://www.bacterio.net/planococcus.html) includes 15 species within the genus *Planococcus* (Table 2.2), while six species previously from this genus have been reclassified to the genera *Planomicrobium* or *Marinococcus*. *Planomicrobium* consists of 10 species, of which five have been reclassified from *Planococcus* (Table 2.2).

The newly proposed genus *Planomicrobium* differs from *Planococcus* in term of cell morphology, which is rod-shaped instead of a coccus. Other characteristics that differentiate *Planomicrobium* from *Planococcus* include its members being motile, non-sporogenous and having lower G+C content (Dai *et al.,* 2015; Yoon *et al*., 2001b). The studies proposed that these two genera can be discriminated through their 16S rRNA gene sequences, specifically the sequence signatures at positions 183 (T for *Planococcus* and C for *Planomicrobium*) and 190 (A for *Planococcus* and G for *Planomicrobium*), following the 16S rRNA gene sequence numbering of *E. coli*. However, since numerous phylogenetic analyses of 16S rRNA gene sequences have shown that these two genera do not form monophyletic clades (Yang *et al*., 2018; Yarza *et al*., 2010), their taxonomic status should be re-assessed.

Table 2.2: Type strains of the genera *Planococcus* and *Planomicrobium.*

Type strain	Isolation source	Origin of isolation source	Reference
Planococcus antarcticus	Antarctic cyanobacterial mat	Marine/cold	Reddy *et al*., 2002
Planococcus citreus	Seawater	Marine	Migula, 1894
Planococcus columbae	Pigeon faeces	Animal	Suresh *et al*., 2007
Planococcus donghaensis	Seawater	Marine	Choi *et al*., 2007
Planococcus faecalis	Antarctic penguin stool	Marine/cold/animal	Kim *et al*., 2015
Planococcus halocryophilus	Arctic permafrost	Cold	Mykytczuk *et al*., 2012
Planococcus kocurri	Fish sample	Marine/animal	Hao & Komagata, 1986
Planococcus maitriensis	Antarctic cyanobacterial mat	Marine/cold	Alam *et al*., 2004
Planococcus maritimus	Seawater	Marine	Yoon *et al*., 2003
Planococcus plakortidis	Marine sponge	Marine	Kaur *et al*., 2012
Planococcus rifietoensis	Algal mat	Marine	Romano *et al*., 2003
Planococcus ruber	Soil	Soil	Wang *et al*., 2017
Planococcus salinarum	Marine solar saltern	Marine	Yoon *et al*., 2010
Planococcus salinus	Saline-alkaline soil	Soil	Gan *et al*., 2018
Planococcus versutus	Antarctic soil	Marine/cold	See-Too *et al*., 2016b
†*Planomicrobium alkanoclasticum*	Intertidal beach sediment	Marine	Dai *et al*., 2005
Planomicrobium chinense	Coastal sediment	Marine	Dai *et al*., 2005
Planomicrobium flavidum	Marine solar saltern	Marine	Jung *et al*., 2009
Planomicrobium glaciei	Glacier	Cold	Zhang *et al*., 2009
Planomicrobium koreense	Fermented seafood	Marine	Yoon *et al*., 2001
†*Planomicrobium mcmeekinii*	Antarctic sea ice	Cold	Yoon *et al*., 2001
†*Planomicrobium okeanokoites*	Seawater	Marine	Yoon *et al.*, 2001

Table 2.2, continued.

Type strain	Isolation source	Origin of isolation source	Reference
†*Planomicrobium okeanokoites*	Seawater	Marine	Yoon *et al.,* 2001
†*Planomicrobium psychrophilum*	Antarctic cyanobacterial mat	Marine	Dai *et al*., 2005
†*Planomicrobium stackebrandtii*	Soil from cold desert	Cold/Soil	Jung *et al*., 2009
Planomicrobium soli	Soil	Soil	Luo *et al*., 2014

† species reclassified from *Planococcus*

2.6.2 *Planococcus* and *Planomicrobium* in cold regions

Members of *Planococcus* and *Planomicrobium* are known for the ability to grow at moderately low temperatures (psychrotolerant) and are moderately halotolerant or halophilic. Members of *Planococcus* have been exploited for biotechnological and industrial applications, for instance through their production of carotenoids, thermophilic and alkaline/salt-tolerant xylanases and the biosynthesis of butanol (Huang *et al*., 2015; Kim *et al*., 2015; See-Too *et al*., 2016a; Unverferth *et al*., 2014).

Numerous *Planococcus* species are psychrotolerant, and able to survive at higher temperatures (>20 °C). The type strain of *Planococcus halocryophilus*, which was isolated from Artic permafrost, was reported to grow, divide and metabolise even at extremely low temperature (-15 °C) (Mykytczuk *et al*., 2013). The first novel *Planococcus* species isolated from a cold environment was *Planococcus antarcticus,* that was isolated from cyanobacterial mat samples from an Antarctic pond (Reddy *et al*., 2002). *Planoccocus* spp. have also been identified in samples collected in various locations in the polar regions or other low temperature environments (Table 2.2). Metagenomic studies of Antarctic ice cores (Shtarkman *et al*., 2013), or bacterial isolation from Antarctic samples (Shivaji *et al*., 1988; Smith *et al*., 2006; Wilkins *et al*., 2013)

indicate that *Planococcus* spp. and *Planomicrobium* spp. are commonly found in cold environments.

Planococcus spp. have also been identified in various marine samples (Table 2.2). Metagenomic studies of marine corals indicate that *Planococcus* is one of the major bacterial genera in healthy corals (Li *et al*., 2014). *Planococcus* strains have also occasionally been isolated from non-marine/cold environments (Table 2.2). They were identified as highly abundant in a metagenomic study of the tick midgut (Yuan, 2010). The abundance of this genus in various contrasting environments make it a good model to study the molecular adaptations required to survive in environmental stresses.

2.7 Genomic studies of *Planococcaceae*

The genomes of a few members of *Planococcaceae* have been studied thoroughly, due to their ability to survive in extreme environments. *Planococcus halocryophilus* strain Or1, isolated from the Arctic, is well-known as the record holder as bacterium that remain active at very low temperature (Mykytczuk *et al*., 2012). Molecular insights from this bacterium has given clues about cold-adaptation. Transcriptomic and proteomic studies have revealed various cold-adaptation strategies involved in changing the expression of metabolism stress response pathways (Raymond-Bouchard *et al*., 2017; Mykytczuk *et al*., 2012). The bacterium changes the expression of fatty acid (FA) synthesis, peptidoglycan, and DNA repair and recombination at lower temperatures. These genes were included in the proposed minimal-gene-set that are essential for survival (Gil *et al*., 2004). The capability of growth down to -15 °C makes *P. halocryophilus* a model organism for the study of survival strategies at sub-zero temperatures.

Adaptation strategies to cope with osmotic stress are another major focus in genomic studies of *Planococcaceae.* Other than the halotolerant *P. halocryophilus*, a comparative transcriptomic study was conducted on *Jeotgalibacillus malaysiensis*, revealing that it can increase the uptake or synthesis of osmoprotectants in high salinity environments (Yaakop *et al*., 2016). The genomic data of this bacterium could be an important source for mining of osmotically stable enzymes.

Even though abundant genomic data are available for representatives of *Planococcaceae* in public repositories such as NCBI, there is a lack of phylogenetic information using other core genes, and also of genomic comparisons at a pan-genomic level that could correlate the lifestyle of all species with their gene conservation and their position in the phylogenetic tree.

2.8 Genome mining of psychrotolerant bacterial genomes

The scientific community, and particularly industrial research organizations, are intrigued with research into cold-adapted enzymes, because their high specific activity at low/moderate temperature can offer considerable advantages in manufacturing process, and particularly in cost saving (Huston, 2008). Cold-adapted enzymes can be rapidly inactivated without elevation to relatively high temperature, and they are low in undesirable enzyme activity (Cavicchioli *et al*., 2011; Gerday *et al*., 2000; Russell, 1998). Such beneficial traits of these enzymes are also particularly useful as an ingredient in low temperature detergents (Furhan *et al.*, 2019; Nielsen *et al*., 1981) , or as bio-catalytic enzymes in the food and other industries, and also as important reagents components in many molecular biology kits (Huston, 2008).

In molecular biology, the heat-labile characteristic of cold-adapted/psychophilic enzymes is a crucial feature that provides unique advantages in various processes. In gene cloning, alkaline phosphatase (AP) is commonly used in the dephosphorylation of the 5′ end of a linearized fragment of DNA to avoid the re-circularization of the DNA fragment. Kobori *et al*. (1984) first isolated the psychrophilic AP, which was subsequently improved by Rina *et al*. (2000) and Koutsioulis *et al*. (2008), into as an easily inactivated AP, without the need of using detergent in the inactivating step that may affect downstream work. Suppliers for life science research such as New England Biolabs (Ipswich, MA, USA) has now commercialized the psychrophilic heat-labile AP. Cold-adapted DNA ligases from *Pseudoalteromonas haloplanktis* maintain a high specific function at 4 °C (Georlette *et al.*, 2000), without interference of other nucleases. Another example is the cold-adapted thermolabile proteinase K-like serine proteinase that is highly active at low temperatures and could be inactivated with only mild heat (Joshi & Satyanarayana, 2013). This feature allows an effective approach to achieve degradation of protein contamination in a sample while avoiding degradation of the targeted product.

Enzymes are also important additives in detergents. In order to perform well at ambient temperature, cold-active proteases, lipases, amylases, and celluloses can be incorporated in low temperature/cold active detergents. The cold-adapted characteristics of these enzymes offer advantages in term of energy saving and reducing overall emissions of carbon dioxide, to protection of the fabric from high heat. Research for the discovery and development of novel cold-adapted lipases that hydrolyse and remove fats/lipids/fatty acids at low temperature is growing (Cheng *et al*., 2011; Ji *et al*., 2015; Jiewei *et al*., 2014; Litantra *et al*., 2013; Park *et al*., 2009; Xuezheng *et al*., 2010). The lipase produced by *Pseudomonas stutzeri* PS59, that was isolated from a cold region, has been incorporated in detergent formulations with optimal activity at 20 °C (Li *et al*., 2014).

In the food and beverage industries, the use of enzymes is crucial for food processing and they have been extensively employed for decades. From cheese production to the processing of variety of food, enzymes serve as food additives to preserve flavors or to enhance taste and appearance. Like other industries, food processing at low temperature provides advantages like energy saving, prevention of contamination and spoilage, minimization of unwanted enzyme reactions at high temperatures, and the current trend is to replace the current enzyme usage with cold-adapted enzymes (Horikoshi, 1999; Pulicherla *et al*., 2011). Pectinases that catalyze degradation of the plant carbohydrate pectin are extensively used in the food processing industry, such as in fruit juice processing for clarification and viscosity reduction, and the extraction of natural oils (Adapa *et al*., 2014; See-Too *et al*., 2017a).

2.9 Background and brief history of bacterial quorum sensing research

Quorum sensing (QS) is a term to define bacterial cell-to-cell communication. Bacteria rely on QS to coordinate gene expression of the whole population when the population cell density attains a particular threshold (Miller & Bassler, 2001). *N*-acylhomoserine lactones (AHLs) are the chemical signalling molecules gaining the most research interest, since many Gram-negative pathogens utilize AHLs for QS (Deep *et al*., 2011; Galloway *et al*., 2009). Generally, AHLs will be produced and released to the extracellular environment, and the accumulation of AHLs will diffuse back to the cells when a critical concentration threshold of AHLs is achieved. At this point cells will have sufficient AHL molecules to bind on the transcriptional regulators and trigger the expression of QS-regulated genes (Taga & Bassler, 2003). QS regulates a large number of bacterial genes, up 10% of the open reading frame and more than 20% of the proteome (Arevalo-Ferro *et al*., 2003; Wagner *et al*., 2003). Thus, the bacterial population behaves uniformly like a

multicellular organism, granting significant advantages (Atkinson & Williams, 2009; Chhabra *et al*., 2003).

The pathogenicity of numerous pathogens including *Pseudomonas aeruginosa* (Lee & Zhang, 2015), *Aeromonas hydrophila* (Bi *et al*., 2007) and *Pectobacterium carotovorum* subp. *carotovorum* (Lee *et al*., 2013) has been shown to be QS-regulated. QS-regulated phenotypes for certain bacteria have been confirmed causing detrimental effects to the environment. For instance, bacteria with AHL-mediated QS are identified to cause black band disease of coral by destroying the structure or functionality of the coral reef (Zimmer *et al*., 2014; Meyer *et al*., 2015). Therefore, disruption of QS, a process known as quorum quenching (QQ), is of great interest for its potential application in solving the detrimental effects associated with AHL producing bacteria.

2.9.1 Disruption of bacterial QS

To date, two AHL-degrading QQ enzyme families have been identified: (1) AHL-lactonase and (2) AHL-acylase (Figure 2.1). AHL-lactonases target the homoserine lactones, by hydrolyzing homoserine lactones ring. AiiA of *Bacillus* sp. (Dong *et al*., 2000), AiiB and AttM from *A. tumefaciens* (Carlier *et al*., 2003; Zhang *et al*., 2002) and QsdA of *Rhodococcus erythropolis* (Uroz *et al*., 2008) are examples of AHL-lactonase. AHL-acylases hydrolyse the amide bond of AHLs, for example AiiD of *Ralstonia* sp. (Lin *et al*., 2003), PvdQ from *P. aeruginosa* (Huang *et al*., 2003) and AhlM of *Streptomyces* sp. (Park *et al*., 2005). These AHL-degrading QQ enzymes are reportedly produced by bacteria, yeast (Wong *et al*., 2013), mammalian sera (Yang *et al*., 2005) and have been identified in a metagenomic library constructed using total soil DNA (Schipper *et al*., 2009). Another family of QQ enzyme, the AHL-oxidoreductases produced by *Rhodococcus erythropolis*, unlike the AHL-degrading enzymes, alter the functional group

of AHLs to disrupt its binding to the cognate receptor (Uroz *et al*., 2005). Numerous plants also produce compounds that act as QS inhibitors. For example, halogenated furanones produced by the macro-alga *Delisea pulchra* enhance the degradation of transcriptional regulators (Link *et al*., 1997; Manefield *et al*., 1999).

2.9.2 Current application of QQ

Antibiotic abuse is a global issue as antibiotics exert a high selective pressure which leads to emergence of antibiotic-resistant bacteria. QQ is an alternative strategy to prevent the onset of bacterial infections (Alanis, 2005; Kumar & Scheweizer, 2005; Rasmussen & Givskov, 2006; Wright, 2005). QS regulates the production of virulence factors (Jones *et al*., 1993), antibiotic biosynthesis (Pierson *et al.,* 1994) and biofilm formation/differentiation (Davies *et al*., 1998). Various pathogens like *A. tumefaciens, Erwinia* spp., *Pectobacterium* spp. and *P. aeruginosa* have been proven to rely on QS to establish the pathogenic cycle (Fuqua *et al*., 1994). Unlike other antibacterial strategies that cause the emergence of multidrug-resistant pathogens (Karam *et al*., 2016), QQ does not affect bacterial viability and, therefore, imposes a lower selective pressure (Tang & Zhang, 2014). It is notable that mammals, including humans, also produce a bacterial-lactonase-like enzyme, the paraoxonase (PON), that hydrolyzes 3-OC_{12}-HSL, a signalling molecule that regulates genes controlling pathogenicity in pathogens like *P. aeruginosa* (Ozer *et al*., 2005; Yang *et al*., 2005). The bacterial-lactonase-like enzyme manipulates the microbiome of the host by modulation of bacterial QS.

In the wastewater treatment industry, QQ has been applied to prevent biofilm formation in membrane bioreactors (Lee *et al*., 2016). A similar strategy has also been applied to prevent biofouling in reverse osmosis (RO) systems (Oh *et al*., 2016). QS-inhibitors such as brominated furanone produced by *D. pulchra* has been used as a

therapeutic drug to antagonize AHL-mediated phenotypes of a number of pathogens (Givskov *et al*., 1996), including in an *in vivo* experiment against *P. aeruginosa* in mice (Hentzer *et al*., 2003). Dong *et al*. (2000) demonstrated that heterologous expression of an AHL lactonase, AiiA, significantly reduced AHL production in the plant pathogen, *Pectobacterium carotovorum*, and attenuated its pathogenicity. This strategy has also been proven effective in controlling plant diseases by co-culturing *P. carotovorum* with the purified QQ enzyme (Lee *et al*., 2013). However, the development of QQ as a therapeutic drug methodology still faces many obstacles. The delivery of the QQ enzyme to the host and ensuring the stability of enzyme remain important challenges before QQ can be applied widely in many industries (Whiteley *et al*., 2017).

2.9.3 QQ of Antarctic bacteria

In the natural environment, QS provides the bacterial population advantages in facilitating population migration towards/away from favourable/unfavourable conditions, and eases symbioses and niche adaptation in the host (Williams *et al*., 2007). Antarctica is one of most challenging environments on Earth (Peck *et al*., 2006; Convey *et al*., 2014). NGS analysis of Antarctic soil DNA has revealed that the complexity of Antarctic microbial communities and diversity was previously underestimated (Lee *et al*., 2012). QS genes have been identified in soil DNA, suggesting that QS confers the QS-mediated Antarctic bacteria a competitive advantage in hostile Antarctic environments (Pearce *et al*., 2012).

QQ in extremophiles is not well characterized, and *Geobacillus kaustophilus* is the only well-studied thermophile for its QQ activity (Chow *et al.*, 2010). To our knowledge, there is no reported characterization of QQ activity in bacteria isolated from the polar

regions. With the ability to disrupt intercellular communication, QQ in bacteria potentially makes an important contribution to competitive ability.

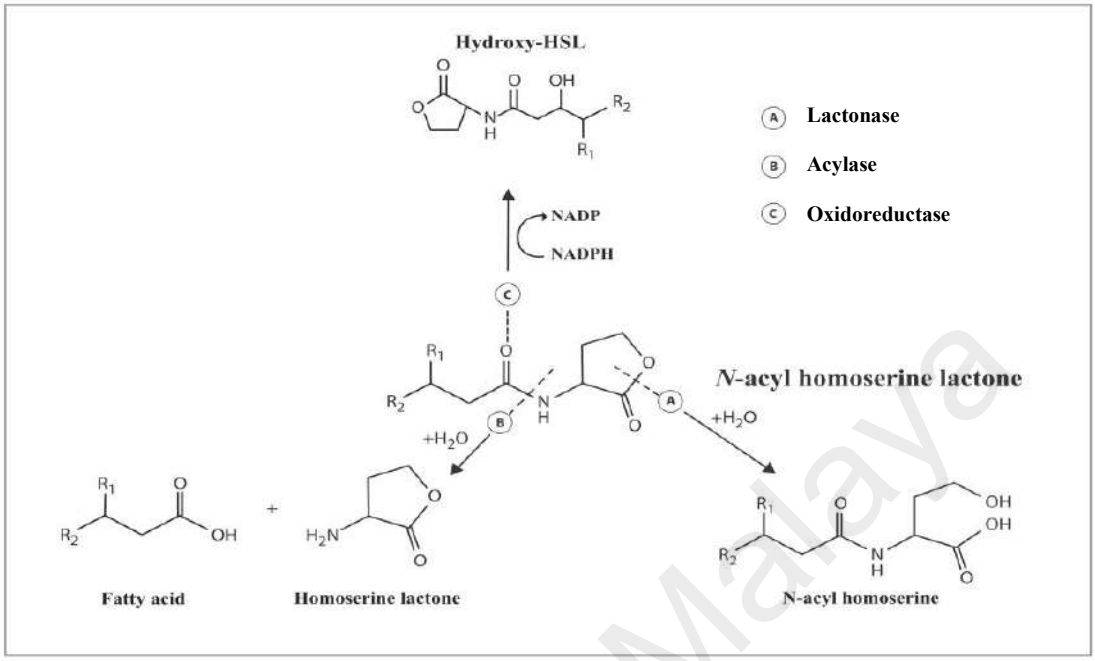


Figure 2.1: Schematic diagram of enzymatic degradation or modification of AHLs. The diagram was adapted from Chen *et al.* (2013) and generated using Adobe Illustrator.

2.9.4 Biochemical properties of AHL lactonases

To date, the phosphotriesterase family AHL lactonases are the most thermophilic QQ enzyme group (Hiblot *et al*., 2015), which remains stable and exhibits high activity at 85–95°C (Hiblot *et al*., 2012; Merone *et al*., 2005). On the hand, the metallo-*β*-lactamase superfamily AHL lactonases remain stable in a lower temperature, and exhibit lower optimal temperature range (30-50°C) (Cao *et al*., 2012; Seo *et al*., 2011; Tang *et al*., 2015; Pedroza *et al*., 2014; Vinoj *et al*., 2014), except the AiiT that was identified in *Thermaerobacter marianensis* that displayed a higher optimal enzyme activity at 60–80°C (Morohoshi *et al*., 2015). The AHL lactonases have optimal functioning pH at 7 and above, because the AHLs will undergo lactonolysis at high pH, and the reaction will reverse at pH 2.0 (Yates *et al.,* 2002). Therefore, pH 7 was used to measure the QQ activity for AHL lactonases (Cao *et al*., 2012; Seo *et al*., 2011; Tang *et al*., 2015; Pedroza *et al*., 2014; Vinoj *et al*., 2014). The AHL lactonases have a broad catalytic spectrum for

AHLs, however, the enzyme activity has always been affected by the acyl chain length or/and substitution of the acyl chain (Wang *et al.,* 2004; Yates *et al.,* 2002).

2.9.5 Potential of QQ enzyme to be applied in industry

Some plant pathogens synchronize the bacterial population in infection of the host by relying on their QS system (Mole *et al*., 2007). The *aiia* gene identified in *Bacillus* sp. was the first QQ gene that was transformed into tobacco and potato (Dong *et al*., 2001), and the transgenic plants showed an increase in tolerance to the plant pathogen that causes soft-rot disease, *P. carotovorum*. Such results spark the hope that QQ can be applied as a potent treatment or strategy to protect plants from bacterial pathogens.

In aquaculture, bacterial infections are a significant obstacle to the development of a sustainable industry (Defoirdt *et al*., 2004; Subasinghe, 1997). Abuse of antibiotics in aquaculture is leading to rapid development of broad range-antibiotic resistant bacteria (Heuer *et al*., 2009; Romero *et al*., 2012), and the use of antibiotics remains inefficient in aquatic disease treatment or prevention (Defoirdt 2004; Subasinghe, 1997). Therefore, alternative strategies like QQ, with a lower possibility of developing resistance, are desirable for application in aquaculture (Defoirdt *et al*., 2004). Major fish pathogens such as *Aeromonas* spp. or *Vibrio* spp. possess a QS system that regulate pathogenicity (Cao *et al*., 2012; Vinoj *et al*., 2014). *In vivo* study by applying AHL lactonase enzymes from *Bacillus* sp. to zebra fish has successfully decreased the level of *A. hydrophila* infection (Cao *et al*., 2012).

In wastewater treatment, bacteria are widely used to remove soluble pollutants in membrane bioreactors (MBR) (Drews, 2010). High pressure applied to the filtration membrane to minimize bacterial growth has inevitably increased the cost of MBR

significantly (Yeon *et al*., 2009). Since the development of biofilms for bacterial colonization relies on QS, QQ has been regarded as a promising strategy to inhibit the formation of biofilms, particularly during the early stages of the biofouling process, and bacteria capable of AHL production were also identified in the filtration membrane (Yeon *et al*., 2009). Thus, applying QQ enzymes in MBR potentially minimizes bacterial colonization of the filtration membrane and thereby reduces maintenance costs. The anti-QS compound, vanillin, has been applied in membrane filters and shown to reduce biofilm formation (Ponnusamy *et al*., 2013).

Since the highly toxic compound tributyltin (TBT) was banned, the search for alternatives to prevent biofouling has been a major concern. Microbiofouling is the formation of bacterial/protozoa colonies in structures in contact with seawater. The current antifouling printing using copper is also regarded as environmental unfriendly and high in cost, and to degrade a developed biofilms is also challenging (Ciriminna *et al*., 2015; Cordeiro & Werner, 2011). In the effort to prevent biofouling, the potential of QQ, which is a non-toxic approach, has been studied (Cordeiro & Werner, 2011; Olsen *et al*., 2007). Moreover, QQ could reduce QS-regulated biofilm development. However, the main concern for the use of QQ enzymes in biofouling reduction is the stability of enzymes within paints and their limited activity in seawater. To counter this, QQ enzymes from extremophile organisms constitute promising candidates for retaining activity in extreme conditions. QQ enzymes are also more environmental friendly as compared to QS inhibitors that could remain active and irreversible if the compound is released to the environment.

2.9.6 QQ enzymes as the basis of QQ strategy

To compensate for the major drawbacks of antibiotics as mentioned above, QQ serves as a promising alternative treatment for bacterial infection as it can be used to inhibit virulence factors (Hentzer *et al*., 2003), but does not affect the bacterial viability (Romero *et al*., 2012; Uroz *et al*., 2009; Waters & Bassler, 2005). Therefore, QQ is a promising strategy that induces less selection pressure, even though the emergence of resistance to QQ should be dependent on the actual use of the strategy. QQ enzymes are exogenous enzymes that are released upon production, and therefore induce lower selection pressure, since they act extracellularly. Moreover, QQ enzymes act independently without the need to bind to a target protein as with anti-QS compounds. Even though resistance to QQ enzymes has been suggested by some studies (Defoirdt *et al*., 2010; García-Contreras *et al*., 2013). These studies suggested that bacteria may have evolved by increasing producion of QS signalling molecules, however, this could be resolved by increasing the total QQ enzymatic activity.

AHL lactonases such as AhlS (Morohoshi *et al*., 2012) and AdeH (Garge & Nerurkar, 2016) have also been proven effective in reducing pathogenicity of *Pectobacterium carotovorum*, a species that causes soft-rot disease in various plants. When a gene encoding AHL-lactonase was transformed into *P. carotovorum*, AHL production was significantly reduced and the genes responsible for pectinolytic activity were not expressed (Dong *et al*., 2000; Mei *et al*., 2010; Torres *et al*., 2017). A transgenic plant with the gene encoding AHL-lactonase also had substantially enhanced resistance against *P. carotovorum* (Dong *et al*., 2001). These findings indicate the potential of AHL lactonases to be used as microbial antagonists.

QQ enzymes are naturally broad spectrum enzymes and can be engineered for altered specificity. They may therefore still effectively degrade the signalling molecule even if the bacteria modify the chemical structure of the autoinducer. Even though there are concerns that the bacteria may be able to increase the affinity of the autoinducer towards LuxR receptors or improve response to AHL (Collins *et al*., 2005; Hawkins *et al*., 2007), enzyme engineering may still offer solutions allowing production of enzymes with higher affinity for AHLs. For bacteria which cease production of quorum regulated factors, described as "social cheaters" by Sandoz *et al*. (2007), such QS-insensitive mutants might interfere with QQ efforts (Tay & Yew, 2013). A recent study, however, suggested that QQ resistance would spread slowly, as these mutants were found to be less fit than their counterparts (Gerdt & Blackwell, 2014).

The scientific community has raised concerns on the approaches used to create disease-resistant transgenic plants or crops. First, resistance may be induced and developed in target bacteria even though AHL lactonases impose lower selection pressure compared to antibiotics (García-Contreras *et al*., 2016). Even though the effect of AHL lactonase is specific and potent (Chen *et al*., 2013), constant expression of the AHL lactonase in a plant or crop might also accelerate the emergence of resistance in the pathogen. Second, AHLs play crucial roles in plant defence responses, growth and development (Bai *et al*., 2012; Schikora *et al*., 2011; Veliz-Vallejos *et al*., 2014). Therefore, expression of AHL lactonase in plant cells may also impact positive effects derived from bacteria-plant interactions. Third, degradation of AHLs within plant cells itself may affect beneficial endophytes which possess AHL-mediated QS systems. Endophytes are known to improve plant rooting and nutrient uptake and enhance chlorophyll production (Ali, 2013). Some endophytes produce AHLs to enhance their colonization on host plants and to counter phytopathogens (Liu *et al*., 2011; Sessitsch *et al*., 2005). Therefore, a strategy using direct

application of AHL lactonases to target specific plant pathogens that produce AHLs could be recommended as treatment of infected plants, mitigating the dependency on antibiotics that could accelerate the development of antibiotic-resistant pathogens.

To date, all studies have been performed in laboratory conditions with relatively high concentrations of QQ enzyme. Field tests are obviously much more challenging and further studies are needed to assess the impact of QQ and balance its drawbacks against its beneficial impact.

CHAPTER 3: MATERIALS AND METHODS

3.1 Media and chemical reagents

All media and chemical reagents utilized in this study were obtained from the following sources (in alphabetical order):

1. Becton Dickinson® (USA)

- Trypticase soy yeast extract broth/agar, Bacto marine broth/agar, Marine Agar (MA), yeast extract, pepton

2. bioMérieux (France)

- *N*,*N*,*N′*,*N′*,-tetramethyl 1,4-phenylenediamine, API NaCl 0.85 % medium

3. Biolog® Inc. (USA)

- Inoculating fluid (IF) B

4. First BASE Laboratories (Malaysia)

- All primers

5. Invitrogen® (USA)

- 1 kb DNA extension ladder

6. Lonza (Switzerland)

- SeaKem® agarose powder

7. Kapa Biosystem® (USA)

8. Macherey-Nagel® (Germany)

- Thin layer chromatography (TLC) on silica gel

9. Merck® (Germany)

- Phosphate buffered saline (PBS) buffer, glycerol, Acetonitrile (ACN), Tween 20

10. New England Biolabs® (USA)

- Protein G Magnetic Beads

11. Pacific Biosciences® (USA)

- Ampure® PB Beads, Magbead

12. Sage Science® (USA)

- BluePippin system with dye-free gel cassettes (PAC20KB)

13. Sigma-Aldrich® (USA)

- isopropyl-ß-D-thiogalactopyranoside (IPTG), phosphotungstic, ethanol (absolute), methanol, Sodium Choloride (NaCl), Isopropyl β- d-1-thiogalactopyranoside (IPTG), Ethylenediaminetetraacetic acid (EDTA), Coomassie brilliant blue R-250, hydrochloric acid (HCL), methyl t-butyl ether, hexane, sodium hydroxide (NaOH), sodium sulfate (Na_2SO_4), sodium hypochorite (NaOCl), Luria-Bertani (LB) broth/agar, Hydrogen peroxide (H_2O_2) solution, *N*-hexanoyl-l-homoserine lactone (C_6-HSL), *N*-heptanoyl-l-homoserine lactone (C_7-HSL), *N*-octanoyl-l-homoserine lactone (C_8-HSL), *N*-(3-oxohexanoyl)-l-homoserine lactone (3-oxo-C_6- HSL), *N*-(3-oxooctanoyl)-l-homoserine lactone (3-oxo-C_8-HSL), *N*-(3-oxodecanoyl)-l-homoserine lactone (3-oxo-C_{10}-HSL)

14. Thermo Fisher Scientific® (USA)

- Tris-borate-EDTA (TBE) buffer,

3.2 Commercial Kits

Commercial kits used in this study are as follows (in alphabetical order):

1. Agilent DNA 12000 Kit (Agilent Technologies, USA)
2. Agilent High Sensitivity DNA Kit (Agilent Technologies, USA)
3. Agilent Protein 230 Kit (Agilent Technologies, USA)
4. Ampure PB MagBead Kit (Pacific Biosciences, USA)
5. BluePippin™ Cassette Kit PAC20KB (Sage Science, USA)

6. Champion™ pET200 Directional TOPO® Expression Kit (Invitrogen, USA)
7. DNA Sequencing Reagent Kit 4.0 v2 (Pacific Biosciences, USA)
8. DNA/Polymerase Binding Kit P6 v2 (Pacific Biosciences, USA)
9. KAPA HiFi™ PCR kit (Kapa Biosystem, USA)
10. MasterPure™ Gram Positive DNA Purification Kit (Epicentre, USA)
11. Ni-NTA Fast Start Kit (Qiagen, Germany)
12. OptiSol™ Protein Solubility Screening kit (DiLYX, USA)
13. PacBio RS II SMRT Cell Oil (Pacific Biosciences, USA)
14. pGEM®-T Easy Vector Systems (Promega, USA)
15. Qubit® dsDNA Broad Range (BR) Assay Kit (Life Technologies, USA)
16. Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, USA)
17. SMRTbell Template Prep Kit 1.0 (Pacific Biosciences, USA)

3.3 Equipments and consumables

Equipments used in this study is as follows (in alphabetical order):

1. Agilent® 1200 Series RRLC (Agilent Technologies, USA)
2. Agilent® 2100 Bioanalyzer (Agilent Technologies, USA)
3. Agilent® 6400 Series Triple Quadrupole LC/MS (Agilent Technologies, USA)
4. Allegra® X-15R Benchtop Centrifuge (Beckman Coulter, USA)
5. BenchMixer™ (Benchmark Scientific Inc., USA)
6. Centrifuge 5424 R (Eppendorf, Germany)
7. DNA LoBind Tubes (Eppendorf, Germany)
8. Force Mini Centrifuge (Select Bioproducts, USA)
9. Galileo Biosciences RapidCast Complete Mini-Gel System (Sage Science, USA)

10. Gel Documentary Image Analyser (UV Products, Canada)
11. Glacier NU-9668 Upright Large Capacity -86 °C Ultra Low Freezer (NuAire, USA)
12. G-TUBE™ (Covaris, USA)
13. HPLC (Thermo-fisher, USA)
14. HVE-50 Autoclave Machine (Hirayama, Japan)
15. Leica DM750 Microscope (Leica Microsystems, Germany)
16. Maxymum Recovery Pipette Tips (Axygen, USA)
17. Mediline Lab Freezer (Liebherr, Switzerland)
18. MilliQ® Integral Water Purification System (Merck Millipore, Germany)
19. Mini-rotator Bio RS-24 (Biosan, Latvia)
20. MixMate® Vortex Mixer (Eppendorf, Germany)
21. NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, USA)
22. New Brunswick™ Innova® 44R Incubator Shaker (Eppendorf, Germany)
23. Pipette Tips (Axygen, USA)
24. Pipettes (Eppendorf, Germany)
25. Pippin Pulse Power Supply (Sage Science, USA)
26. PowerPac™ Basic Power Supply (Bio-Rad Laboratories Inc., USA)
27. Qubit 2.0 Fluorometer (Life technologies, USA)
28. Scanning Transmission Electron Microscope (Carl Zeiss AG, Germany)
29. Single Molecule Real Time (SMRT) RSII Sequencer (Pacific Biosciences, USA)
30. Sub-Cell® GT Agarose Gel Electrophoresis System (Bio-Rad Laboratories Inc., USA)
31. T100™ Thermal Cycler (Bio-Rad Laboratories Inc., USA)
32. TM3030 Scanning Electron Microscope (Hitachi, Japan)

33. Thermomixer Comfort (Eppendorf, Germany)

34. ThermoStat™ C (Eppendorf, Germany)

3.4 Bacterial strains, media and plasmids

In taxonomic and genomic studies, a total of 9 *Planococcus* type strains and 1 in-house novel *Planococcus* type strain were included. The culture conditions and media used to cultivate all the strains are listed in Table 3.1, along with isolation source. Other bacterial strains involved in the quorum quenching study are listed in Table 3.2, along with culture conditions and roles in the study of each strain. The cell suspensions for all bacterial strains were kept in 20 % w/v glycerol stock for long-term storage at -80 °C. All plasmids used in the quorum quenching study are listed in Table 3.4.

Table 3.1: List of *Planococcus* type strains used in this study. The composition of all media are given in Appendix A.

Name	Isolation source	Growth temperature (°C)	Medium
† *Planococcus antarcticus* DSM 14505^T	Cyanobacterial mat sample (Antarctica)	20	Trypticase soy yeast extract broth/agar
† *Planococcus donghaensis* DSM 22276^T	Deep sea sediment (East sea of South Korea)	28	Trypticase soy yeast extract broth/agar
‡ *Planococcus faecalis* CECT 8759^T	Faeces of Antarctic penguins (Antarctica)	25	Bacto marine broth/agar
† *Planococcus halocryophilus* DSM 24743^T	Permafrost active-layer soil (Arctic)	20	Trypticase soy yeast extract broth/agar
† *Planococcus kocurii* DSM 20747^T	Skin of cod fish (country of origin unknown)	28	Seawater yeast peptone medium
† *Planococcus maritimus* DSM 17275^T	Sea Water (Yellow Sea of South Korea)	28	Bacto marine broth
† *Planococcus plakortidis* DSM 23997^T	Marine sponge *Plakortis simplex* (India)	37	Trypticase soy yeast extract medium
† *Planococcus rifietoensis* DSM 15069^T	Algal mat from sulfurous mineral water spring (Italy)	28	*Planococcus* medium

Table 3.1, continued.

Name	Isolation source	Growth temperature (°C)	Medium
† *Planococcus salinarum* DSM 23820^{T}	sediment from marine solar saltern (Yellow Sea of South Korea)	28	Bacto marine broth/agar
§ *P. versutus* L10.15^{T}	Soil (Antarctica)	25	Luria Bertani broth/agar

† Strain obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen-German Collection of Microorganisms and Cell Cultures (DSMZ-GmbH)
‡ Strain obtained from Spanish Type Culture Collection (CECT)
§In-house isolate

Table 3.2: List of other bacterial strains used in the QQ study.

Name	Roles	Growth temperature (°C)	Medium
†*Escherichia coli* DH5α™	Cloning host, negative control for AHL inactivation assay	37	Luria (low salt) broth/agar
†*Escherichia coli* BL21 Star™ (DE3)	Expression host for heterologous expression quorum quenching protein	37	Luria (low salt) broth/agar
‡*Pectobacterium carotovorum* subp. *carotovarum* strain GS101	Positive control in pectinolytic assay	25	Luria Bertani broth/agar
‡*Pectobacterium carotovorum* subp. *carotovarum* strain PNP22	Negative control in pectinolytic assay	25	Luria Bertani broth/agar

† Strains obtained from Invitrogen®, USA
‡ Strains obtained Prof. Paul Williams's lab (University of Nottingham, UK)

Table 3.3: List of plasmids used in the QQ study.

Name	Roles
†pET200/D-TOPO®	Expression vector for expression of recombinant quorum quenching protein with an *N*-terminal tag containing a 6xHis tag
‡pGEM®-T Vector	Cloning vector for gene sequence verification

† Plasmid obtained from Invitrogen®, USA
‡ Plasmid obtained from Promega®, USA

3.5 Chemotaxonomic, Physiological and biochemical tests

Chemotaxonomic, physiological and biochemical tests were performed according to Bergey's manual of Systematics of Archaea and Bacteria (Shivaji, 2015).

3.5.1 Respiratory lipoquinones analysis

Respiratory lipoquinones analysis was performed at Korean Agricultural Culture Collection (KACC, South Korea). Generally, strain L10.15^T was cultivated for 24 h on TSA plates (Becton Dikinson, BBL) at 28 °C to obtain the cell mass required for quinone analysis, which was carried out from freeze-dried cells as described by Tindall (1990a, b). Briefly, respiratory quinones were extracted with mixture of methanol:hexane (1:2 v/v) from 100 mg of freeze-dried cells followed by phase separation into their different classes (menaquinones, ubiquinones, etc.) by thin layer chromatography (TLC) on silica gel (Macherey-Nagel Art. No. 805 023), using hexane t-butylmethylether (9:1 v/v) as solvent. UV-absorbing bands corresponding to different quinone classes at (269 nm) were purified and further analyzed using HPLC (Thermo-fisher, USA) using polymeric reversed-phase column (Supelcosil LC-PAH, 250 × 2.1 mm, 5 µm, 120 Å; Supelco, Deisenhofen, Germany) with a flow rate of 200 µL/min using a gradient of water containing methanol (5%, vol/vol) (A) and pure methanol (B) as mobile phase.

3.5.2 Polar lipid analysis

Polar lipid analysis was performed at Korean Agricultural Culture Collection (KACC, South Korea). Generally, the polar lipids of strain L10.15^T were extracted and analyzed by two-dimensional TLC following Embley & Wait (1994). Molybdophosphoric acid was used for the detection of total polar lipids, ninhydrin for amino lipids, molybdenum blue for phospholipids, Dragendorff reagent for choline-containing lipids and α-naphthol/sulphuric acid reagent for glycolipids.

3.5.3 Catalase and oxidase activity

Catalase activity was qualitatively assayed using 3% (v/v) H_2O_2 and determined by assessing the production of bubbles, and oxidase activity was determined using 1% (w/v) *N*,*N*,*N′*,*N′*,-tetramethyl 1,4-phenylenediamine (bioMérieux) as described by Smibert & Krieg (1994).

3.5.4 Salt tolerance, pH and temperature growth range test

The pH range for growth of strain L10.15^T was determined on LBA plates adjusted to various pH values between 4 to 12 with 1 pH unit increments. Tolerance of salt was determined by growing on LBA media supplemented with 0-25 % (w/v) NaCl at increments of 1%. Both salt tolerance and pH range tests were conducted by incubating the LBA plates at 26 °C for up to 14 d, using buffer system as in Appendix A. The temperature range for growth was determined by plating on LBA and incubation at 4-37 °C with increments of 1 or 2 °C over 14 d.

3.5.5 Cellular fatty acid analysis

Cellular fatty acid analyses of L10.15^T and other reference type strains were determined in parallel. The cellular fatty acids were extracted following manufacturer's instruction (Sherlock™ Microbial Identification System) with reference to Kuykendall *et al*. (1988) and Miller (1982) using Sherlock 6.1 and the library RTSBA6, according to the technical instructions provided by this system (Sasser, 1990). The cellular fatty acids were harvested after growth on MA plates (Becton Dickinson, USA) for 24 h at 28 °C in late log growth phase. Briefly, around 40 mg of bacterial cells were scraped from the third quadrant on the fresh agar plate culture and transferred into a glass test tube. One millilitre of reagent 1 (45g sodium hydroxide, 150 mL methanol, and 150 mL distilled water) was added to perform saponification. The mixture was boiled for 30 min and cooled to room

temperature before addition of 2 mL of reagent 2 (325 mL of 6 N hydrochloric acid and 275 mL methyl alcohol) for the methylation process by heating for 10 min at 80 °C. Extraction of Fatty acid methyl esters (FAME) was then performed by addition of 1.25 mL of reagent 3 (200 mL hexane and 200mL methyl t-butyl ether) and the test tubes were rapidly cooled and mixed for 10 min in a clinical rotator. Finally, sample clean-up was performed by discarding the lower aqueous phase before addition of 3 mL of reagent 4 (10.8 g sodium hydroxide dissolved in 900 mL distilled water). The sample was then subjected to gas chromatography analysis using a 6890N gas chromatograph (Agilent Technologies, USA) equipped with a 5% phenyl-methyl silicone capillary column (0.2 mm × 25 m), a flame ionization detector and a 7683A automatic sampler (Agilent Technologies, USA). Peaks were automatically integrated and fatty acid names as well as percentages were calculated by the MIS Standard Software (Microbial Identification, MIDI, USA). Ultra-high purity hydrogen was used as the carrier gas; column head pressure of 60 kPa; column split ratio of 100:1; septum purge at 5 mL/min; column temperature from 170 °C to 270 °C at 5 °C/min; injection port temperature of 240 °C; detector temperature at 300 °C. The sample injection volume was 2 μL.

3.6 Phenotypic characterization

API 20E (bioMérieux, France), API ZYM (bioMérieux, France) and GEN III MicroPlate^{TM} (Biolog Inc., USA) were used for phenotypic characterization following the manufacturer's instructions with modification. All reference type strains and the in-house strain were run in parallel under the same conditions to minimize any outcome bias.

3.6.1 API biochemical assay

For the API 20E assay, the inoculum was prepared using API NaCl 0.85 % medium with a single bacterial colony from a 24 h culture on an agar plate. For the API ZYM

assay, the inoculum was prepared with a single bacterial colony from a 24 h culture on an agar plate using the API Suspension Medium provided in the kit, and the turbidity was adjusted to 5-6 McFarland units. The homogeneous bacterial suspensions were then filled into the cupule of the strip. Since most of the reference strains including the in-house strain do not grow at 37 °C, which is the incubation temperature recommended by the manufacturer's instructions, all sample were incubated at 26 °C. The results were interpreted by referring to the Reading Table provided in the kit (Appendix B) *via* visual inspection.

3.6.2 GEN III MicroPlate™

The GEN III MicroPlate™ test was conducted using an OmniLog® incubator/reader. The results were assessed by OmniLog® Data Collection software. Briefly, a fresh bacterial colony was inoculated into inoculating fluid (IF) B (Biolog Inc., USA) and adjusted to 98%T cell density using a turbidimeter (Biolog Inc., USA). The cell suspension was inoculated into the GEN III MicroPlate, 100 μL per well, and the MicroPlate was incubated at 26 °C to allow phenotypic fingerprint (a characteristic pattern or "metabolic fingerprint" from discrete test reactions performed within a 96 well microplate) to form.

3.7 Bacterial colony and cell morphologies

Colony morphologies of strain L10.15^T were observed after 48 h of incubation on LB agar. Gram-staining was conducted using a Difco Gram stain set followed by observation under a Leica DM750 microscope (Leica Microsystems, Germany). Type of flagellation and cell morphology were documented using a TM3030 scanning electron microscope (SEM, Hitachi, Japan) and a scanning transmission electron microscope (STEM, LIBRA 120; Carl Zeiss AG, Germany). For SEM, a sample was prepared as described by Vali *et*

al. (2004). For STEM, overnight suspension cells were stained using 1% phosphotungstic acid on a Formvar grid and observed at an operating voltage of 80 kV. For STEM, cells were grown on LB broth overnight at 25 °C, harvested by centrifugation and resuspended in PBS.

3.8 Molecular identification and phylogenetic analysis

In order to confirm the identity of the bacteria isolate, the 16S ribosomal RNA (rRNA) gene of the isolate was amplified and sequenced. The sequence similarity of the 16S rRNA gene of the isolate with other type strains was analyzed using the EzBioCloud database (Yoon *et al*., 2017).

3.8.1 DNA extraction and amplification of 16S rRNA gene

The DNA of strain L10.15^{T} was extracted using the QIAamp DNA mini kit (Qiagen, Germany). Universal primers 27F (Weisburg *et al*., 1991) and 1525R (Dewhirst *et al*., 1989) were used for 16S rRNA gene amplification. The thermal cycling temperature profile for PCR is given in Table 3.4.

Table 3.4: Thermal cycling temperature profile for 16S rRNA gene amplification.

Step	Temperature (°C)	Time (s)
Initial denaturation	95	120
30 cycles		
Denaturation	95	30
Annealing	68	120
Extension	72	60
1 cycle		
Final extension	72	300

PCR products were purified and ligated into pGEM-T following the manufacturer's protocol (Promega, USA). DNA sequencing was performed by First Base Laboratories (Malaysia), in which standard T7 forward and SP6 reverse primers were used.

3.9 Molecular evolution analyses and amino acid profile analysis

All gene sequences were aligned in MEGA 7 (Kumar *et al*., 2016) using MUSCLE (Edgar, 2004). The multiple sequence alignment analysis was conducted using Jalview version 2 (Waterhouse *et al*., 2009). In order to investigate the presence of selective forces acting on *aidP* genes, the branch-site analysis for detection of positive selection using the CodeML program within PAML 4.7 (Yang, 2007) was performed. The ML phylogenetic tree inferred in the analysis was constructed using sequences obtained from a BLASTn search using the *aidP* gene sequence from *P. versutus* L10.15^{T} (WP_049694637.1) (Supplementary Figure 1). Likelihood ratio tests (LRT) were used to test for significance between the null and alternative models (χ^2 distribution, p value < 0.05). The Bayes empirical Bayes method (BEB) was used to identify positively selected sites when significant results were found (Yang *et al*., 2005). The amino acid composition was calculated from the aligned region of gene sequences using CodeML analysis, and the heatmap was created using MORPHEUS (https://software.broadinstitute.org/morpheus/). The 3D structure of AidP was modeled using the automated SWISS-MODEL server (Biasini *et al*., 2014). The model was examined and the positively selected sites highlighted using PyMOL.

3.10 Whole genome sequencing and functional gene annotation

The Pacific Biosciences (PacBio) RSII sequencing platform was used to perform whole genome sequencing using C4 chemistry in a Single Molecule Real Time (SMRT) cell.

3.10.1 DNA extraction for whole genome sequencing

Prior to DNA extraction, the cells of an overnight culture were pelleted by centrifugation (5,000 *g*) and the pellet washed at least three times using 1× PBS buffer. Genomic DNA of all *Planococcus* strains was isolated using the MasterPure™ Gram-positive DNA purification kit (Epicentre Technologies) following the manufacturer's recommended protocol. First, the samples were subjected to cell lysis. Briefly, each sample was added to a mixture of Ready-Lyse Lysozyme (1 μL) and TE buffer (150 μL) and was mixed thoroughly before being incubated at 37 °C for 30 min. Then, a mixture of Proteinase K (50 μg/μL) and Gram Positive Lysis Solution (150 μL) was added to the culture pellet and mixed thoroughly. Each sample tube was then incubated at 65 °C along with vortex agitation every 5 min. The samples were cooled to 37 °C and were subsequently mixed following addition of RNase A (1 μL, 5 μg/μL). A final incubation at 37 °C was performed for 30 min and the samples were chilled for 3 to 5 min prior to DNA precipitation. For precipitation of gDNA, the lysed samples were firstly combined with 175 μL of MPC protein precipitation reagent followed by vigorous mixing. The mixture was subsequently pelleted via centrifugation (4 °C, 10 min, ≥ 10,000 × *g*) and the resultant supernatant was transferred to a new tube and the pelleted debris discarded. Subsequently, 500 μL of isopropanol was added to each tube of recovered supernatant and the tubes were inverted 30 to 40 times prior to centrifugation (4 °C, 10 min). The isopropanol was subsequently discarded and the DNA pellets were rinsed twice with freshly prepared 70 % (v/v) ethanol. Finally, all residual ethanol was removed by air-dried in room temperature prior to resuspension in EB buffer (Qiagen, Germany)

3.10.2 SMRTbell™ template library construction

Genomic DNA concentration was assessed using a Qubit® Fluorometer (Life Technologies, USA) following the manufacturer's protocol of the Qubit® dsDNA broad range assay kit. The purity of genomic DNA was assessed using a NanoDrop® spectrophotometer 2000 (Thermo Scientific, USA) along with the corresponding Nanodrop® software (version 1.5) (Thermo Scientific, USA), by determining the ratio of absorbance at wavelength of 260 nm and 280 nm (A260/280) and the ratio of absorbance at wavelength of 260 nm and 230 nm (A260/230) of each sample. Only samples with approximately 1.8 reading of A260:A280 ratio and 2.0-2.2 of A260:A230 ratio were used for SMRTbell template library construction. Around 20 μg of gDNA DNA was then sheared to size of 17 to 20 kb using Covaris g-TUBE following the manufacturer's protocol. Briefly, gDNA was subjected to several spin cycles of 4800 rpm (Eppendorf centrifuge model 5424 R) for 2 min and eluted in 100 μL EB buffer (Qiagen, Germany). The gDNA fragment size assessments were made using (1) Agilent 2100 bioanalyzer with DNA 12000 kit (Agilent Technologies, USA) following the manufacturer's protocol, and (2) pulsed-field gel electrophoresis (PFGE) utilizing the Pippin Pulse electrophoresis instrument (Sage Science, USA). The use of Pippin Pulse electrophoresis enables accurate size estimation of the large DNA fragments. For Pippin Pulse electrophoresis, 1 % TBE gel was used to perform a 14 h gel run and 0.5× TBE buffer was used as the running buffer. The 1 % (w/v) TBE gel (pH 8.3) was prepared by melting 1.1 g of Lonza SeaKem® agarose powder in 110 mL of 0.5× TBE buffer and a 1 kb DNA extension ladder (Invitrogen, USA) was used as the DNA marker. A pre-set protocol which target a DNA size range of 5 to 80 kb was selected on the Pippin Pulse software (version 1.32) (Sage Science, USA) to control the run voltage, forward and reverse time steps and number of steps per cycle of the pulsed field electrophoresis gel run. The sheared gDNA was then purified using 0.45× volume ratio of Ampure® PB Beads followed by several

steps leading to the final SMRTbell™ library construction following the "Procedure & Checklist-20 kb Template Preparation using BluePippin™ Size Selection" protocol using SMRTbell™ template prep kit 1.0 (Pacific Biosciences, USA). The BluePippin system with dye-free gel cassettes (PAC20KB) was used in the size-selection step for the purified SMRTbell™ template as recommended by the protocol. Cassette definition "0.75 % DF Marker S1 high-pass 6-10 kb vs3" and pre-set protocol "SMRTbell 20kb, 7000 bp High-Pass" were used to perform the size selection run. The eluted size-selected SMRTbell™ was purified with 1.0× volume ratio of Ampure® PB beads prior to eluting in EB buffer (Qiagen, Germany).

3.10.3 Whole genome sequencing

Single Molecule Real Time (SMRT) sequencing was carried out on a PacBio RSII sequencer (Pacific Biosciences, USA) using a Magbead loading protocol 1 SMRT cell. The annealing and binding calculator version 2.3.1.1 were used to set up the annealing and binding reactions for the 20 kb SMRTbell™ library. In the primer annealing step, the primer was first diluted and conditioned by going through an 80 °C melting step and was subsequently mixed with the SMRTbell™ template to a final concentration of 0.8333 nM. P6 DNA polymerases were bound to the primer-annealed template with the presence of binding buffer, DTT and nucleotides. Stage-start was set with 180 min movie collection time.

3.10.4 Genome assembly and annotation

The reads were *de novo* assembled using hierarchical genome assembly process (HGAP) algorithm version 2 through the SMRT Portal user interface (Chin *et al*., 2013) into a circular contig. Briefly, raw reads obtained from sequencing were pre-processed and were further filtered using default parameters to generate subreads. The filtered

subreads were subsequently used for *de novo* assembly. Genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 2.5 and Rapid Annotation using Subsystem Technology (RAST) version 3.0 (Aziz *et al*., 2008; Brettin *et al*., 2015; Overbeek *et al*., 2014).

3.11 Genome relatedness analysis

In addition to the 16S rRNA gene sequence and MLSA analyses, in order to provide support for the classification of L10.15^T as a novel species, average nucleotide identity (ANI) analysis was carried out. An ANI value of 95–96% has been accepted as the cutoff threshold for bacterial species delineation, corresponding to 70% DNA relatedness (Goris *et al.,* 2009; Ricther & Rossello-Mora, 2009). Ortho ANI analysis was performed using the orthologous average nucleotide identity tool, OAT (Lee *et al.,* 2015). The genome of strain L10.15^T was used as the query genome. The NCBI Genbank Genome accession number for L10.15^T is CP016540. The reference genome sequence accession numbers for the closely-related type strains included in this study are: *Planococcus* halocryophilus Or1^T (CP016537), *Planococcus donghaensis* JH1^T (CP016543), *Planococcus antarcticus* DSM 14505^T (CP016534), *Planococcus plakortidis* AS/ASP6 (II)^T (CP016539), *Planococcus maritimus* TF-9^T (CP016538), *Planococcus salinavum* ISL-6^T (MBQG00000000), *Planococcus faecalis* AJ003^T (CP019401) and *Planococcus kocurii* NCIMB 629^T (CP013661).

3.12 Cloning and confirmation of QQ activity of the candidate gene

The candidate QQ gene (locus tag: I858_011945) from L10.15^T was amplified using the KAPA HiFi^{TM} PCR kit (Kapa Biosystem, USA). KAPA HiFi^{TM} polymerase is a high-fidelity DNA polymerase from the B-family of DNA polymerase which has proofreading ability and 100x lower error rates than wild-type Taq DNA polymerase. The candidate

QQ gene was then cloned into *E. coli* BL21 Star™ (DE3) using the Champion™ pET200 Directional TOPO™ Expression Kit to confirm its function. The expression vector pET200/ D-TOPO® allows the expression of the protein with a 6xHis tag at the N-terminal. The protein was also further purified and characterized and the potential of the enzyme to be applied in field was also examined.

3.12.1 Heterogous expression of candidate QQ gene

The primer to amplify the candidate QQ gene was designed following the manufacturer's protocol. The forward PCR primer places the candidate QQ gene of interest in frame with the N-terminal tag, which means the forward primer included the start codon (ATG in this case) and part of the following sequences of the candidate QQ gene, which directly follows the 5′ CACC overhang (to express the protein using the vector ribosome binding site). For the reverse PCR primer, the native stop codon in the candidate QQ gene was removed to preserve the reading frame through the C-terminal tag.

The sequences of the primer pairs were:

Forward: 5′-**CACC**ATGACTGGTATTATCAAGCC

Reverse: 5′- TTATTCGTAGTATCCTTCAGTCGACT

PCR reaction was set as according to manufacturer's recommendation and using the following cycling parameters: 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min, for 35 cycles. The amplicons were purified using the AMPure XP-PCR purification system (Beckman Coulter, USA) following the manufacturer's protocol. The purified PCR product was cloned into the pET200/D-TOPO® (Figure 3.1) and was designated as pET-AidP. The recombination system was transformed into cloning host *E. coli* TOP10 using

One Shot® TOP10 Chemically Competent *E. coli* kit (Invitrogen, USA) following the manufacturer's protocol. The pET-AidP was then extracted from the cloning host using the Qiagen Plasmid Mini kit (Qiagen, Germany) following the manufacturer's protocol prior to transformation into the expression host *E. coli* BL21 Star™ provided in the Champion™ pET200 Directional TOPO™ Expression Kit. The QQ activity of the recombinant system was assessed as described in Section 3.12, except that a temperature of 16 °C was used to accelerate the growth of *E. coli*, and IPTG (0.4 mM) was added to induce the production of recombinant protein.

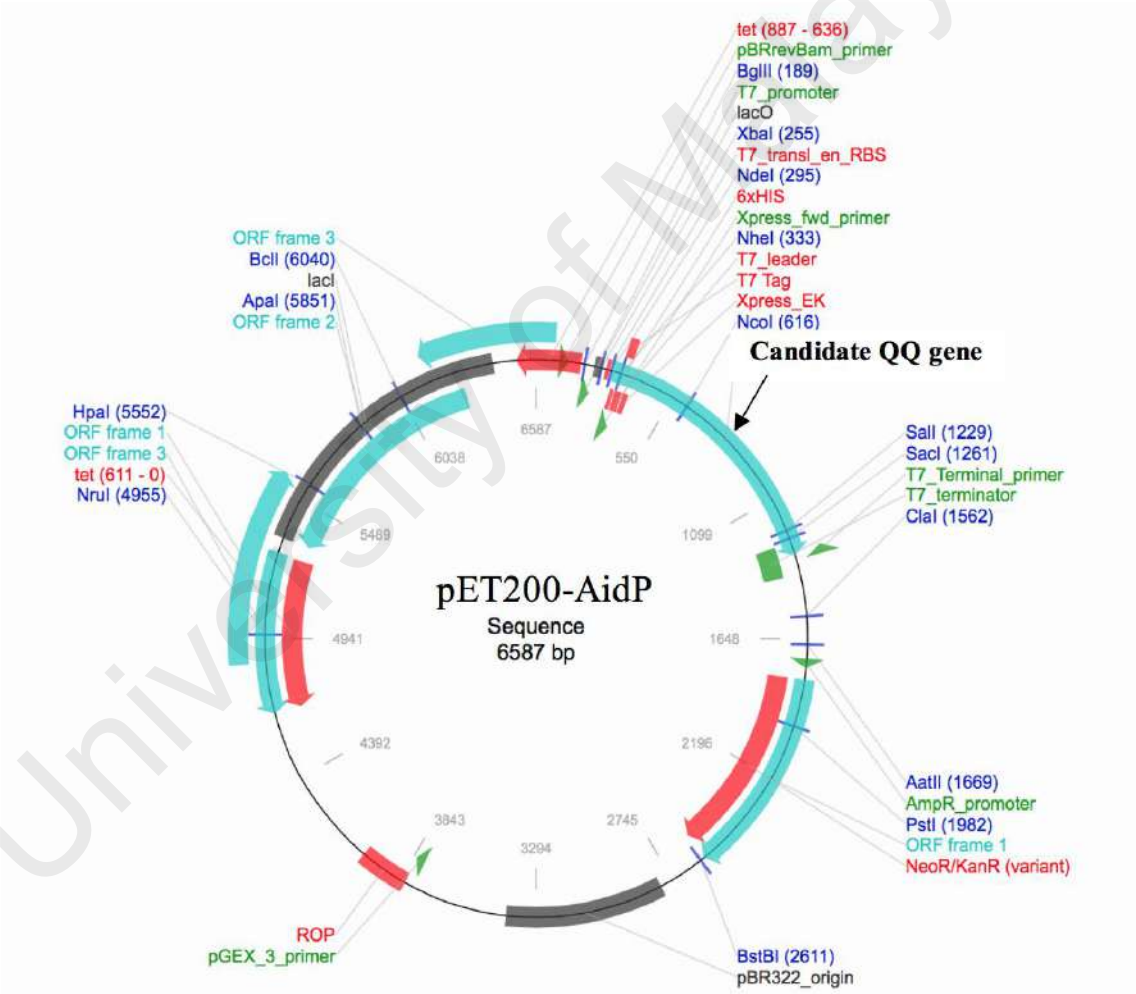


Figure 3.1: The circular map and features of the expression vector with candidate QQ gene. The candidate QQ gene is gene encoded 282 aa and annotated as "MBL fold metallo-hydrolase" by PGAP of NCBI. The circular map for pET200/ D-TOPO® is in Appendix C.

3.12.2 Expression and purification of AidP protein

BL21 Star^{TM} harboring pET-AidP were grown in LB broth at 16 ˚C overnight following the manufacturer's alternative instruction to increase the protein yield (Invitrogen, USA). Another purpose of using 16 ˚C instead 37 ˚C to grow the heterologous expression system is to assure the integrity of the cold-adapted protein. Protein expression was induced by the addition of 0.5 mM IPTG. The cultures were further grown in 16 mM IPTG (150 rpm) for 16 h. Cells were harvested by centrifugation at 5,000 *g* for 10 min at 4 ˚C. The His_6-AidP protein was partially purified from the harvested cells using the Ni-NTA Fast Start Kit (Qiagen, Germany) according to the manufacturer's instructions. The solubility of the protein was optimized using OptiSol^{TM} Protein Solubility Screening kit (DiLYX, USA) according to the manufacturer's instructions. Next, protein in buffer containing 200 mM imidazole was dialyzed into 1 mL of reaction buffer [500 mM Na_2SO_4, and 1 % Tween 20 (w/v), pH6].

3.12.3 Protein analysis

The apparent molecular mass of the purified protein was determined by SDS-PAGE (12% [w/v]) after staining it and molecular mass standards with Coomassie brilliant blue R-250. Protein concentrations were routinely determined by the Bradford method (Bradford 1976) and Qubit® 2.0 Fluorometer (Life Technologies, USA). A Bioanalyzer 2100 (Agilent Technologies, USA) with High Sensitivity Protein Screening Chip was also used to access the purified protein. Briefly, the purified protein was normalized to 1 µM then immunoprecipitated using Protein G Magnetic Beads (New England Labs, UK).

3.12.4 Detection of QQ activity and mechanism of action for AidP

To assess quenching activity on AHLs, an RRLC analysis (Agilent Technologies, USA) was performed as previously described (Todd & Gomez, 2001;). Briefly, the

residue AHL from the assay was analysed by RRLC 6400 series using an Agilent Poroshell 120 EC-C18 column (4.6 mm × 100 mm, 2.7 μm particle size) with the elution procedure consisting of an isocratic profile of acetonitrile/water (35:65, v/v), and a constant flow rate of 0.7 mL/min, with UV detection set at 205 nm. Briefly, the synthetic AHL was dispensed into 1.5 mL sterile tubes and the solvent evaporated, after which 100 μL of either partially purified AidP or *E. coli* DH5α cell suspension in PBS were added to rehydrate the dried AHL (100 μM final concentration). The mixtures were then incubated at 4 °C for AidP or 37 °C for *E. coli* DH5α with shaking (220 rpm). Samples were withdrawn at 0 h and 24 h and subjected to heat inactivation as described by Chan *et al.* (2009) and McClean *et al*., (1997). Residue AHLs were extracted twice using an equal volume of ethyl acetate, followed by evaporation to dryness. The AHLs were then reconstituted in 0.1 mL HPLC grade acetonitrile (ACN) before being subjected to RRLC analysis. Control experiments were carried out using suspension buffer (PBS 10 mM, pH 6.5) and *E. coli* DH5α.

For identification of AHL lactonase activity, the method of Yates *et al*. (2002) was followed. Samples withdrawn from the AHL inactivation assay were divided into two aliquots of equal volume (50 μL), and one was acidified with 100 mM HCL to pH 2.0 to induce recyclization of the lactone ring, while the other untreated aliquot was used as control. The acidified sample was incubated at 26 °C for 48 h before adjusting to pH 6.0 with MOPS buffer (0.5 M, pH 7.5). Residue AHLs were extracted twice using an equal volume of ethyl acetate after 0 h, 24 h or 48 h incubation, followed by evaporation to dryness. The AHLs were reconstituted in 0.1 mL ACN and separated using a symmetry EC-C_{18} column (2.7 × 100 mm) (Agilent Poroshell 120 EC-C18).

The mechanism of action of the QQ enzyme was confirmed by ultra performance liquid chromatography (UPLC) and electrospray ionization-mass spectorometry (ESI-MS). ESI-MS was performed with a 6460 triple-quadrupole instrument (Agilent Technologies, USA). Briefly, the residue AHL extracted from above method were analyzed by LC using an Agilent Poroshell 120 EC-C18 column (4.6 mm × 100 mm, 2.7 μm particle size) with a mobile phase of acetonitrile-water (0.1% formid acid; a linear gradient [v/v] of acetonitrile from 5 to 95% over 1.5 min at a flow rate of 1.8 mL min^{-1}. The separated fractions were further analyzed by ESI-MS. AHLs tested are listed in Appendix D.

3.12.5 Characterization of enzymatic activity of AidP

The effects of various metal ions and the metal chelating reagent EDTA on AidP activity were examined both *in vitro* and *in vivo*. For the *in vitro* assay, 1 mM EDTA or 1 mM of each metal ion (Cu^{2+}, Ca^{2+}, Fe^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}) were mixed with 1 μM concentration of purified AidP protein in reaction buffer and $3OC_6$-HSL. After incubation at 25 ºC for 3 h, the assay was measured using RRLC as described above. For the *in vivo* assay, an overnight culture of *E. coli* harbouring the plasmid pET-AidP was diluted into fresh LB medium with 1 mM EDTA or 1 mM concentration of each selected metal ion. $3OC_6$-HSL and IPTG were added to induce AidP over-expression after 6 h cultivation. After an additional 6 h cultivation, crude cell extracts were prepared and adjusted to the same concentration before being subjected to RRLC analysis as describe in Section 3.12. Positive control (PC) was defined as 100% relative activity in the reaction buffer (pH 7) at 25 °C.

3.12.6 Pectinolytic inhibition assay

The assay was performed on Chinese cabbage as described by Lojkowska *et al*. (1995) and Uroz *et al.* (2003). Chinese cabbage was gently washed with sterile H_2O, wiped with sodium hypochorite and dried under sterile conditions. *Pectobacterium carotovorum* subp. *carotovarum* strain GS101 was used in this assay and QS mutant of strain GS101, *Pectobacterium carotovorum* subp. *carotovarum* strain PNP22 as a negative control. Strains were grown overnight at 25 °C in LB medium, suspended and diluted in PBS. The bacterial suspensions were introduced into the cabbage (pathogen alone, or with AidP enzyme, PBS as negative control) using sterile pipette tips. Chinese cabbage was incubated at 25 °C under atmosphere with 90% humidity for 3 d. The result was assessed by visual inspection.

3.13 Phylogenomic analysis of *Planococcus* within *Planococcaceae*

A total of 103 genome sequences of bacterial strains from the family of *Planococcaceae* were obtained from the NCBI database (accession numbers detailed in Table 3.5). The completeness of the genome was assessed using BUSCO v3 (Simão *et al*., 2015), and a threshold of 95% completeness was used as the criterion for genome selection. The genome relatedness analyses were performed using orthologous average nucleotide identity tool, OAT (Lee *et al.,* 2015), Average Amino acid Identity (AAI) was performed using AAI-matrix tool (Rodriguez-R & Konstantinidis, 2016), Percentage of Conserved Protein (POCP) was performed based on Qin *et al*. (2014). The general features and overview of the pan-genome were generated using Anvi'o (version 5.2) platform1. Generally, the headers of the fasta files for bacteria genomes were converted to Anvi'o compatible format using "anvi-script-reformat-fasta" script, and a contig database was created from each bacteria genome using the default setting of "anvi-gen-contigs-database" script. Open Reading Frames (ORF) of each gene in a bacterial genome

were identified using Prodigal (version 2.6.3)2. Genes in each contig database were annotated with functions from NCBI's COG using "anvi-run-ncbi-cogs" script and aligning tool, DIAMOND (version 0.9.22.123)3 (Buchfink *et al*., 2015). Hidden Markov model (HMM) hits and were added to each contig database using "anvi-run-hmms" script. Numbers of each amino acid in each bacterial genome were counted using "anvi-get-aa-counts" script. Genome storage was generated from annotated contig databases using "anvi-gen-genomes-storage" script, external genome option. Pan-genomic overview was performed on genome storage using "anvi-pan-genome" script. Similarity of each amino acid sequence in every bacterial genome against the other bacterial genomes stored in genome storage was calculated using DIAMOND 3. Clusters in amino acid sequence similarity search results were identified using Markov Cluster Algorithm (MCL) algorithm 4. MCL inflation parameter was set to 1.5. Gene clusters that occurred in less than four genomes in the genome storage were removed from the data. Functional and geometric homogeneities of the gene clusters were estimated using "anvi-compute-gene-cluster-homogeneity" script. ANIb scores were computed between each bacterial genome in the genome storage using "anvi-compute-ani" script. Results from the pan-genome analysis were viewed on the user interface of Anvi'o platform1. Anvi'o was only used to generate a visualization of the pan-genome data. The pan-genome data was obtained as illustrated in Figure 3.2, the workflow for the phylogenomic study of *Planococcaceae,* in which the parameter of the pan-genome pipeline was set as the same as for Anvio's analysis.

3.13.1 Protein clustering and genome-scale phylogenetic inference

The panX pipeline (Ding *et al*., 2017) was used for pan-genome analysis. The annotated genomic data in Table 3.5 were obtained from NCBI RefSeq database. The pipeline further processed the annotated genomic data using DIAMOND for all-against-

all protein alignment, and clustered it into OG using MCL. Adaptive phylogenetic post-processing was then applied to split and filter the paralogous genes. The core genes were then aligned using MAFFT version 7 (Katoh & Standley, 2013), and the poorly aligned regions were removed using trimAL (Capella-Gutiérrez *et al*., 2009). The best-fit evolution model of all genes from the concatenated supermatrices were determined using ModelTest-NG (https://github.com/ddarriba/modeltest) before being subjected to phylogenetic inference using RaxML-NG (Kozlov, 2018). The RAST annotation server (Aziz *et al*., 2008) was used for automatic annotation and ORFs prediction. All protein sequences were compared reciprocally by alignment (*e*-value cutoff 1e-10) and filtering (70% coverage) using blastp (Altschul *et al*., 1990). The core GCs were also subjected to COG annotation (Tatusov *et al*., 2000), and KEGG mapper was used for KEGG pathways analysis (Kanehisa *et al*., 2012). The ML phylogeny generated in Section 3.14.1 was used to assess the positive selection of all core OGs using codeML from the PAML 4.6 package (Yang, 2007). Briefly, positive selection on branches within *Planococcus* cluster was examined using a neutral evolution model (null hypothesis) and a model specifying a proportion of sites undergoing positive selection (alternative hypothesis). Comparisons of likelihood ratio test between the two models were made using the χ^2 distribution with one degree of freedom (Xu *et al*., 2011).

Table 3.5: Genome sequences of bacterial strains from the family of *Planococcaceae* obtained from NCBI.

Strain	Isolation source	Genome status	Genome accession number
Bhargavaea cecembensis DSE10^T	deep-sea sediment	Contig	AOFT00000000.1
Bhargavaea cecembensis B-C2	human gut	Scaffold	CDGP01000000.1
Bhargavaea cecembensis T14	hot Spring	Contig	LQNT01000000.1
Caryophanon latum DSM 14151^T	cow dung	Contig	MATO00000000.1
Caryophanon tenue DSM 14152^T	cow manure	Contig	MASJ00000000.1

Table 3.5, continued.

Strain	Isolation source	Genome status	Genome accession number
Chryseomicrobium excrementi ET03^T	Cast from soil	Contig	PCGR00000000.1
Jeotgalibacillus alimentarius YKJ-13^T	fermented seafood	Contig	JXRQ00000000.1
Jeotgalibacillus campisalis SF-57^T	marine saltern	Contig	JXRR00000000.1
Jeotgalibacillus malaysiensis D5^T	sea water	Complete	CP009416.1
Jeotgalibacillus soli P9^T	soil	Contig	JXRP00000000.1
Jeotgalibacillus sp. 22-7	Ocean sediment	Contig	PREZ00000000.1
Kurthia massiliensis JC30^T	human stool	Scaffold	CAEU00000000.1
Kurthia senegalensis JC8E^T	human fecal flora	Scaffold	CAEW00000000.1
Kurthia sp. 11kri321	geothermal spring	Complete	CP013217.1
Kurthia huakuii LAM0618	biogas slurry samples	Contig	AYTB00000000.1
Kurthia sibirica ATCC 49154^T	stomach and intestinal contents from woolly Magadan mammoth	Contig	QFVR00000000.1
Kurthia zopfii NCTC10597^T	intestinal contents of poultry	Contig	UGNP00000000.1
Paenisporosarcina indica PN2^T	soil near to glacier	Contig	MPTA00000000.1
Paenisporosarcina sp. TG-14	sediment-laden stratified basal ice	Contig	AMGD01000000.1
Paenisporosarcina sp. TG20	sediment-laden stratified basal ice from Taylor Glacier	Contig	ALJG00000000.1
Paenisporosarcina sp. OV554	*Populus* root and rhizosphere	Contig	QBUC00000000.1
Planococcus antarcticus DSM 14505^T	Antarctic cyanobacterial mat	Complete	CP016534.2
Planococcus citreus DSM 20549^T	Seawater	Scaffold	RCCP00000000.1
Planococcus donghaensis DSM 22276^T	deep sea sediment	Complete	CP016543.2
Planococcus donghaensis MPA1U2	coastal water	Contig	AEPB01000000.1
Planococcus kocurii ATCC 43650^T	skin of cod fish	Complete	CP013661.2
Planococcus maritimus Y42	crude oil-contamination soil	Complete	CP019640.1
Planococcus halocryophilus DSM 24743^T	permafrost active-layer soil	Complete	CP016537.2

Table 3.5, continued.

Strain	Isolation source	Genome status	Genome accession number
Planococcus maritimus DSM 17275^T	seawater	Complete	CP016538.2
Planococcus plakortidis DSM 23997^T	marine sponge	Complete	CP016539.2
Planococcus rifietoensis M8^T	sulfurous mineral water spring, algal mat	Complete	CP013659.2
Planococcus versutus L10.15^T	Antarctic soil	Complete	CP016540.2
Planococcus massiliensis ES2^T	human feces sample	Scaffold	CCXS00000000.1
Planococcus maritimus MKU009	marine water	Contig	LTZG01000000.1
Planococcus maritimus SAMP	coastal water	Contig	MINM01000000.1
Planococcus faecalis AJ003^T	penguin feces	Complete	CP019401.1
Planococcus sp. CAU13	soil	Contig	JRGN01000000.1
Planococcus sp. PAMC21323	Antarctic soil	Complete	CP009129.1
Planococcus halotolerans SCU63^T	Saline soil	Contig	QLZR00000000.1
Planococcus maitriensis S1^T	Antarctic cyanobacterial mat	Contig	QLZQ00000000.1
Planococcus salinarum ISL-16^T	Marine solar saltern sediment	Contig	QQRT00000000.1
Planococcus sp. MB-3u-03	Artic Sea ice	Complete	CP025135.1
Planococcus sp. Urea-3u-39	Artic Sea ice	Contig	PJCB01000032.1
Planococcus sp. Urea-trap-24	Artic Sea ice	Contig	PJCC01000031.1
Planomicrobium glaciei UCD-HAM	Hammock	Contig	LGAF00000000.1
Planomicrobium glaciei CHR43	river water of cold deserts	Contig	AUYR01000000.1
Planomicrobium okeanokoites Marseille-P2029	human gut	Contig	FWYH00000000.1
Planomicrobium okeanokoites IFO 12536^T	Marine sediment	Contig	QQRS00000000.1
Planomicrobium flavidum S5-TSA-19	soil	Contig	NHTN00000000.1
Planomicrobium sp. MB-3u-38	Artic Sea ice	Contig	PJBO00000000.1
Planomicrobium soli CGMCC 1.12259^T	Soil	Scaffold	PYAT00000000.1

Table 3.5, continued.

Strain	Isolation source	Genome status	Genome accession number
Rummeliibacillus stabekisii PP9	Antarctic soil	Complete	CP014806.1
Rummeliibacillus pycnus DSM 15030^T	Soil	Scaffold	NJAS00000000.1
Rummeliibacillus sp. POC4	Sewage sludge	Contig	QWUA00000000.1
Solibacillus isronensis B3W22^T	Cryotubes air samples from high altitudes	Contig	AMCK01000000.1
Solibacillus isronensis Marseille-P3605	Unknown	Contig	FVZN00000000.1
Solibacillus silvestris StLB046	Potato leaf	Complete	AP012157.1
Solibacillus silvestris DSM 12223^T	Forest soil	Complete	CP014609.1
Solibacillus silvestris MROC3	Domestic chicken feces	Contig	LZRJ00000000.1
Solibacillus kalamii ISSFR-015^T	HEPA filter from International Space Station	Contig	NHNT00000000.1
Solibacillus sp. R5-41	Soil	Complete	CP024123.1
Sporosarcina koreensis S-K12	human gut	Scaffold	CDGU01000000.1
Sporosarcina psychrophila DSM 6497^T	soil/river water	Complete	CP014616.1
Sporosarcina sp. EUR3 2-2-2	permafrost	Contig	AXVE01000000.1
Sporosarcina newyorkensis 2681	human blood sample	Scaffold	AFPZ01000000.1
Sporosarcina sp. D27	sediment from creek	Contig	AZUC01000000.1
Sporosarcina sp. P37	base of single tree in sidewalk	Complete	CP015349.1
Sporosarcina sp. P33	soil	Complete	CP015027.1
Sporosarcina ureae P8	soil	Complete	CP015207.1
Sporosarcina ureae P17a	soil near to a tree	Complete	CP015109.1
Sporosarcina ureae P32a	public walkway	Complete	CP015348.1
Sporosarcina ureae S204	soil	Complete	CP015348.1
Sporosarcina pasteurii NCTC4822^T	soil	Contig	UGYZ00000000.1
Sporosarcina sp. P1	Base of eucalyptus tree	Contig	PDYS00000000.1
Sporosarcina sp. P2	Base of eucalyptus tree	Contig	PDYY00000000.1
Sporosarcina sp. P3	Base of eucalyptus tree	Contig	PDZE00000000.1
Sporosarcina sp. P7	Garden soil	Contig	PDZF00000000.1
Sporosarcina sp. P10	Base of telephone pole	Contig	PDYK00000000.1
Sporosarcina sp. P12	Base of pine tree	Contig	PDYL00000000.1
Sporosarcina sp. P13	Base of tree	Contig	PDYM00000000.1
Sporosarcina sp. P16b	Base of oak tree	Contig	PDYO00000000.1
Sporosarcina sp. P16a	Base of oak tree	Contig	PDYN00000000.1

Table 3.5, continued.

Strain	Isolation source	Genome status	Genome accession number
Sporosarcina sp. P17b	Base of tree	Contig	PDYP00000000.1
Sporosarcina sp. P18a	Base of terrace	Contig	PDYQ00000000.1
Sporosarcina sp. P19	Base of eucalyptus tree	Contig	PDYR00000000.1
Sporosarcina sp. P20a	Base of oak tree	Contig	PDYT00000000.1
Sporosarcina sp. P21c	Base of tree	Contig	PDYU00000000.1
Sporosarcina sp. P25	Dirt at foot	Contig	PDYV00000000.1
Sporosarcina sp. P26b	Dirt of walkway	Contig	PDYW00000000.1
Sporosarcina sp. P29	Dirt alley	Contig	PDYX00000000.1
Sporosarcina sp. P30	Front yard	Contig	PDYZ00000000.1
Sporosarcina sp. P31	Front yard	Contig	PDZA00000000.1
Sporosarcina sp. P32b	Public walkway	Contig	PDZB00000000.1
Sporosarcina sp. P34	Base of shrub	Contig	PDZC00000000.1
Sporosarcina sp. P35	Base of shrub	Contig	PDZD00000000.1
Sporosarcina sp. PTS2304	Feces	Complete	CP031230.1
Tetzosporium hominis VT-49	Dental calculus	Scaffold	NOKQ00000000.1
Ureibacillus thermosphaericus str. Thermo-BF	Hot spring water	Contig	AJIK00000000.1
Viridibacillus arenosi FSL R5-0213	Milk	Contig	ASQA01000000.1
Viridibacillus sp. FSL H7-0596	Milk	Contig	MSPV01000000.1
Viridibacillus sp. FSL H8-0123	Milk	Contig	MSPU01000000.1
Viridibacillus sp. OK051	Populus root and rhizosphere	Contig	PHTX00000000.1
Viridibacillus arvi DSM 16317^{T}	Soil	Scaffold	LILB00000000.1
Rummeliibacillus pycnus DSM 15030^{T}	Soil	Scaffold	NJAS00000000.1
Rummeliibacillus sp. POC4	Sewage sludge	Contig	QWUA00000000.1
Solibacillus isronensis B3W22^{T}	Cryotubes air samples from high altitudes	Contig	AMCK01000000.1
Solibacillus isronensis Marseille-P3605	Unknown	Contig	FVZN00000000.1
Solibacillus silvestris StLB046	Potato leaf	Complete	AP012157.1
Solibacillus silvestris DSM 12223^{T}	Forest soil	Complete	CP014609.1
Solibacillus silvestris MROC3	Domestic chicken feces	Contig	LZRJ00000000.1
Solibacillus kalamii ISSFR-015^{T}	HEPA filter from International Space Station	Contig	NHNT00000000.1

Table 3.5, continued.

Strain	Isolation source	Genome status	Genome accession number
Solibacillus sp. R5-41	Soil	Complete	CP024123.1
Sporosarcina koreensis S-K12	human gut	Scaffold	CDGU01000000.1
Sporosarcina psychrophila DSM 6497^{T}	soil/river water	Complete	CP014616.1
Sporosarcina sp. EUR3 2-2-2	permafrost	Contig	AXVE01000000.1
Sporosarcina newyorkensis 2681	human blood sample	Scaffold	AFPZ01000000.1
Sporosarcina sp. D27	sediment from creek	Contig	AZUC01000000.1
Sporosarcina sp. P37	base of single tree in sidewalk	Complete	CP015349.1
Sporosarcina sp. P33	soil	Complete	CP015027.1
Sporosarcina ureae P8	soil	Complete	CP015207.1
Sporosarcina ureae P17a	soil near to a tree	Complete	CP015109.1
Sporosarcina ureae P32a	public walkway	Complete	CP015348.1
Sporosarcina ureae S204	soil	Complete	CP015348.1
Sporosarcina pasteurii NCTC4822^{T}	soil	Contig	UGYZ00000000.1
Sporosarcina sp. P1	Base of eucalyptus tree	Contig	PDYS00000000.1
Sporosarcina sp. P2	Base of eucalyptus tree	Contig	PDYY00000000.1
Sporosarcina sp. P3	Base of eucalyptus tree	Contig	PDZE00000000.1
Sporosarcina sp. P7	Garden soil	Contig	PDZF00000000.1
Sporosarcina sp. P10	Base of telephone pole	Contig	PDYK00000000.1
Sporosarcina sp. P12	Base of pine tree	Contig	PDYL00000000.1
Sporosarcina sp. P13	Base of tree	Contig	PDYM00000000.1
Sporosarcina sp. P16b	Base of oak tree	Contig	PDYO00000000.1
Sporosarcina sp. P16a	Base of oak tree	Contig	PDYN00000000.1
Sporosarcina sp. P17b	Base of tree	Contig	PDYP00000000.1
Sporosarcina sp. P18a	Base of terrace	Contig	PDYQ00000000.1
Sporosarcina sp. P19	Base of eucalyptus tree	Contig	PDYR00000000.1
Sporosarcina sp. P20a	Base of oak tree	Contig	PDYT00000000.1
Sporosarcina sp. P21c	Base of tree	Contig	PDYU00000000.1
Sporosarcina sp. P25	Dirt at foot	Contig	PDYV00000000.1
Sporosarcina sp. P26b	Dirt of walkway	Contig	PDYW00000000.1
Sporosarcina sp. P29	Dirt alley	Contig	PDYX00000000.1
Sporosarcina sp. P30	Front yard	Contig	PDYZ00000000.1
Sporosarcina sp. P31	Front yard	Contig	PDZA00000000.1
Sporosarcina sp. P32b	Public walkway	Contig	PDZB00000000.1
Sporosarcina sp. P34	Base of shrub	Contig	PDZC00000000.1
Sporosarcina sp. P35	Base of shrub	Contig	PDZD00000000.1
Sporosarcina sp. PTS2304	Feces	Complete	CP031230.1
Tetzosporium hominis VT-49	Dental calculus	Scaffold	NOKQ00000000.1

Table 3.5, continued.

Strain	Isolation source	Genome status	Genome accession number
Ureibacillus thermosphaericus str. Thermo-BF	Hot spring water	Contig	AJIK00000000.1
Viridibacillus arenosi FSL R5-0213	Milk	Contig	ASQA01000000.1
Viridibacillus sp. FSL H7-0596	Milk	Contig	MSPV01000000.1
Viridibacillus sp. FSL H8-0123	Milk	Contig	MSPU01000000.1
Viridibacillus sp. OK051	Populus root and rhizosphere	Contig	PHTX00000000.1
Viridibacillus arvi DSM 16317^{T}	Soil	Scaffold	LILB00000000.1

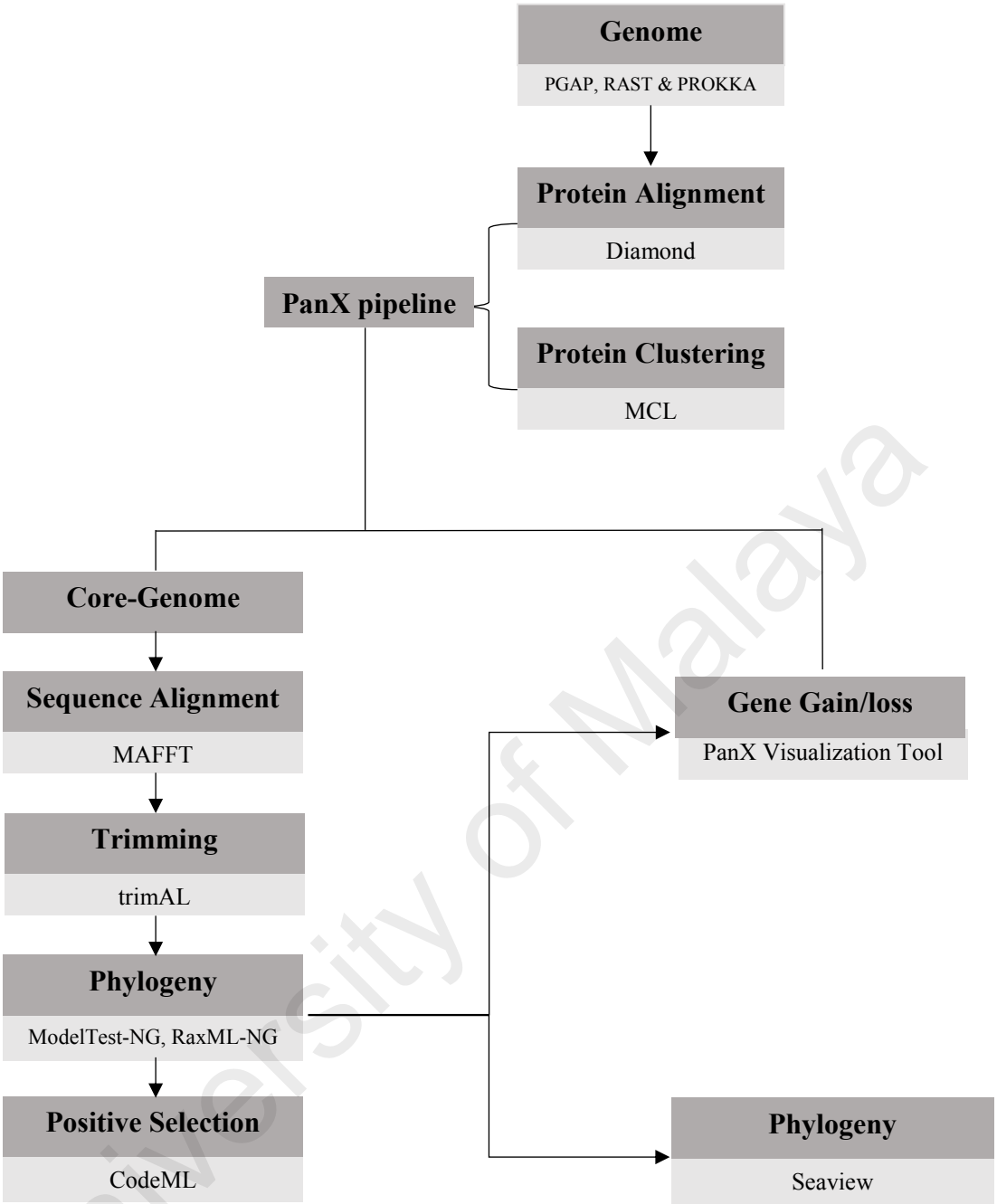


Figure 3.2: Schematic diagram of phylogenomic study workflow.

CHAPTER 4: RESULTS

4.1 4.1 General genome features

The data generated from WGS consisted of 135,526 polymerase reads with N50 of 9333 bp. The reads were subjected to filtering to remove low quality reads, and 167,910 subreads with N50 of 7768 bp were subsequently produced for *de novo* genome assembly using HGAP 2. The final assembly had mean coverage of 219.3x, and consisted of three contigs with self-overlapping ends (the indication of contig circularity). The sizes of the contigs were 3.2 Mb (chromosome), 70.7 kb (plasmid) and 9.8 kb (plasmid) (Figure 4.1, Appendix E). The preliminary identity assignment for each contig was determined based on sequence similarity in BLASTn searches. The identification of the 3.2 Mb contig as a chromosome was further supported by identification of an oriC region that contained 9 DnaA boxes. Therefore, the dnaA gene (1-1344) was set as the beginning of chromosomal contig and the oriC region (3248918-3249625) as the end. The completeness of the chromosome was assessed by a Z-curve plotted against the chromosomal contig. AT- and GC- disparity curves achieved a maximum value on the replication terminus site, and minimum at diametrically opposite locations corresponding to the predicted oriC (Appendix F). The presence of the predicted origin and terminus of replication strongly supports the completeness of the chromosome. Classification of pPS15-1 and pPS15-2 as plasmids was supported by identification of the replication module of plasmids. In pPS15-1, *parA* genes (WP_007723335.1) were identified, together with genes encoding replication proteins for the plasmid. For pPS15-2, the plasmid replication initiator protein gene, *RepB* (WP_049694148.1), was identified, which is a common feature of plasmids from cold-active bacteria. The 70.7 kb and 9.8 kb contigs were designated as pPS15-1 and pPS15-2, respectively. The general genome features based on NCBI PGAP are listed in Table 4.1.

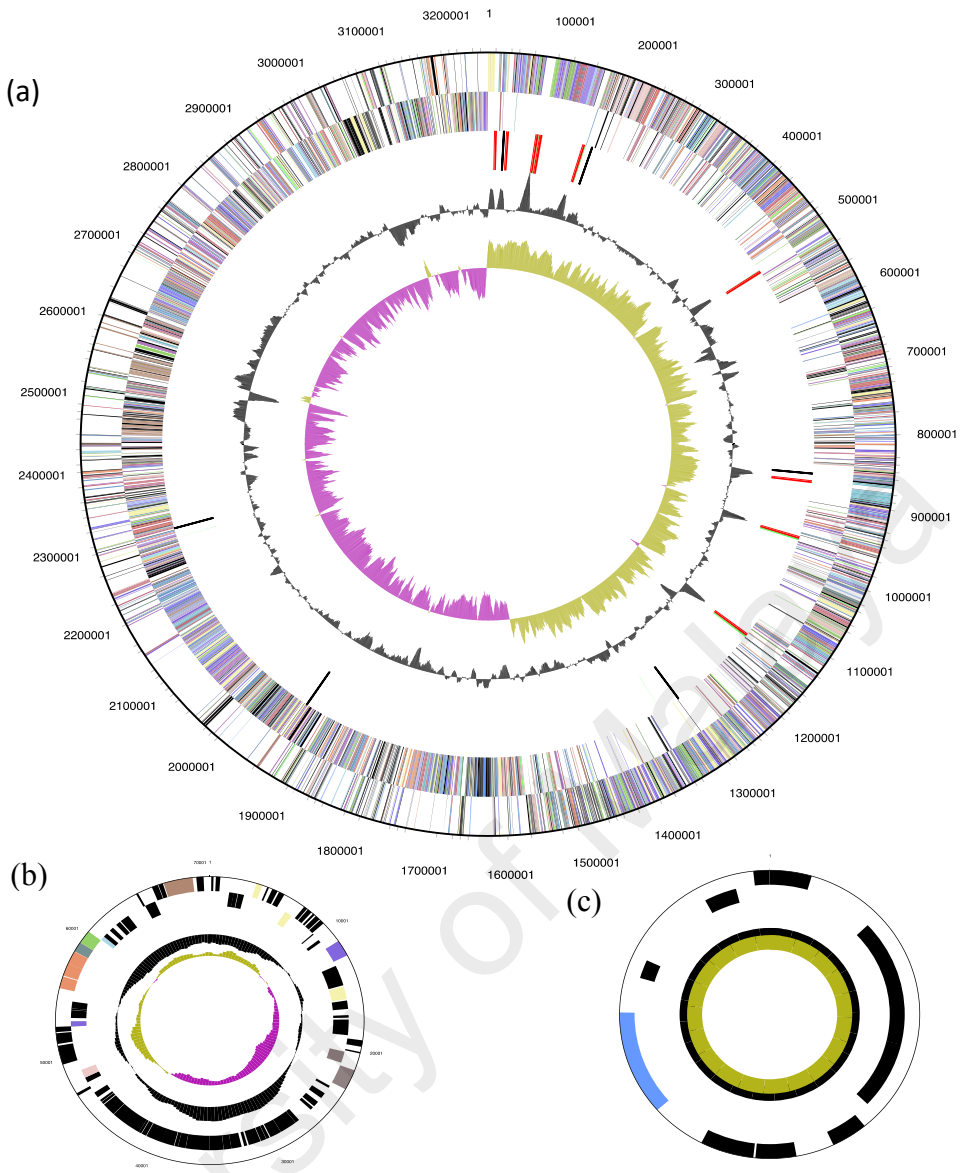


Figure 4.1: (a) The genomic ideogram of the chromosome and plasmids (b, c) of strain L10.15^{T}. From the outside to the centre: genes on forward strand (coloured by COG categories, Appendix E), genes on reverse strand (coloured by COG categories), RNA genes (tRNAs green, rRNAs red, other RNAs black), GC content and GC skew.

Table 4.1: General genome features of strain L10.15^{T}.

Features	Number
Chromosome	3249625 bp
pPS15-1	70675 bp
pPS15-2	9775 bp
mol% G+C content	39.4 %
rRNA genes (5S, 16S, 23S)	27 (9, 9, 9)
tRNA genes	71
Predicted coding sequences (CDS)	3153

4.2 Taxonomic study of strain L10.15^T

Strain L10.15^T was identified as *Planococcus* sp., a member of the family *Planococcaceae* which belongs to the class Firmicutes. Phenotypic, chemotaxonomic, and phylogenetic studies were conducted to confirm that this strain represents an undescribed species of *Planococcus,* and the name *P. versutus* sp. nov. is proposed.

4.2.1 Morphology of strain L10.15^T

Colony morphology of strain L10.15^T was orange-pigmented, circular, entire, smooth, convex and 1-2 mm in size on LB agar after 48 h incubation at 26 °C. Cells were observed to be motile and Gram-positive with no spore formation. Under SEM, cells were coccoid, typically 1.0-1.5 μm in diameter, mostly arranged as diplococci, but cells in single coccoid or tetrad were also observed (Figure 4.2). Results of all physiological tests, and comparison with closely related species, are presented in Table 4.2.

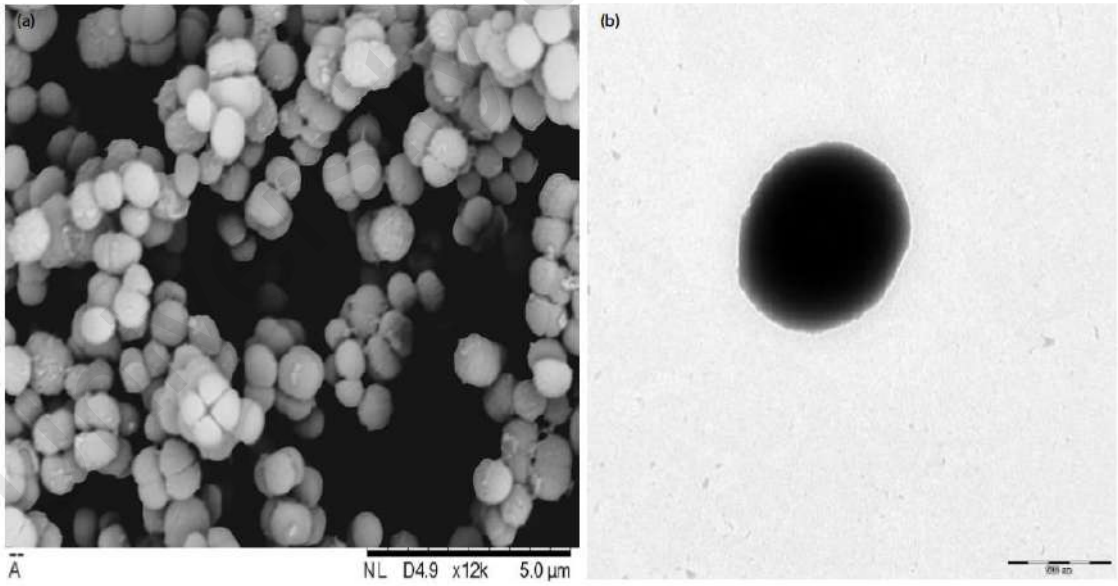


Figure 4.2: SEM (a) and STEM (b) micrographs of cells of strain L10.15^T grown at 26 °C. Most of the cells are observed as diplococci and cell division septa at different stages were also observed. Scale bars: a, 5 μm; b, 0.5 μm.

Table 4.2: Distinctive phenotypic characteristics of *P. versutus* L10.15^T and its phylogenetically closest related species. Strains: 1, L10.15^T; 2, *P. donghaensis* JH 1^T; 3, *P. halocryphilus* Orl^T; 4, *P. antarcticus* DSM 14505^T; 5, *P. kocurii* DSM 20747^T; 6, *P. maritimus* JCM 11543^T; 7, *P. plakortidis* DSM 23997^T and 8, *P. salinarum* ISL-16^T. All data were obtained in this study.

Characteristics	1	2	3	4	5	6	7	8
Growth:								
at pH	6.0-12.0	6.0-10.0	6.0-11.0	6.0-12.0	6.0-12.0	5.0-8.0	6.0-10.0	5.5-12.0
NaCl tolerance (%, w/v)	15	12	19	12	8	17	9	13
up to °C	30	37	37	26	37	41	37	38
From GenIII plate								
Assimilation of:								
D-Maltose	-	+	+	+	+	-	-	+
D-Trehalose	-	+	-	+	-	-	-	-
D-Cellobiose	-	-	-	+	-	-	-	-
Gentiobiose	-	+	-	-	-	-	-	-
Sucrose	-	+	-	-	-	-	-	-
D-Raffinose	-	-	-	+	-	-	-	-
α-D-Lactose	-	-	-	-	+	+	-	-
D-Melibiose	-	-	-	-	+	+	-	-
β-Methyl-D- Glucoside	-	+	+	+	-	-	-	-
D-Salicin	-	+	+	-	-	+	-	-
N-Acetyl-Glucosamine	+	+	+	-	+	+	-	-
N-Acetyl-*β*-D-Mannosamine	-	+	+	-	+	+	-	-
α-D-Glucose	+	+	+	-	+	-	-	-
D-Galactose	-	-	-	-	+	+	-	-
D-Fucose	-	+	-	-	-	-	-	-
L-Fucose	-	-	-	+	-	-	-	-

Table 4.2, continued.

Characteristics	1	2	3	4	5	6	7	8
L-Rhamnose	-	-	-	-	-	+	-	-
Inosine	+	+	+	-	+	+	+	-
D-Mannitol	+	+	+	+	+	+	-	-
D-Arabitol	-	-	-	+	-	-	-	+
myo-Inositol	-	-	-	+	-	-	-	-
Glycerol	+	+	+	+	+	+	-	-
D-Fructose-6-PO_4	+	+	+	-	+	+	+	-
D-Aspartic Acid	-	+	+	+	-	+	-	-
D-Serine	-	-	+	+	-	-	-	+
Gelatin	-	+	+	+	+	+	-	-
Glycyl-L-Proline	+	+	+	+	+	+	-	-
L-Arginine	-	+	+	+	+	+	-	+
L-Aspartic Acid	+	+	-	+	+	+	-	+
L-Histidine	-	+	+	+	-	-	-	-
L-Pyroglutamic Acid	+	+	+	-	+	+	-	+
L-Serine	+	+	+	-	+	+	+	-
L-Galactonic Acid Lactone	+	+	+	-	+	+	+	+
D-Gluconic Acid	+	+	+	+	+	+	-	+
D-Glucuronic Acid	+	+	-	-	+	+	+	+
Mucic Acid	+	+	+	+	+	+	-	+
Quinic Acid	-	+	+	+	+	+	-	+
D-Saccharic Acid	+	+	+	-	+	+	-	-
Methyl Pyruvate	-	-	-	+	-	-	-	-
L-Lactic Acid	-	+	+	+	-	+	-	-
α-Keto-Glutaric Acid	+	+	+	-	+	+	-	+

Table 4.2, continued.

Characteristics	1	2	3	4	5	6	7	8
D-Malic Acid	+	+	+	+	+	+	-	+
L-Malic Acid	+	+	+	-	+	+	-	+
Tween 40	+	+	+	+	+	+	+	+
γ-Amino-Butryric Acid	-	-	-	+	-	-	-	-
α-Hydroxy- Butyric Acid	-	-	+	+	-	+	-	-
α-Keto-Butyric Acid	-	-	+	-	-	+	+	-
Acetoacetic Acid	+	+	-	+	+	-	+	+
Propionic Acid	-	-	-	+	-	+	+	-
Acetic Acid	+	+	+	-	+	+	+	+
Formic Acid	+	+	+	-	+	-	-	+
Chemical Sensitivity:								
Fusidic Acid	+	-	-	-	-	-	-	-
D-Serine	-	-	+	-	-	-	-	-
Lincomycin	-	-	-	+	-	-	-	-
Guanidine HCl	-	-	-	+	-	-	+	-
Tetrazolium Violet	W	+	w	w	+	+	+	+
Tetrazolium Blue	-	-	-	w	-	-	-	-
Nalidixic Acid	+	+	-	w	+	-	+	+
Lithium Chloride	+	+	+	-	+	+	+	+
Potassium Tellurite	-	+	-	-	+	+	+	+
Sodium Bromate	W	w	-	+	-	-	+	-
API ZYM test:								
Alkaline phosphatase	-	+	-	-	+	+	+	+
Esterase	-	w	-	+	w	+	+	+
Leucine arylamidase	+	w	-	+	+	+	+	+

Table 4.2, continued.

Characteristics	1	2	3	4	5	6	7	8
Valine arylamidase	-	+	-	w	+	+	w	+
Cystine arylamidase	+	-	-	+	+	+	+	+
α-chymotrypsin	+	+	-	w	-	-	-	+
β- galactosidase	-	w	-	+	+	+	-	-
β-glucosidase	-	+	+	-	-	-	-	-
Genome features:								
Genome size (Mb)	3.37	3.32	3.42	3.83	3.49	3.29	3.28	NA
DNA G+C content (mol %)	39.4	40.1	40.1	43.2	40.9	47.2	50.0	NA
Number of genes #	4639	4417	4598	5040	4631	4609	4889	NA
Number of coding sequences #	4425	4196	4276	4811	4460	4365	4718	NA

4.2.2 Molecular identification and phylogenetic analysis

The 16S rRNA gene sequence pairwise similarity analysis confirmed that L10.15^T belongs the genus *Planococcus*, with higher sequence pairwise similarity to the type strain of *P. halocryophilus* (Table 4.3) based on the EzBiocloud database.

Based on the numbering of the *E. coli* 16S rRNA gene, the 16S rRNA gene sequence of strain L10.15^T included 183T and 190A, which are the signature nucleotides of *Planococcus* distinguishing it from *Planomicrobium* (Dai *et al.*, 2005; Yoon *et al.,* 2001). All 16S rRNA phylogenies concordantly demonstrated that strain L10.15^T clustered within *Planococcus*, but formed a distinct clade separate from *P. halocryophilus*, *P.*

donghaensis, *P. antarticus*, *P. plakortidis, P. maritimus*, *P. salinavum*, and *P. kocurii* (Figure 4.3).

Table 4.3: 16S rRNA sequence pairwise similarity of L10.15^T with the closest relatives in the EzBiocloud database.

Type Strain	16S rRNA Sequence Pairwise Similarity (%)
P. halocryophilus DSM 24743^T	99.3
P. donghaensis DSM 22276^T	99.0
P. antarcticus DSM 14505^T	98.3
P. plakortidis DSM 23997^T	97.6
P. maritimus DSM 17275^T	97.5
P. salinavum DSM 23820^T	97.5
P. kocurii DSM 20747^T	97.5

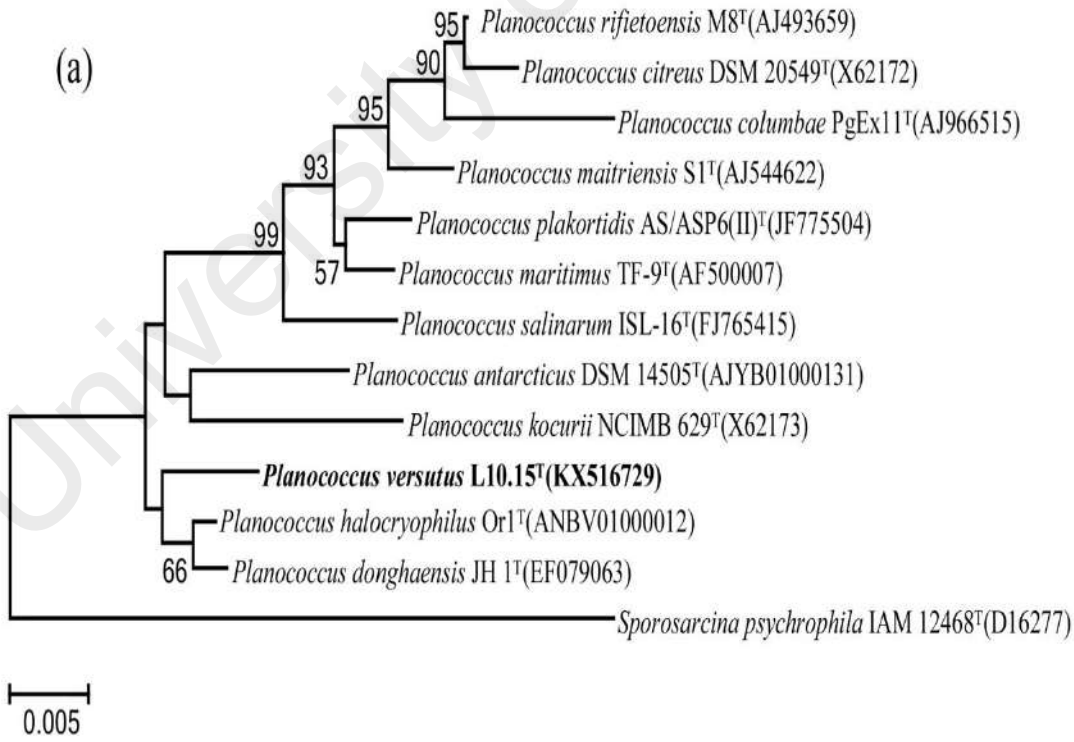


Figure 4.3: 16S rRNA gene phylogenetic trees of strain L10.15^T with related type species of the genus *Planococcus*, generated using (a) neighbour-joining and (b) maximum likelihood algorithms. Scale bar represents evolutionary distance as 0.005 change per nucleotide position. Only bootstrap values (%) > 50 from 1,000 replicates are shown.

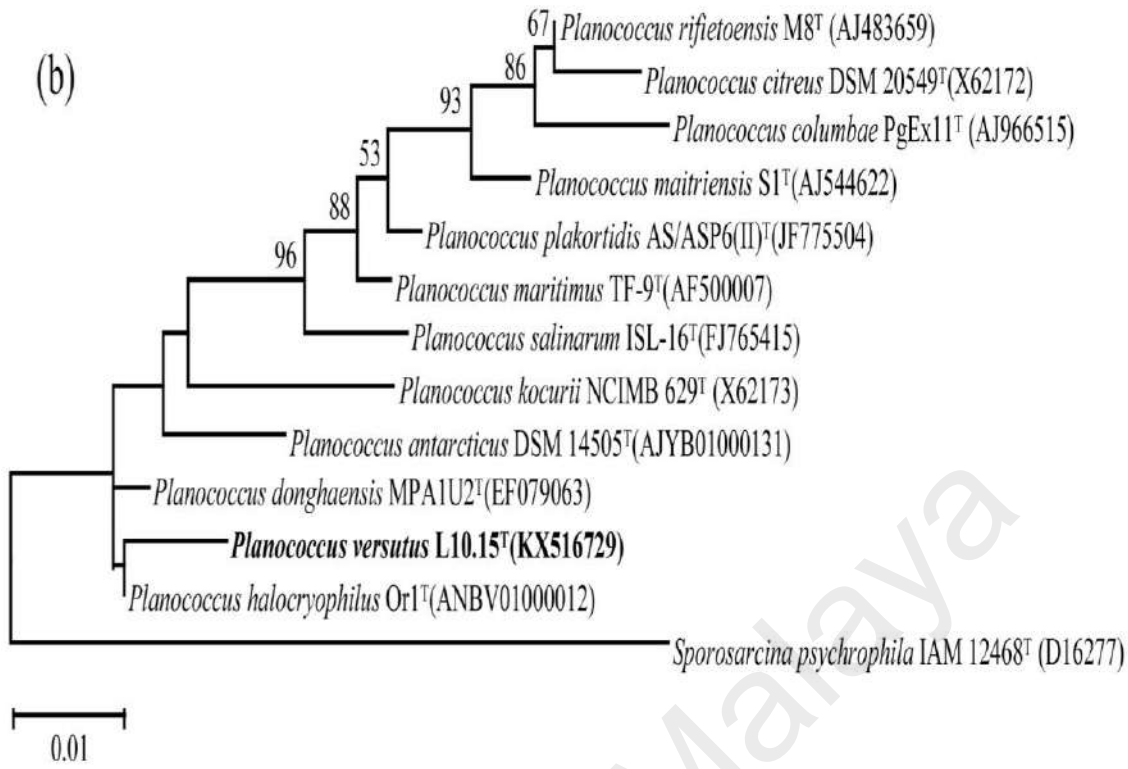


Figure 4.3, continued.

4.2.3 Genome relatedness analysis of strain L10.15^T

OrthoANI analysis gave values ranging from 71.9-82.3% for strain L10.15^T to the other type strains (Figure 4.4). ANIm and ANIb also gave ANI values ranging from 84.0-89.9% and 71.1-89.0%, respectively. These analyses confirm that strain L10.15^T does not belong to any of these related species, with values much lower than the accepted threshold of 94–96 % for bacterial species delimitation.

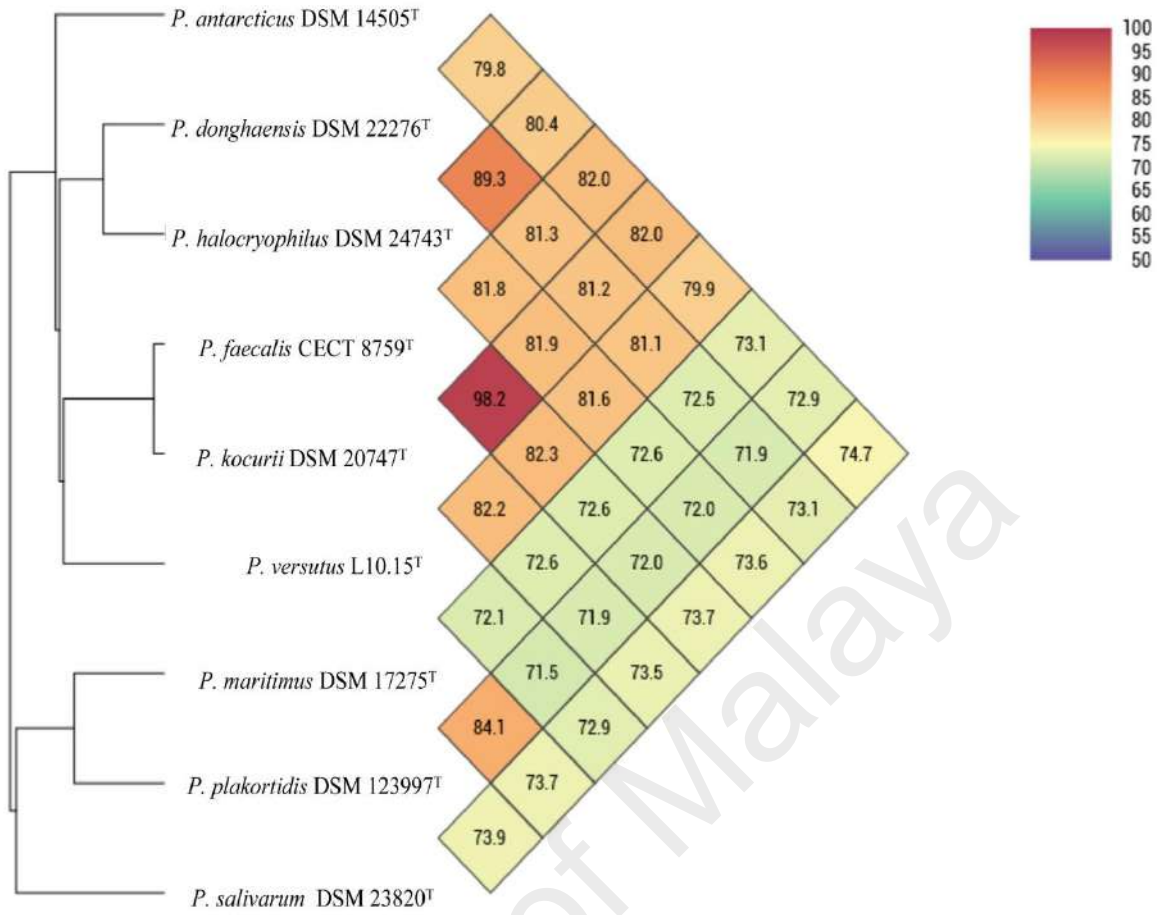


Figure 4.4: OrthoANI analysis of L10.15^T and other type strains of *Planococcus* sp.

4.2.4 Isoprenoid quinones and polar lipid analyses

The predominant menaquinones of strain L10.15^T were MK-5 (48 %), MK-6 (6 %) and MK-7 (44 %). The isoprenoid quinone profile was distinct from other *Planococcus* type strains, in which the predominant menaquinones were MK-6, MK-7 and MK-8 (Appendix G). The predominant polar lipids of strain L10.15^T were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and aminophospholipid. However, strain L10.15^T also displayed a complex polar lipid profile, with a number of uncharacterized polar lipids including an aminophospholipid, two lipids and four aminolipids (Figure 4.5)

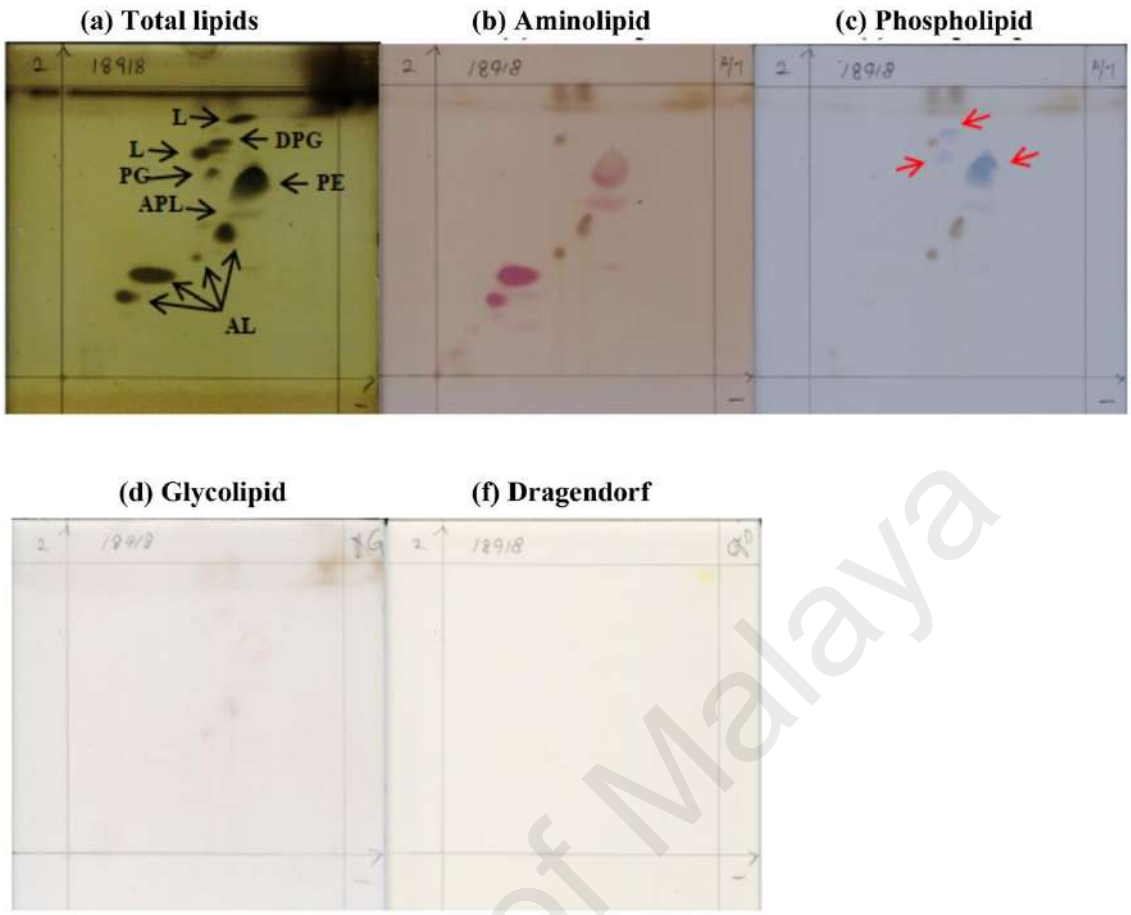


Figure 4.5: TLC chromatograms of polar lipid distribution of *P. versutus* L10.15^T visualized with 5 % ethanolic molybdatophosphoric acid for total lipids (a), ninhydrin (Sigma) for amino lipids (b), molybdenum blue (Sigma) for phospholipids (c), α-naphthol/sulphuric acid reagent for glycolipids (d), and Dragendorff reagent for cholinecontaining lipids (e). The polar lipid profile consisted of a mixture of phosphatidylethanolamine (PE), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), an unidentified aminophospholipid (APL), two unidentified lipids and four unidentified aminolipids (AL).

4.2.5 Cellular fatty acid analysis

The fatty acid composition of L10.15^T mainly consisted of anteiso-$C_{15:0}$ (46.2 %) and anteiso-$C_{17:0}$ (10.7 %), with other fatty acids as listed in Table 4.4. The major fatty acid composition of L10.15^T is similar to most of the *Planococcus* type strains, although there were differences amongst the trace fatty acids in the fatty acid profile.

Table 4.4: Cellular fatty acid profile of strain L10.15^T and close related species. Strains: (1) *P. versutus* sp. nov. L10.15^T; (2) *P. donghaensis* JH1^T; (3) *P. halocryphilus* Orl^T; (4) *P. antarcticus* DSM 14505^T; (5) *P. kocurii* DSM 20747 ^{T}; (6) *P. maritimus* JCM 11543 ^{T}; (7) *P. plakortidis* DSM 23997^T and (8) *P. salinarum* ISL-16 ^{T}. Values are percentages of the total fatty acids, with only fatty acids contributing > 0.5 % shown.

Fatty acid	1	2	3	4	5	6	7	8
Straight chain								
$C_{14:0}$	0.6	1.2	0.5	0.7	0.6	-	0.6	1.2
$C_{15:0}$	1.2	0.9	0.6	1.6	4.1	1.1	1.9	-
$C_{16:0}$	4.0	12.6	6.8	4.1	2.6	1.5	4.4	3.5
$C_{17:0}$	0.7	1.9	0.5	1.2	2.9	1.9	-	0.8
$C_{18:0}$	1.0	4.8	0.9	1.4	0.6	1.2	1.9	1. 8
Branched chain								
anteiso-$C_{13:0}$	0.6	-	0.5	-	-	-	-	-
iso-$C_{14:0}$	3.4	2.4	2.2	1.5	2.1	3.4	2.4	3.2
iso-$C_{15:0}$	1.9	2.3	2.5	3.6	3.6	9.8	5.2	2.5
anteiso-$C_{15:0}$	46.2	35.0	44.4	44.7	43.0	32.3	43.4	32.1
iso-$C_{16:0}$	5.5	4.6	4.9	3.7	4.0	4.2	6.5	3.7
iso-$C_{17:0}$	1.9	3.2	3.6	7.5	5.3	5.5	-	2.9
iso-$C_{17:1}$ $^{\omega 10c}$	1.3	0.9	-	3.5	2.7	4.1	-	3.3
anteiso-$C_{17:0}$	10.7	14.1	15.7	11.9	9.6	5.9	-	9.3
iso-$C_{18:0}$	0.7	1.0	0.4	-	0.6	4.7	1.5	-
Unsaturated								
$C_{16:1}$ $^{\omega 7c}$ alcohol	6.5	1.8	2.9	2.2	2.9	6.6	4.8	10.1
$C_{16:1}$ $^{\omega 11c}$ alcohol	5.6	5.8	4.6	2.9	3.8	1.5	2.8	1.8
$C_{17:1}$ $^{\omega 7}$	0.8	1.1	0.3	0.7	3.0	4.1	-	-
$C_{18:1}$ $^{\omega 9c}$	0.7	2.1	0.8	0.9	1.0	1.6	1.8	0.8
Summed feature 3†	0.6	-	-	-	0.4	-	-	1.0
Summed feature 4††	6.0	3.3	6.1	5.3	6.0	5.4	2.9	8.6

†Summed feature 3 contains $C_{16:1}^{\omega 7c}$ and/or $C_{16:1}$, which could not be separated by GC with the MIDI system.

††Summed feature 4 contains iso-$C_{17:1}$ and/or anteiso-$C_{17:1}$, which could not be separated by GC with the MIDI system.

4.3 Genomic data selection

Among the 140 genomes obtained from NCBI of the *Planococcaceae*, 36 were not included for further analyses due to mis-identification based on the 16S rRNA gene sequence identity from Ezbiocloud, or that the data did not contain any 16S rRNA gene sequences. The 104 selected genomes were further subjected to 'Benchmarking Universal Single-Copy Orthologs' (BUSCO) analysis. Only one genome had < 95% BUSCO value,

that of *Planococcus* sp. MB-3u-03, with the other 103 genomes having > 95% BUSCO values (Appendix H). These were used for further analysis. The 16S rRNA gene sequence similarity analysis using Ezbiocloud identified a number of misclassifications within the family *Planococcaceae*: (1) *Sporosarcina* sp. EUR-3-2-2 should be classified as *Paenisporosarcina* sp. EUR3-2-2, (2) *Planococcus* sp. CAU13 should be classified as *Planomicrobium* sp. CAU13 (Yoon *et al*., 2001) (3) *Planomicrobium soli* strain CGMCC 1.2259^T should be classified as *Planococcus soli* strain CGMCC 1.2259^T (Yoon *et al*., 2001).

4.3.1 Genome relatedness based on OrthoANI, POCP and AAI

To assess the reliability of the taxonomic classification among the genera, all the selected genomic data were subjected to OrthoANI analysis. The 103 selected strains can be classified into 71 species (Figure 4.6). According to both analysis, at least 12 strains were originally assigned to an incorrect taxon. Even though the dataset contains three *Bhargavaea cecembensis* strains, both *B. cecembensis* BC-2 and *B. cecembensis* T-14 have an ANI value lower than the 95% threshold, therefore should be classified as *Bhargavaea* sp. BC-2 and *Bhargavaea* sp. T-14. The type strain of *Planococcus faecalis*, has a 98% ANI value in both analyses with *P. kocurii*, and should therefore be reclassified into the latter taxon. Even though *P. donghaensis* MPA1U2 has a relatively high ANI value (92%) with the type strain of *P. donghaensis,* this still falls below the threshold of 95%, and the strain should therefore be reclassified as *Planococcus* sp. MPA1U2. *Planococcus maritimus* Y42 has only a 73% ANI value with the type strain *P. maritimus* DSM 17275^T, and should be classified as *Planococcus* sp. Y42. *Planomicrobium* sp. MB-3u-38 should be reclassified as *P. okeanokoites* MB-3u-38 since the strain has a 97% ANI value with the type strain of *P. okeanokoites,* strain IFO12536. The genomes of the type strains *Solibacillus silvestris* DSM12223^T, *S. kalamii* ISSFR-015^T and *S. isronensis*

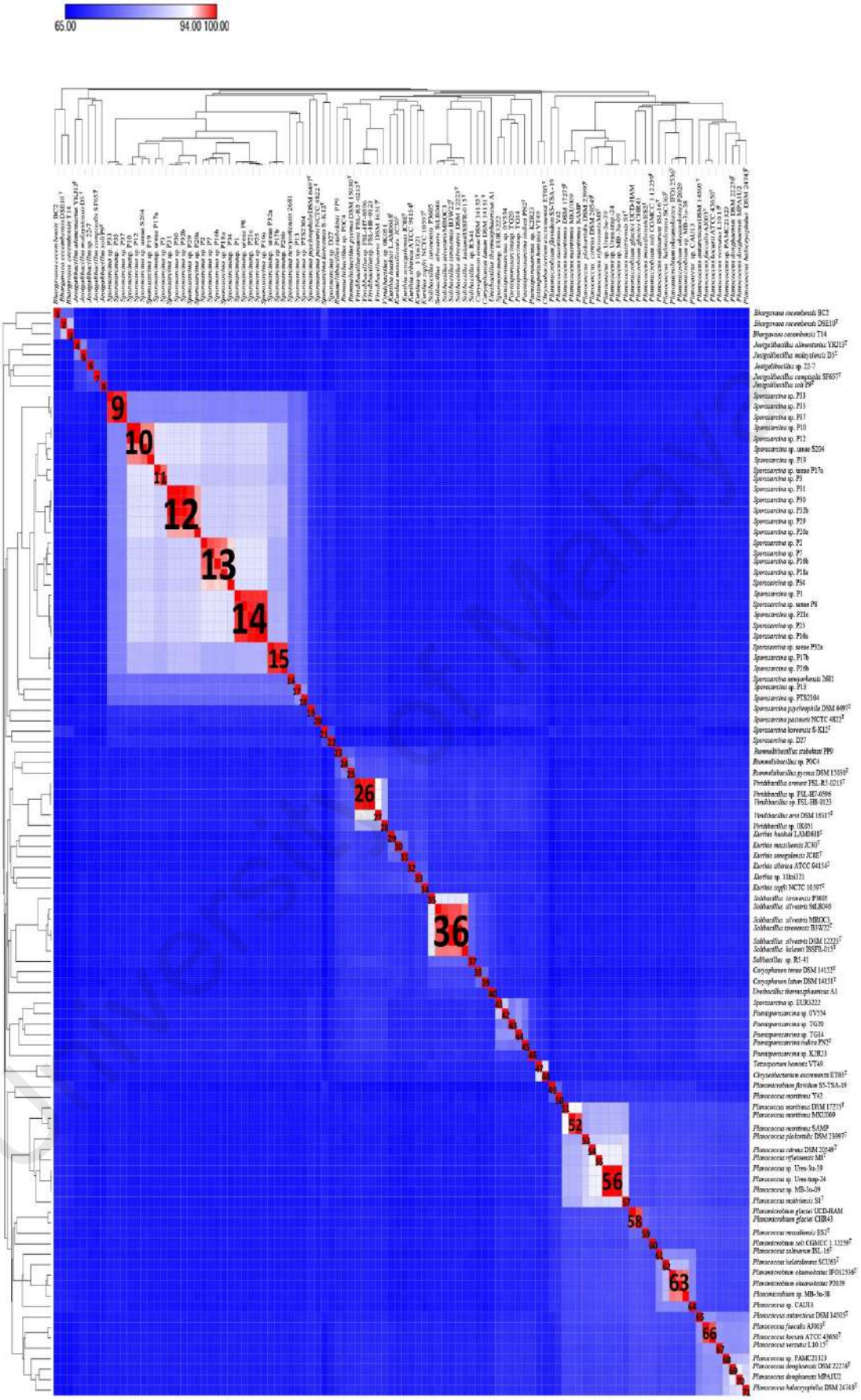


Figure 4.6: OrthoANI analysis of strains from family *Planococcaceae.* The numbers represent the number of species of the dataset.

B3W22^T represent the same taxon, since the ANI value is 97% and greater for each. *Solibacillus isronensis* Marseille P3605, however, may represent a novel species and should be classified as *Solibacillus* sp. Marseille P3605.

Representatives of the 71 species were selected and subjected to AAI and POCP to analyze generic delineation in *Planococcaceae.* The overall POCP values of the representatives from each genus were > 50%, contradict with POCP value of < 50% to delineate the bacteria genus as proposed by Qin *et al*. (2014) (Figure 4.7a). Generally, the POCP values among representatives from each genus ranged from 45-60%, excepting *Planomicrobium* and *Planococcus* which had a high POCP value of 74.4% for the type species *Planococcus citreus* DSM 20549^T and type strain *Planomicrobium okeanokoites* IFO 12536^T. The POCP values among strains from both genera were further examined, and all strains had POCP values of 60% and above. The AAI analysis results were consistent with the POCP outcomes, with all representatives from each genus having low AAI values to each other, ranging from 54-63%, except again for *Planomicrobium* and *Planococcus* which had a high AAI value of 73 (Figure 4.7b).

Further POCP and AAI analyses of *Planococcus* and *Planomicrobium* were conducted. These supported the finding that strains from the two genera are closely related, with POCP values ranging from 60.1-90.2% (Figure 4.8a), and AAI values of 65-100% (Figure 4.8b). Even though *P. maritimus* DSM 17275, *P. maritimus* Y42 and *P. flavidum* S5-TSA-19 had AAI values ranging from 65-67% to other strains of both *Planococcus* and *Planomicrobium,* these strains had high AAI value (77-100) from each other. This result is also consistent with the POCP analysis, which these three strains again have lowest POCP values from other strains. These results suggest that *Planomicrobium* should be reclassified into *Planococcus,* and a 60% POCP value is

proposed to delineate the genus *Planococcus.* The POCP and AAI analyses for each genus are given in Appendix I.

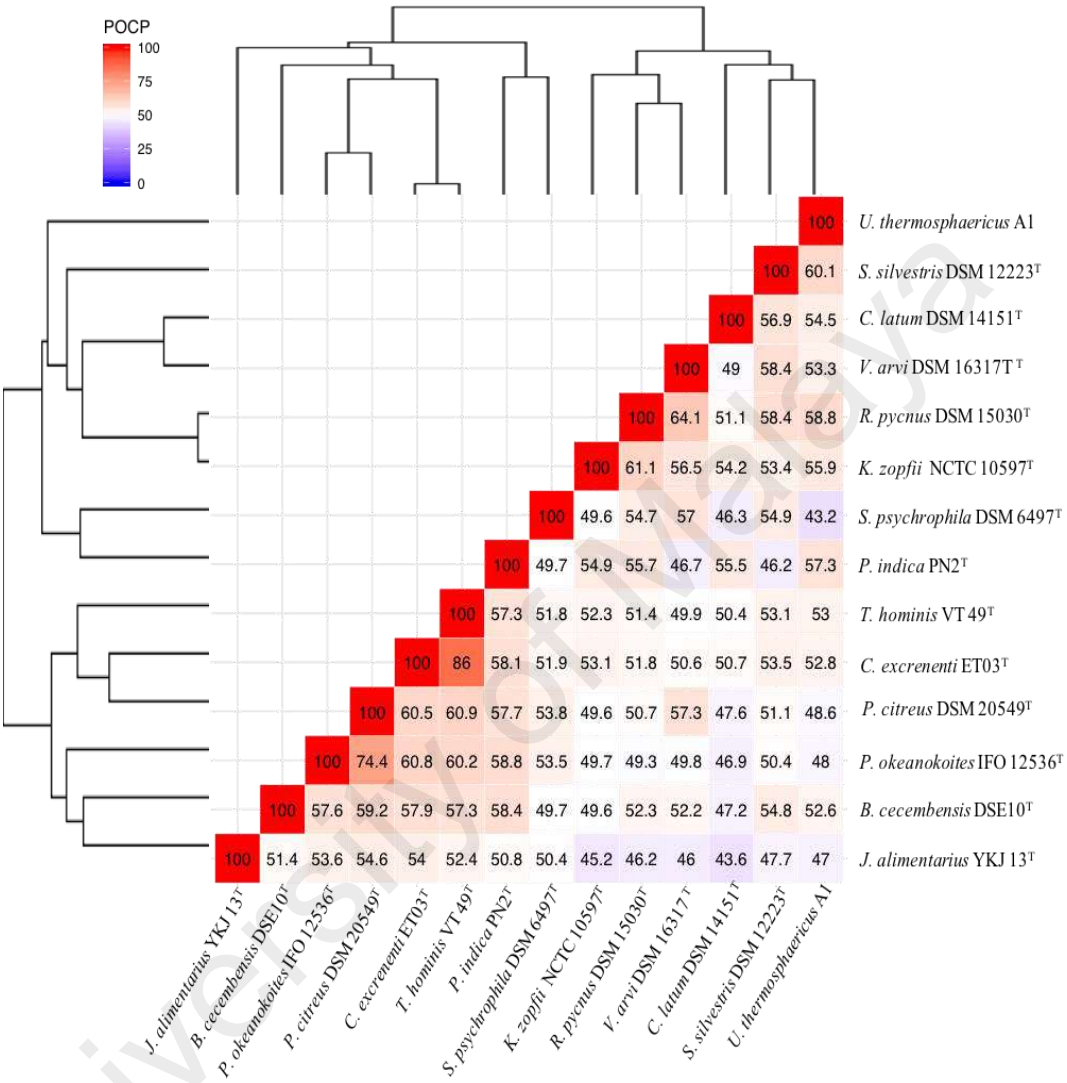


Figure 4.7: (a) POCP and (b) AAI analyses of representatives of each bacterial species from the dataset of *Planococcaceae.*

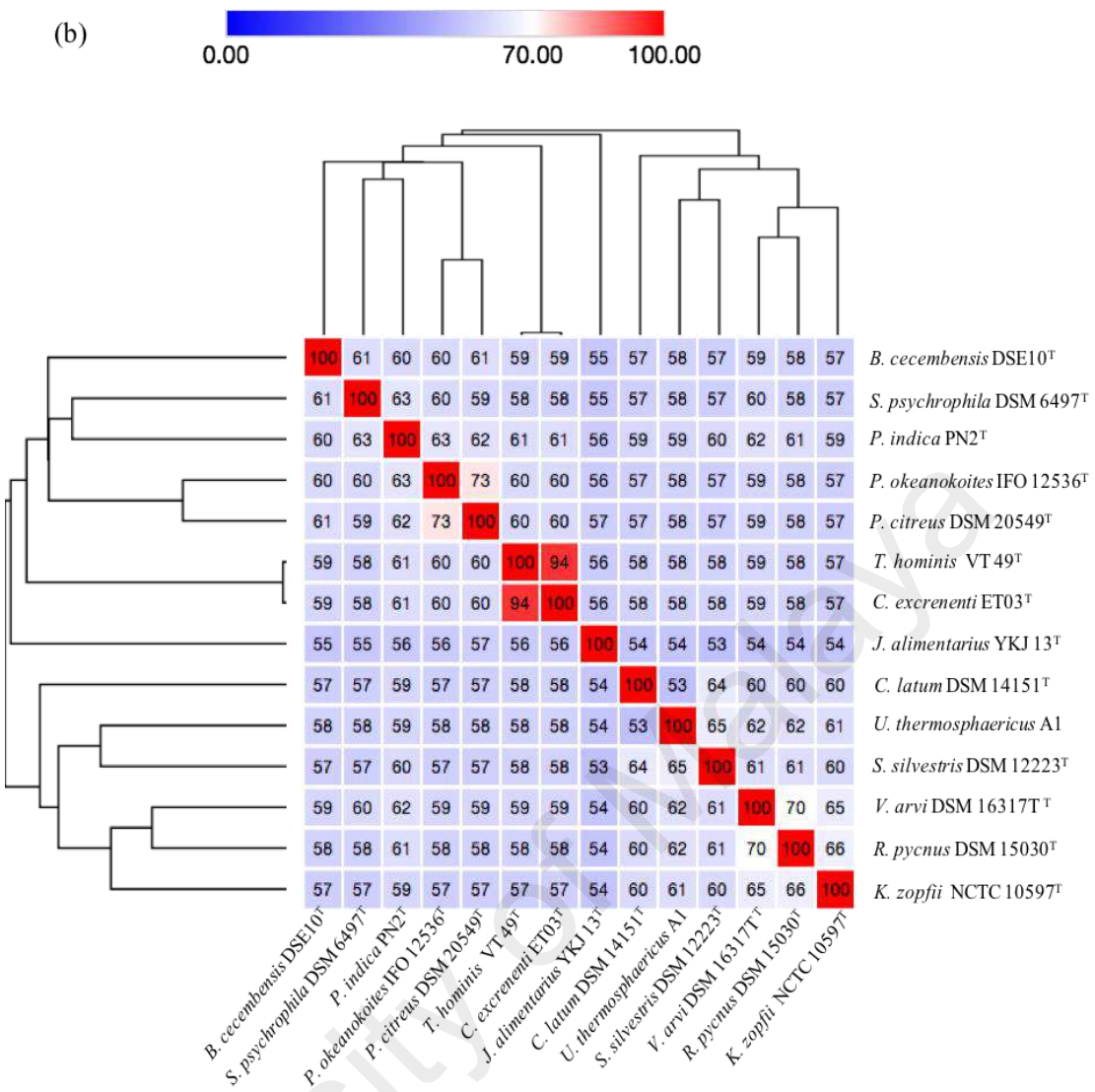


Figure 4.7, continued

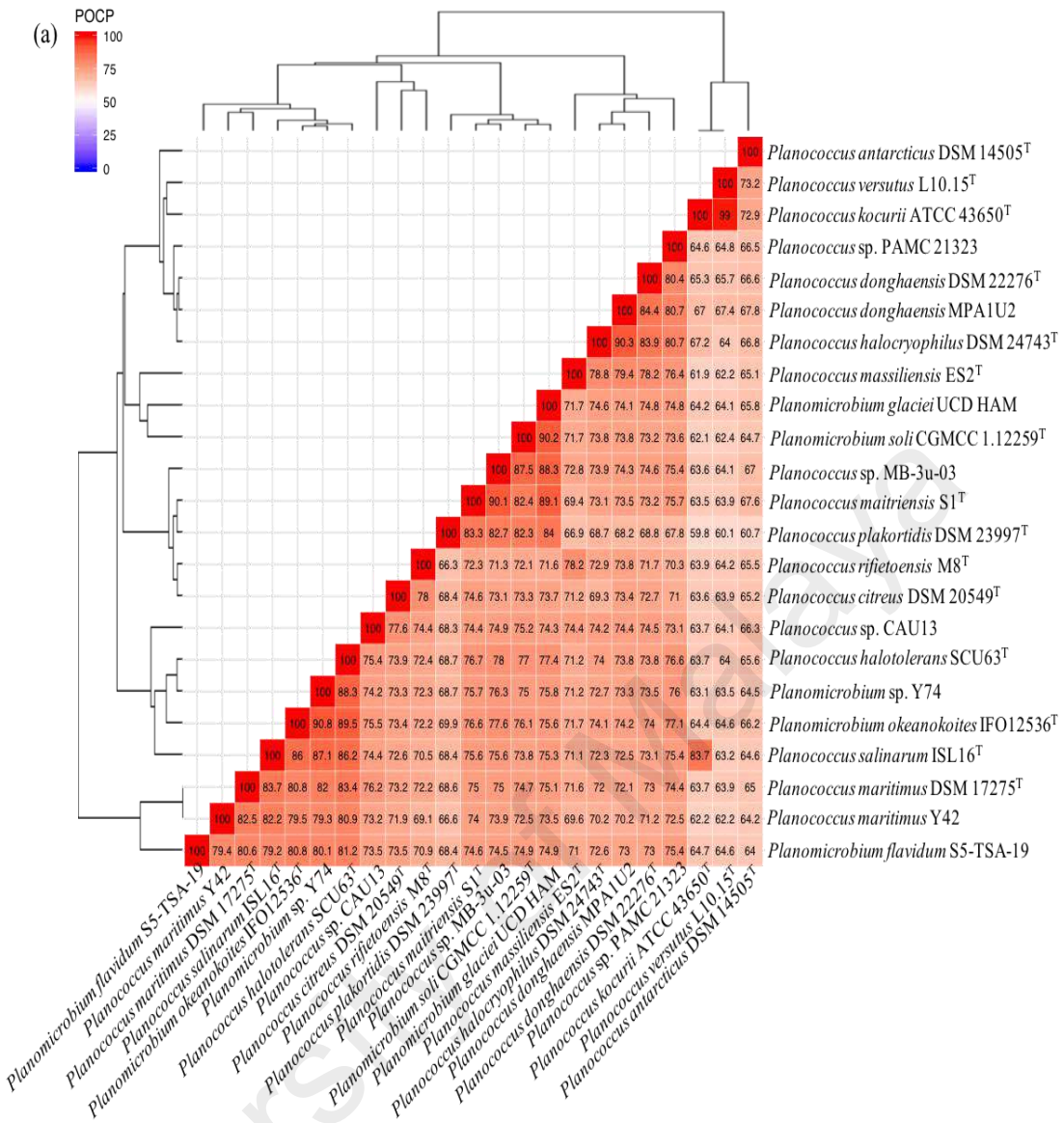


Figure 4.8: (a) POCP and (b) AAI analyses of representatives of each bacterial species from the genera *Planococcus* and *Planomicrobium*.

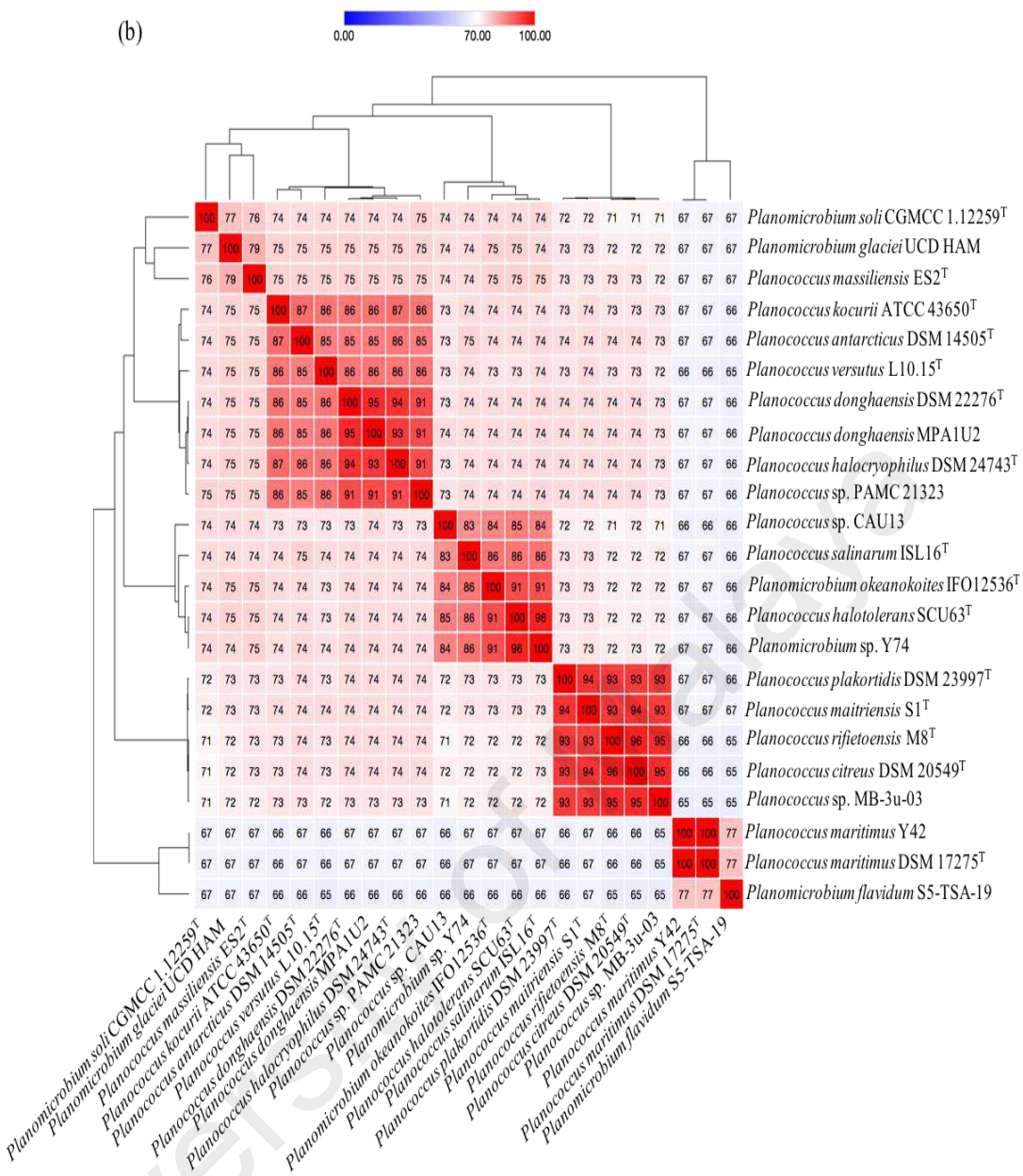


Figure 4.8, continued.

4.3.2 The pan-genome of *Planococcaceae*

The pan-genomic analysis of 103 *Planococcaceae* genomes reveals that these genomes contain 31,667 CDS that are clustered into 16389 gene clusters (GCs), with a core-genome consisting of 503 genes that are clustered into 352 GCs. The overall pan-genome results and the general genome features of *Planococcaceae* are presented in Figure 4.9.

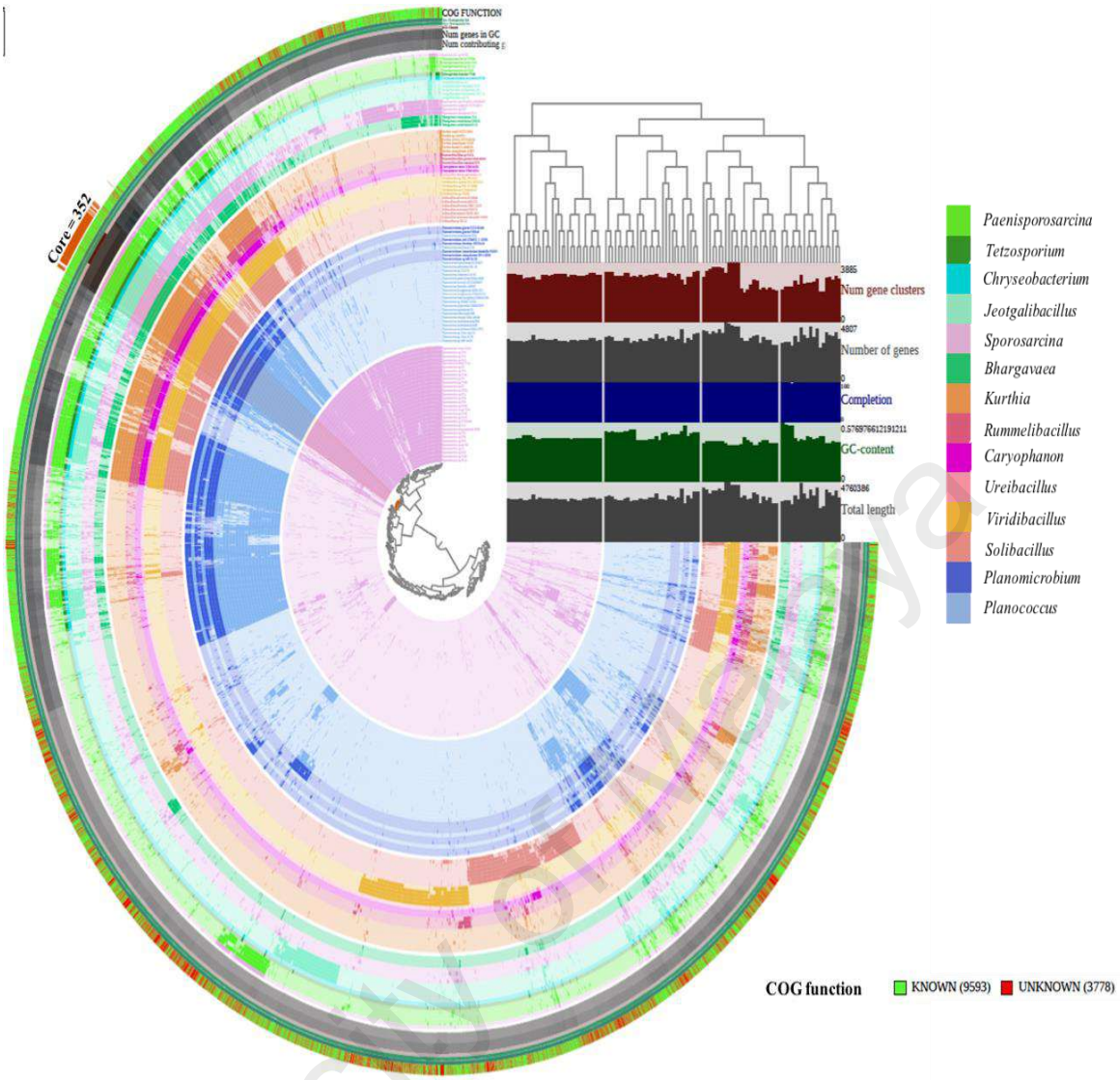


Figure 4.9: The overall pan-genome results and the general genome features of *Planococcaceae.*

4.3.3 Phylogenomic reconstruction of *Planococcaceae*

Using the concatenated nucleotide sequence alignment of 352 GCs obtained from the panX analysis, a well resolved phylogenetic tree with strong bootstrap support (> 99%) on all nodes was constructed (Figure 4.10). The 16S rRNA ML phylogenetic tree, however, gave a poorly-resolved phylogenetic tree with low support on numerous nodes (Figure 4.11). The phylogenomic tree reveals that all genera are monophyletic, except for *Planomicrobium* that was previously split from *Planococcus*. Both *Planomicrobium* and *Planococcus* formed 4 different clades in the phylogenomic tree. Clade 1 included *P. maritimus* strain Y42 and *P. flavidum* strain S5-TSA-19. Clade 2 included *P. maritimus*

Figure 4.10: Phylogenomic tree of the core-genome of family *Planococcaceae.*

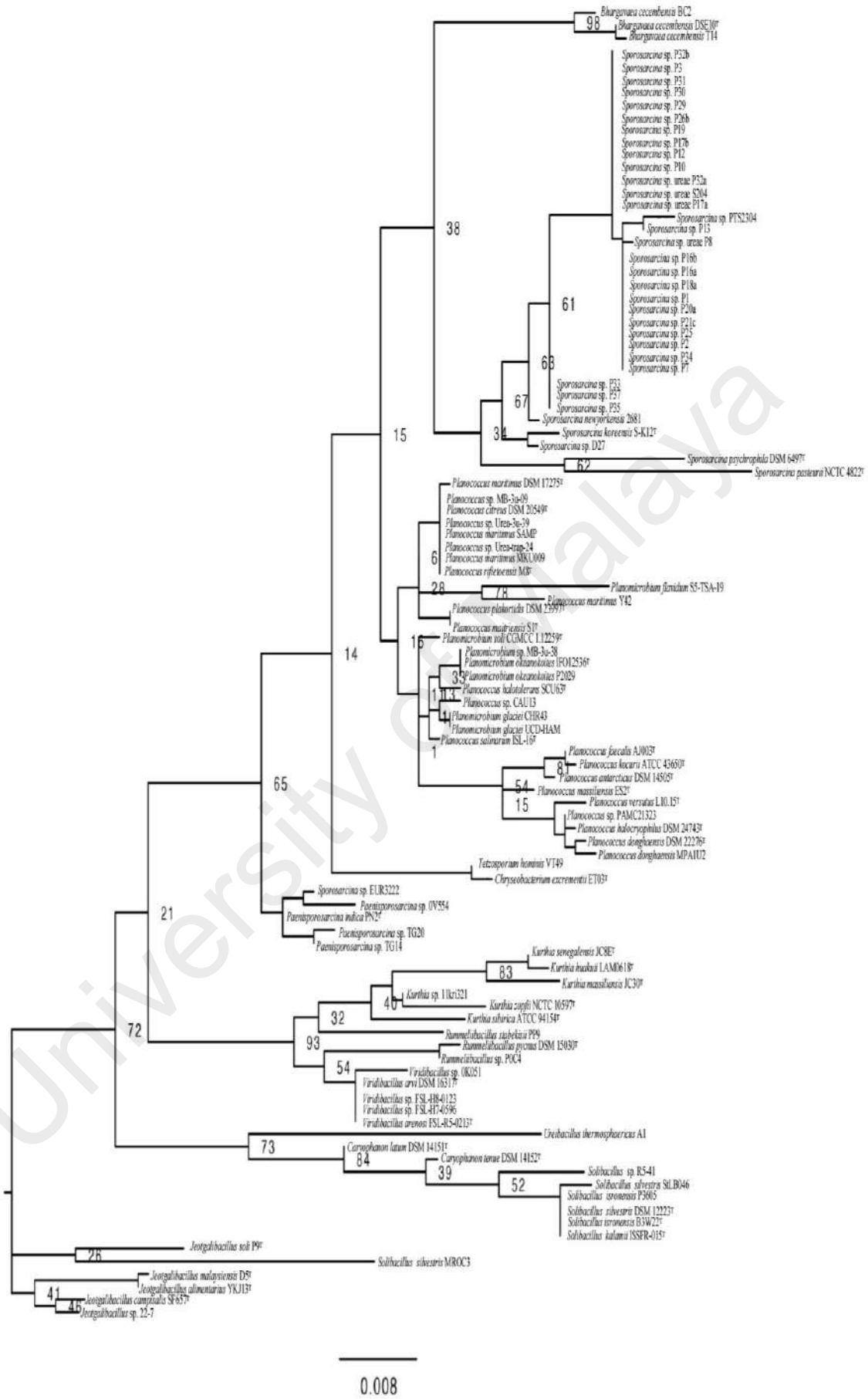


Figure 4.11: ML phylogenetic tree of family *Planococcaceae* generated using the 16S rRNA gene.

DSM 17275^T, *P. maritimus* SAMP, *P. maritimus* MKU009, *P. maitriensis* strain S1^T, *P. plakortidis* DSM 23997^T, *P. citreus* DSM 20549^T, *P. rifietoensis* M8^T, *Planococcus* sp. MB-3u-39, *Planococcus* sp. urea-3u-39 and *Planococcus* sp. urea-trap-24. Clade 3 included *Planococcus* sp. CAU13, *P. salinarum* ISL-16^T, *P. halotolerans* SCU63^T, *Planomicrobium* sp. MB-3u-38, *P. okeanokoites* IFO12536^T, *P. okeanokoites* Marseilie-P2029. Clade 4 included *Planomicrobium soli* CGMCC 1.12259, *P. massiliensis* ES^T, *P. glaciei* CHR43, *P. glaciei* UCD-HAM, *Planococcus antarcticus* DSM 14505^T, *P. faecalis* AJ003^T, *P. kocurii* ATCC 43650^T, *P. versutus* L10.15^T, *Planococcus* sp. PAMC 21323, *P. halocryophilus* DSM 24743^T, *P. donghaensis* DSM 22276^T and *P. donghaensis* MPA1U2.

Based on the method of identifying the conserved signature nucleotide in the 16S rRNA gene to differentiate *Planococcus* and *Planomicrobium* (Yoon *et al*., 2001), two taxa were wrongly assigned to the different genera. *Planococcus* sp. CAU13 is supposedly *Planomicrobium* sp. CAU13, and the type strain *Planomicrobium soli* CGMCC 1.12259^T is supposedly *Planococcus soli* CGMCC 1.12259^T. Therefore, with the exception of clade 2 that only consists of members of *Planococcus* with marine origin, all other clades include taxa from both genera. This finding suggests that the taxonomic status of *Planomicrobium* should be re-examined, and that its members may need to be reassigned to the genus *Planococcus.*

The 16S rRNA phylogenetic analysis yielded a poorly-resolved tree for a number of taxa. Paraphyly has also observed between *Rummeibacillus* and *Kurthia,* which formed two distinct clades in the phylogenomic tree. The branch length of intergenera taxa for 16S rRNA phylogenetic analysis also poorly resolved the taxonomic position of each taxon. The taxonomic position of type strains from *Planococcus, Planomicrobium,*

Solibacillus and *Viridibacillus*, which have high similarity of the 16S rRNA gene, did not formed a distinct branch in the phylogenomic tree. This suggests that further phylogenomic analyses are required in taxonomic studies of *Planococcaceae*, including the description of new genera.

4.3.4 The core-genome of family *Planococcaceae*

The GCs of the core-genome mostly contribute to essential functions including tricarboxylic acid (TCA) cycle, glycolysis and glyconeogenesis, carbon metabolism, pyruvate metabolism, DNA replication, cell division, transcription, translation, and even QS (Figure 4.12). The core-genome of *Planococcaceae* includes 194 (31.5%) of GCs categorized in metabolic responses, mostly involved in energy production and conversion (Table 4.5). A total of 85 GCs are present that are involved in RNA translation and protein synthesis (ribosomal structure and biogenesis), contributing 22.9% of the total core GCs. Genes encoding proteins essential for DNA transcription, including genes involved in the basic replication machinery for DNA repair, modification, and restriction were also identified in the core-genome, contributing 46 GCs (12.4%). Twenty-eight GCs (7.5%) were identified as genes encoding proteins for protein processing, folding and secretion. The COG annotation indicated only 46 GCs (12.4%) were poorly characterized (Table 4.5). Twenty-four GCs were categorized into more than one COG category, leading to a total number of annotated genes of 371, more than the total 352 of OGs.

Table 4.5: COG category of OGs from the core-genome.

COG CATEGORY	Number	%
CELLULAR PROCESSES AND SIGNALING		
[T] Signal transduction mechanisms	19	5.12
[U] Intracellular trafficking, secretion, and vesicular transport	5	1.35
[O] Post-translational modification, protein turnover, and chaperones	28	7.55
[V] Defense mechanisms	5	1.35
[M] Cell wall/membrane/envelope biogenesis	9	2.43
[D] Cell cycle control, cell division, chromosome partitioning	11	2.96
METABOLISM		
[C] Energy production and conversion	26	7.01
[P] Inorganic ion transport and metabolism	14	3.77
[F] Nucleotide transport and metabolism	22	5.93
[Q] Secondary metabolites biosynthesis, transport, and catabolism	4	1.08
[I] Lipid transport and metabolism	19	5.12
[H] Coenzyme transport and metabolism	13	3.50
[G] Carbohydrate transport and metabolism	11	2.96
[E] Amino acid transport and metabolism	8	2.16
INFORMATION STORAGE AND PROCESSING		
[L] Replication, recombination and repair	12	3.23
[J] Translation, ribosomal structure and biogenesis	85	22.91
[K] Transcription	33	8.89
[B] Chromatin structure and dynamics	1	0.27
POORLY CHARACTERIZED		
[R] General function prediction only	18	4.85
[S] Function unknown	28	7.55
	371	100.00

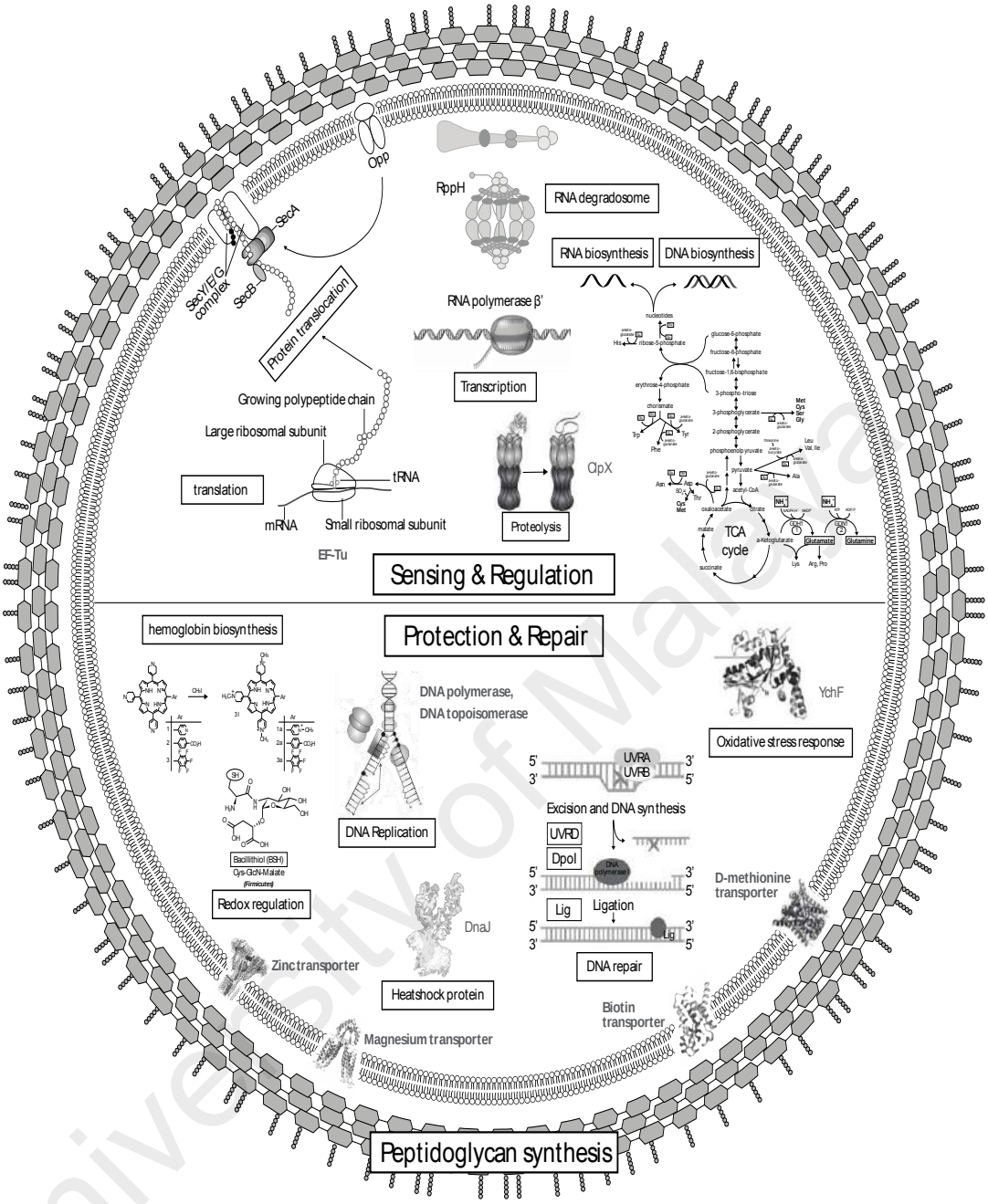


Figure 4.12: Schematic representation of the core-genome of *Planococcaceae.* The schematic representation was created using Adobe Illustrator.

4.3.5 Assessment of positive selection of core-genome

Positive selection of amino acids in the core protein cluster was assessed using the constructed phylogenomic tree, and the branch including *Planococcus* spp. isolated from cold environments was set as foreground branch (Appendix J). Among 352 core protein clusters, 70 clusters were detected with positive sites (>0.95) based on Bayes Empirical Analysis (BEB) of codeML analysis. However, only 52 genes (Table 4.6) passed the

Likelihood Ratio Test with $p < 0.05$ (Appendix K). Based on the KEGG analysis, the core protein clusters with positive sites are mostly involved in metabolic pathways (16 clusters) and biosynthesis of secondary metabolites. Some proteins that serve crucial functions in biological processes including DNA replication, DNA repair, TCA cycle, glycolysis and QS were also detected with positive sites.

Table 4.6: List of core GCs with positive selection pressure amino acids. Locus tag and amino acid position are based on the genome of *P. versutus* L10.15 (CP016540.2) and the amino acid position is based on the position of the site patterns after deleting the gaps identified in alignment by codeML. BEB analysis with value 0.95 and above are shown.

Gene	Locus Tag	Amino acid position	Bayes Empirical Bayes analysis
Threonine--tRNA ligase	I858_RS06605	73S	0.999
Methionine adenosyltransferase	I858_RS05975	307K	0.990
NAD-dependent DNA ligase LigA	I858_RS04470	14L	0.958
		17E	0.999
		77Y	0.963
		87N	0.979
		91L	0.961
		229T	0.976
		234A	0.953
		236N	0.974
		238M	0.952
		475Q	0.998
		568R	0.971
		642T	0.976
		645E	0.986
MBL fold metallo-hydrolase	I858_RS06245	277R	0.993
Ferrochelatase	I858_RS05180	63C	0.976
30S ribosomal protein S10	I858_RS00625	57V	0.951
DNA repair protein RadA	I858_RS00450	34D	0.990
CTP synthase	I858_RS13440	288S	0.998
Protein translocase subunit SecDF	I858_RS07050	334N	0.999
		345K	0.999
		431L	0.999
Phosphate acyltransferase PlsX	I858_RS10890	44E	0.963
		81R	0.959
		90T	0.991

Table 4.6, continued.

Gene	Locus Tag	Amino acid position	Bayes Empirical Bayes analysis
Threonine--tRNA ligase	I858_RS06605	73S	0.999
Methionine adenosyltransferase	I858_RS05975	307K	0.990
NAD-dependent DNA ligase LigA	I858_RS04470	14L	0.958
		17E	0.999
		77Y	0.963
		87N	0.979
		91L	0.961
		229T	0.976
		234A	0.953
		236N	0.974
		238M	0.952
		475Q	0.998
		568R	0.971
		642T	0.976
		645E	0.986
MBL fold metallo-hydrolase	I858_RS06245	277R	0.993
Ferrochelatase	I858_RS05180	63C	0.976
30S ribosomal protein S10	I858_RS00625	57V	0.951
DNA repair protein RadA	I858_RS00450	34D	0.990
CTP synthase	I858_RS13440	288S	0.998
Protein translocase subunit SecDF	I858_RS07050	334N	0.999
		345K	0.999
		431L	0.999
Phosphate acyltransferase PlsX	I858_RS10890	44E	0.963
		81R	0.959
		90T	0.991
YihA family ribosome biogenesis GTP-binding protein	I858_RS06815	105E	0.991
		191S	0.974
DNA polymerase I	I858_RS06565	421K	0.977
		441N	0.996
		472Q	0.998
		522I	0.951
		795D	0.952
Pur operon repressor	I858_RS00220	113E	0.975
Phenylalanine--tRNA ligase subunit beta	I858_RS06670	384Q	0.995
		531E	0.979
		579S	0.974
		584E	0.986
		585E	0.965
		790Q	0.958
Histidine--tRNA ligase	I858_RS07090	74S	1.000
		346E	0.978
		349G	0.998

Table 4.6, continued.

Gene	Locus Tag	Amino acid position	Bayes Empirical Bayes analysis
Alanine--tRNA ligase	I858_RS07140	250E	0.976
		367K	0.974
		756Q	0.970
		786T	0.968
		799G	0.956
		804A	0.964
		859V	0.972
Thioredoxin	I858_RS04975	83E	0.983
		150L	0.994
tRNA epoxyqueuosine(34) reductase QueG	I858_RS04945	232E	0.999
		360N	0.966
DNA-binding protein	I858_RS02430	84A	0.998
Phosphoglucosamine mutase	I858_RS00905	200S	0.969
		264V	1.000
2-oxoglutarate dehydrogenase E1 component	I858_RS09410	105P	0.989
		210K	0.998
DNA repair protein RecO	I858_RS07365	141V	0.960
rRNA maturation RNase YbeY	I858_RS07345	51A	0.979
		58R	0.968
Bifunctional UDP-N-acetylglucosamine diphosphorylase/glucosamine-1-phosphate *N*-acetyltransferase GlmU	I858_RS00235	275Q	0.962
		313V	0.999
		331L	0.978
Phosphatidyl-glycerophosphatase A	I858_RS11705	104C	0.973
Isoprenyl transferase	I858_RS10740	9K	0.983
tRNA pseudouridine(38-40) synthase TruA	I858_RS00785	67T	0.959
		225Q	0.955
Metal-dependent hydrolase	I858_RS04705	238S	0.995
ABC transporter ATP-binding protein	I858_RS04725	95D	0.998
		302V	0.994
Elongation factor G	I858_RS00570	202E	0.952
2-oxo acid dehydrogenase subunit E2	I858_RS03115	79P	0.996
DEAD/DEAH box helicase	I858_RS08950	41S	1.000
1-deoxy-D-xylulose-5-phosphate synthase	I858_RS08705	87E	0.991
Valine--tRNA ligase	I858_RS06850	150K	0.998
		153R	0.951
		596K	0.996
		851A	0.998
Isoleucine--tRNA ligase	I858_RS11090	706N	0.998
GTPase Era	I858_RS07360	102F	0.954
		166Y	0.999

Table 4.6, continued.

Gene	Locus Tag	Amino acid position	Bayes Empirical Bayes analysis
Signal recognition particle protein	I858_RS10850	15N	0.998
		399K	0.986
1-aspartate—tRNA ligase	I858_RS07095	4T	0.983
		93I	0.964
		397K	0.999
		398K	0.992
		445Y	0.978
Phospho-N-acetylmuramoyl-pentapeptide-transferase	I858_RS11155	84F	0.953
		125K	0.960
		256L	0.999
Glycine cleavage system protein GcvH	I858_RS05510	75K	0.999
		107D	0.995
SsrA-binding protein SmpB	I858_RS05445	41K	1.000
		66S	1.000
		81S	0.999
		97H	0.999
Phosphoglycerate kinase	I858_RS05410	27E	0.977
		50Q	0.998
		66Q	0.997
		247K	0.992
		267T	0.998
		325E	0.977
Hydroxymethylbilane synthase	I858_RS06830	235H	0.979
		302Q	0.996
3-deoxy-7-phosphoheptulonate synthase	I858_RS06310	135S	0.973
Aromatic acid exporter family protein	I858_RS04755	98S	0.984
		114S	0.974
		119T	0.998
Aconitate hydratase AcnA	I858_RS08165	254G	0.988
		272V	0.982
		338V	0.992
		350T	0.987
		360I	0.992
		430V	0.982
		485D	0.984
		493E	0.985
		556P	0.993
		588K	0.986
Fructose-bisphosphate aldolase	I858_RS13425	47C	0.981
		261G	0.986
Metal-sulfur cluster assembly factor	I858_RS11880	14N	0.977
		60D	0.986
		64T	0.997

Table 4.6, continued.

Gene	Locus Tag	Amino acid position	Bayes Empirical Bayes analysis
Endopeptidase La	I858_RS06810	556G	0.995
2-oxoglutarate dehydrogenase complex dihydrolipoyllysine-residue succinyltransferase	I858_RS09405	187V	0.996
		188K	0.996
		318N	0.998
Cell wall metabolism sensor histidine kinase WalK	I858_RS16195	54N	0.955
		305D	0.969

4.4 Ancestral character-state reconstruction and analysis of trait evolution

Using the panX pipeline, applied to the phylogenomic tree generated using SNPs in the core-genome (Figure 4.13), numerous traits that were evolutionarily related to cold-adaptation were identified. Carotenoid biosynthesis protein was identified in almost all members of *Planococcus*, *Planomicrobium, Jeotgalibacillus* and *Kurthia* (Figure 4.14a). Two types of carotenoid degradation genes were identified in *Planococcaceae, β,β-carotene 15,15'-monooxygenase* and *carotenoid oxygenase. β,β-carotene 15,15'-monooxygenase*, which cleave the *β-carotene* possessed by all members of *Bhargavaea, Viridibacillus,* and some members of *Planococcus, Planomicrobium,* and *Kurthia.* Interestingly, some members of the *Planococcus* that possess the carotenoid biosynthesis protein seem to have undergone a gene loss event for *β,β-carotene 15,15'-monooxygenase* (Figure 4.14b). Another carotenoid degradation gene, *carotenoid oxygenase*, was identified in a group of *Sporosarcina* (Figure 4.14c), and this gene was not detected in other genera of *Planococaceae.* These findings imply that those members which have undergone a gene loss event for the carotenoid degradation gene will accumulate carotenoid protein in the cell.

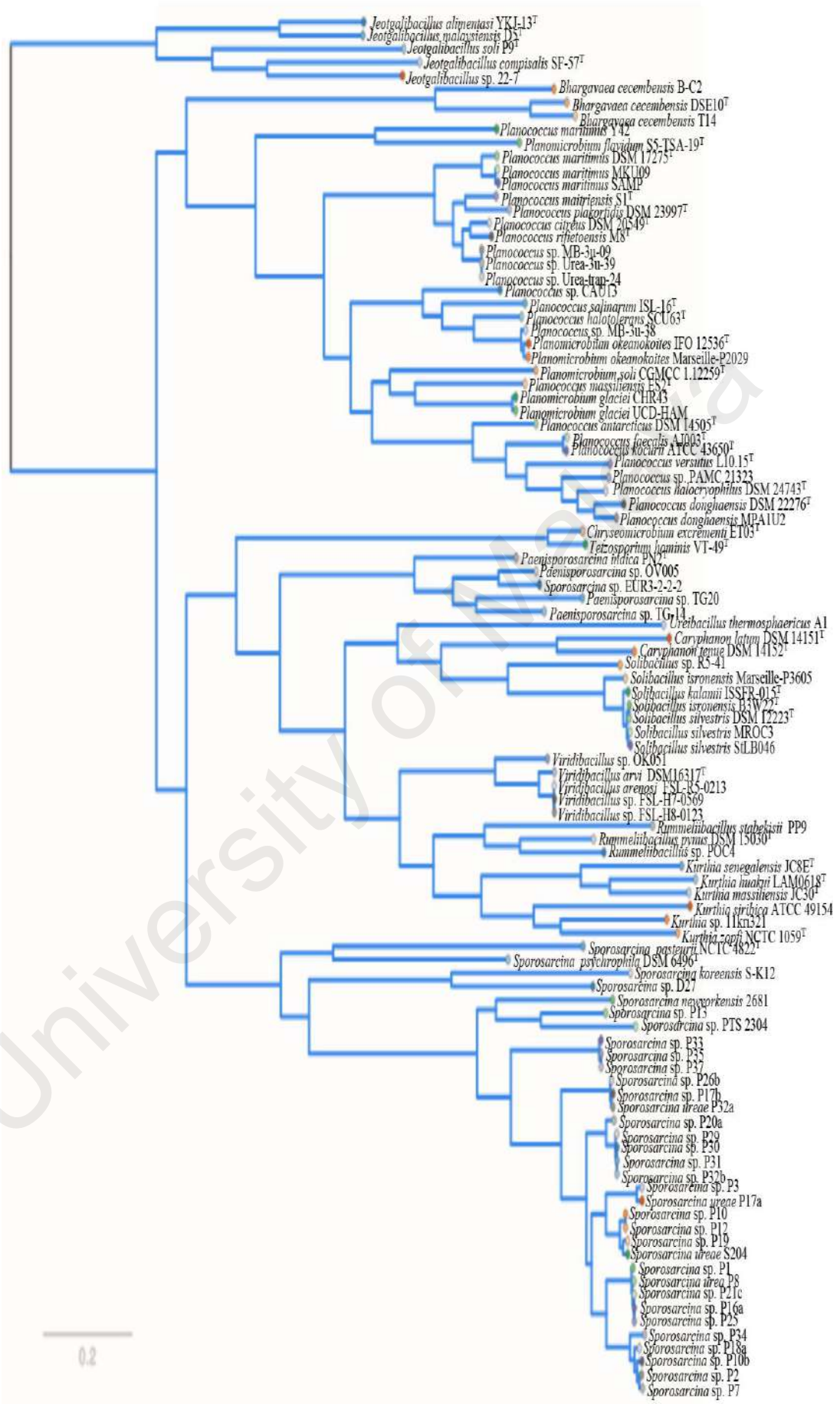


Figure 4.13: Phylogenomic tree generated using SNPs in the core-genome.

Glycine/betaine ABC transporter was identified in all genera except *Sporosarcina.* The gene duplication event has occurred 16 times among strains with this gene in this dataset. Antifreeze protein was only identified in *Sporosarcina*.

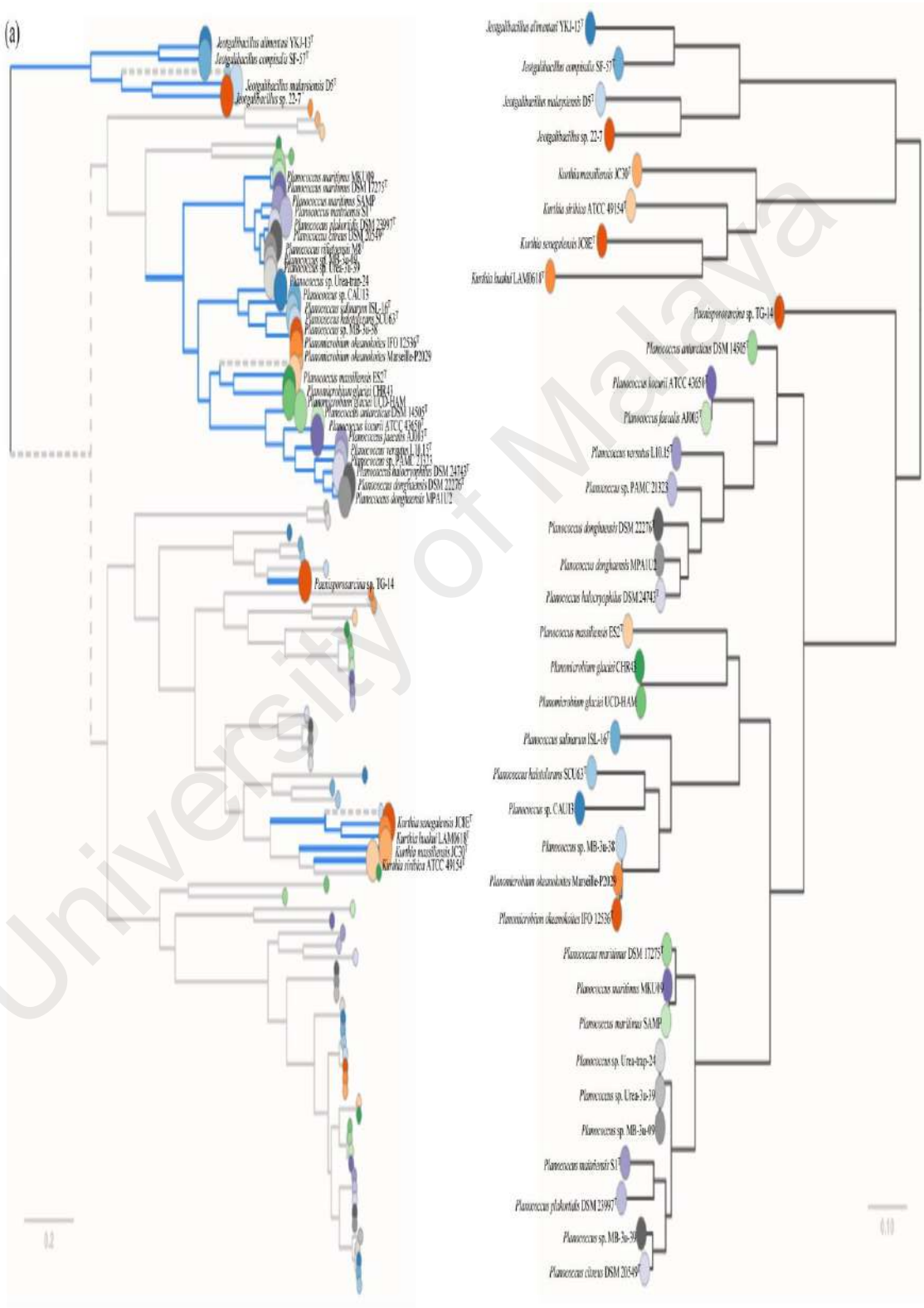


Figure 4.14: Gene gain/loss events in *Planococcaceae* for (a) carotenoid biosynthesis, (b) β-carotene monooxgenase, and (c) carotenoid oxgenase.

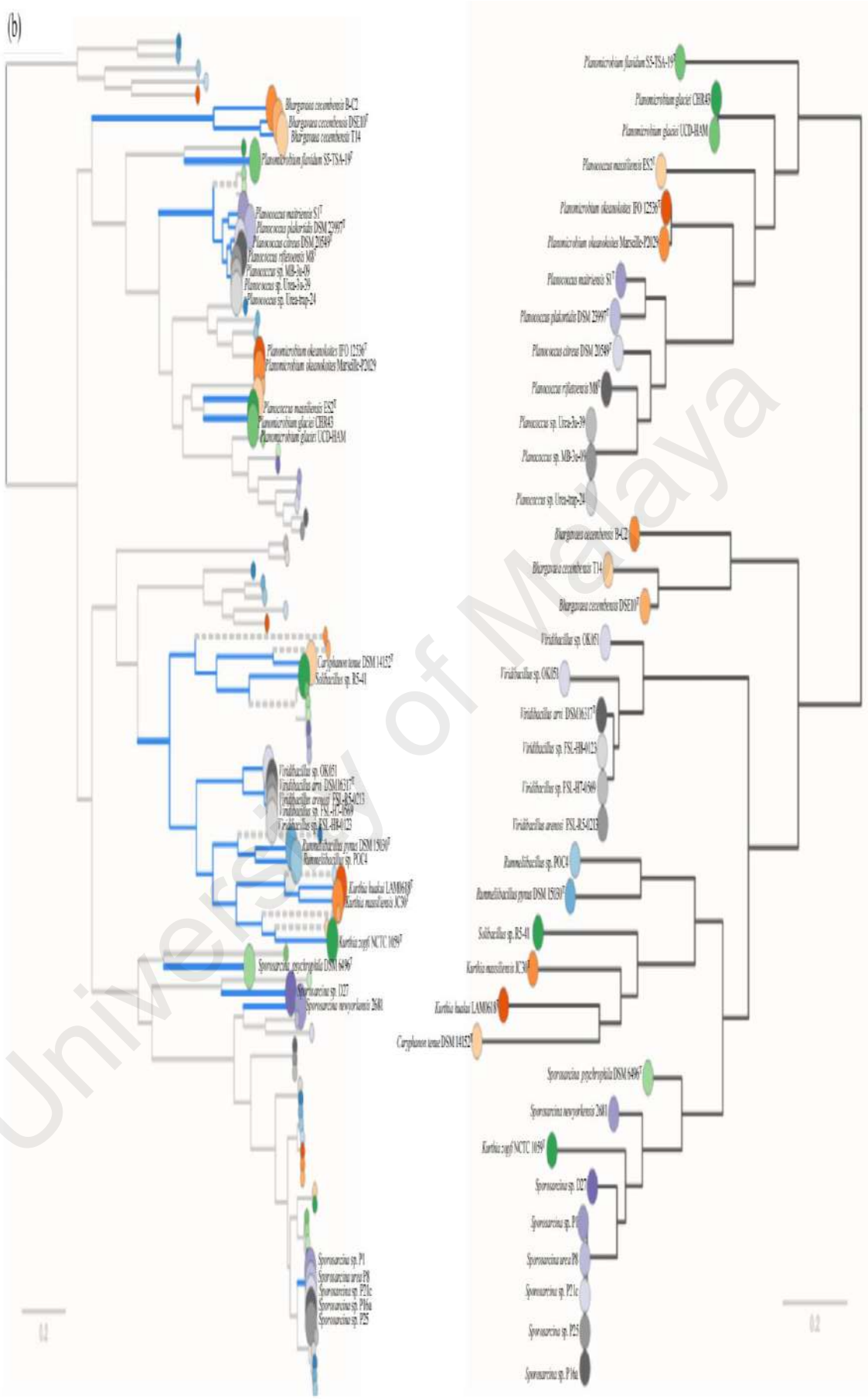


Figure 4.14, continued.

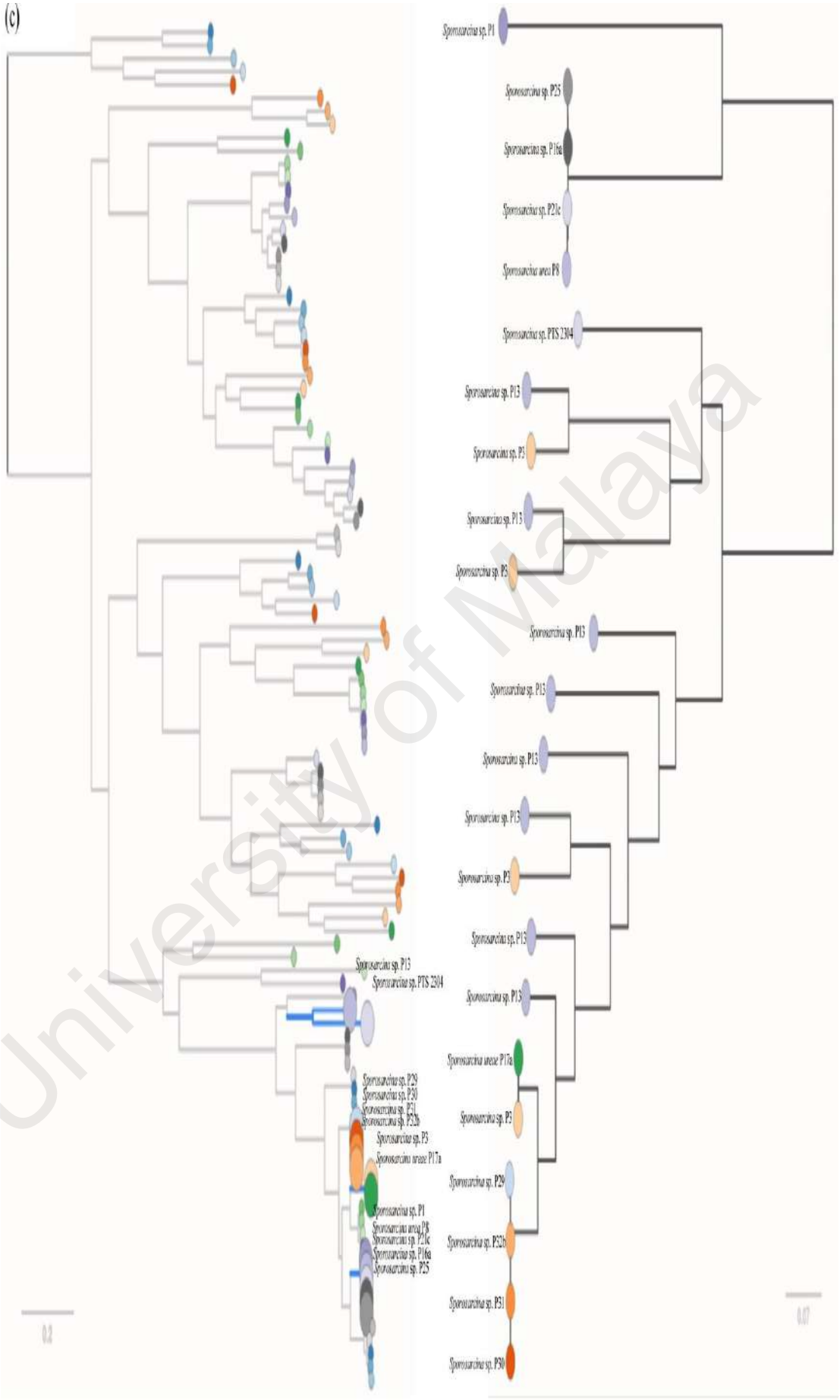


Figure 4.14, continued

4.5 Amino acid contents of *Planococcaceae*

Different strains from *Planococcaceae* exhibited distinct amino acid content profiles of the translated genome. Generally, the amino acid usage clustered according to the genus. However, a few strains of *Planococcus* including *P. versutus* L10.15^T, *P. antarcticus* DSM 14505^T, *P. halocryophilus* DSM 24743^T, *P. donghaensis* DSM 22276^T, *P. donghaensis* MPA1U2, *P. faecalis* AJ003^T, *P. kocurii* ATCC 43650^T and *Planococcus* sp. PAMC 21323, were clustered into *Sporosarcina* rather than other *Planococcus* strains or *Planomicrobium* (Figure 4.15). Most of the *Sporosarcina* sp. were isolated from sea ice, and the overall amino acid usage is clearly similar to that of representatives of *Planococcus* with cold origin. These two groups of bacteria exhibit amino acid profiles consistent with other psychrophilic bacteria, with low percentages of Pro and Arg but a high percentage of Ser.

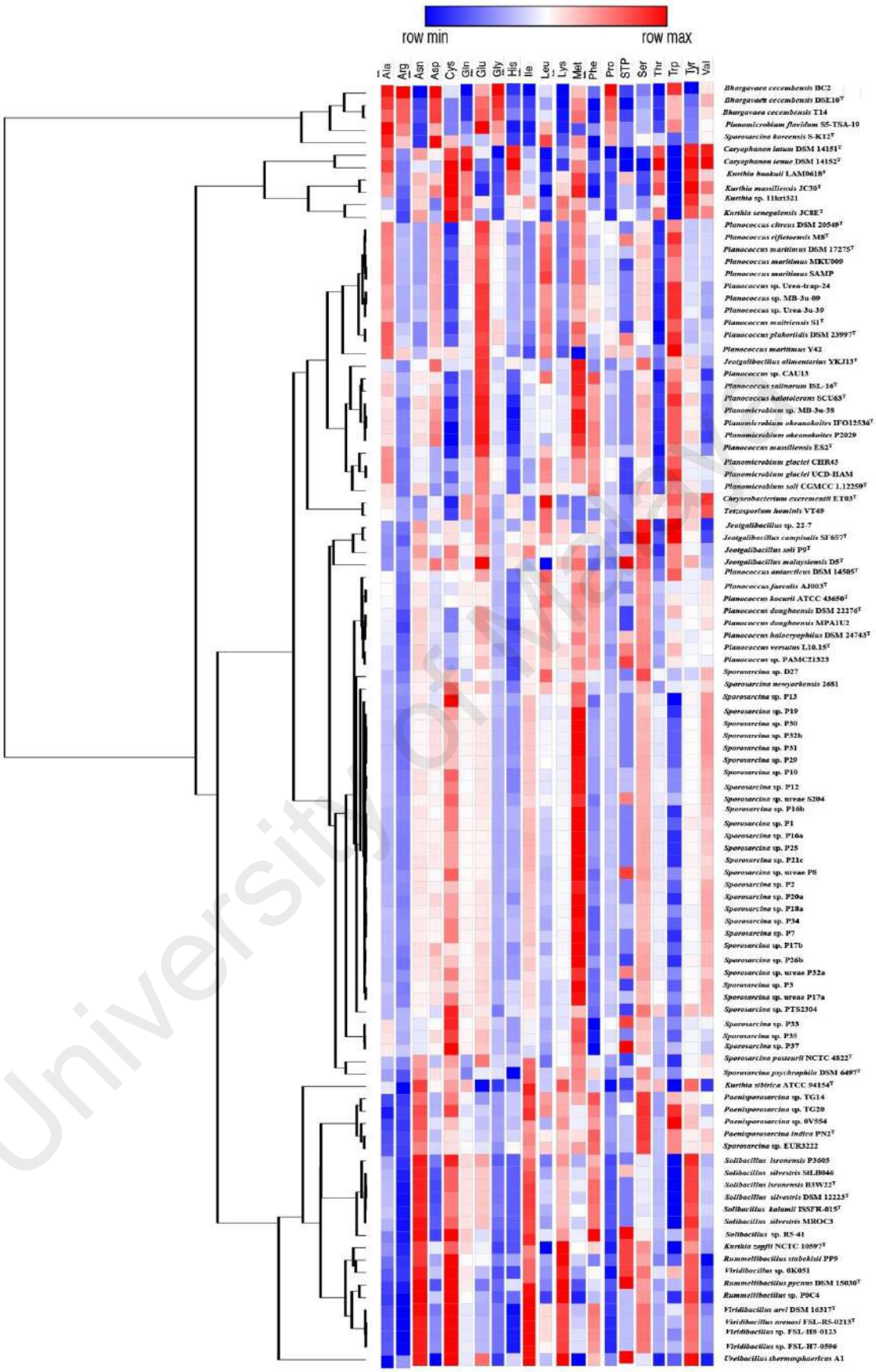


Figure 4.15: Amino acid usage in *Planococcaceae.*

4.6 Identification of AHL-lactonase gene of strain L10.15^{T}

A candidate QQ gene was identified from analysis of the RAST annotation. The candidate gene, coding for 282 amino acids, was annotated as *N*-acyl homoserine lactonase. The BLASTp search revealed that this candidate QQ gene was annotated as an MBL fold metallo-hydrolase from NCBI PGAP in the genome of L.10.15^{T}, with 98% identity to MBL fold metallo-hydrolases in the genomes of *P. antarticus, P. faecalis,* and *P. kocurii*, confirming that this enzyme is conserved across numerous *Planococcus* spp. BLASTp search also revealed that homologous genes with lower identity (<85%) were present in several genera from the Order Bacillales, including strains of *Bacillus, Jeotgalibacillus, Panaenibacillus,* and *Lysinibacillus.* Among these homologous genes, only one AHL lactonase gene, namely *adeH*, was confirmed as a QQ gene. The NCBI Conserved Domain Database (CDD) also identified the AHL lactonase MBL-fold, the conserved domains of AHL lactonase from metallo-*β*-lactamase family of the candidate QQ gene from strain L10.15^{T} (Appendix L). The candidate gene was designated as '*autoinducer degradation gene from Planococcus species*', *aidP*.

4.6.1 Sequence analysis of *aidP* gene of strain L10.15^{T}

HXHXDH~H zinc-binding domains were identified in the multiple sequences alignment analysis, corresponding to ^{117}HLHLDH^{122}~H^{197} in *aidP*. The zinc-binding domains are the signature of AHL lactonase from metallo-*β*-lactamase, and also several functional crucial amino acids based on crystallographic studies of other AHL lactonases from metallo-*β*-lactamase (Figure 4.16, Appendix M). The functional crucial amino acids include active-site residues Tyr^{222}, Leu^{120}, and Asp^{121}, and Asp^{219}, that form bridges with the ligand, His^{265} and His^{122} that interact with the zinc metal ion and the active-site residue Tyr^{222} as an H^{+} donor. Finally, a non-conservative residue, Gly^{235}, was also identified in the *N*-binding region. A number of amino acid substitutions were identified in AidP. F^{107}

and Glu^{136} in AiiA, were substituted by Leu^{120} and Ala^{156} in AidP. Both amino acids are important in forming hydrogen bonds with the substrate and facilitate the interaction of the substrate with the H_2O molecule. Other than amino acids that are involved in catalytic action, Pro^{203} in AidC which is important in maintaining the protein structure was substituted by Glu^{186} in AidP.

Amino acid substitutions at non-crucial sites could effectively change the flexibility of proteins. Therefore, amino acid composition analysis of AidP and the nearest homologous gene including the thermophilic counter was conducted, to detect the presence of common amino acid composition in cold-adapted enzymes. The amino acid composition analysis revealed that AidP has high frequencies of Gly, Ser, Thr and Leu and low frequencies of Arg, Phe and Met compared to the thermally-stable homologous gene identified in thermophilic bacteria including *Geobacillus* and *Themoaerobacter* (Figure 4.17).

The phylogenetic tree of AidP and selected AHL lactonases from different families confirmed that AidP was clustered into the metallo-*β*-lactamase superfamily (Figure 4.18). However, it formed a distinct branch from other members of the metallo-*β*-lactamase superfamily. The amino acid sequence similarity of *aidP* is also low with respect to other members of the metallo-*β*-lactamase superfamily (Table 4.7).

Phylogenetic analysis was conducted for *aidP* and the genes with highest similarity based on NCBI BLASTp search, confirming that *aidP* was clustered with MBL fold metallo-hydrolases from all *Planococcus* spp. mentioned in Section 4.4.1 (Figure 4.10). These genes have amino acid sequence similarity > 98% with *aidP,* strongly suggesting that the enzymes encoded serve the same function as *aidP.* We therefore designated these as *aidP*-homologous genes. *aidP* is also closely related to AHL lactonase from

Lysinibacillus sp. (AdeH) and *Geobacillus kaustophilus* (GcL). GcL is a thermophilic enzyme that retains high QQ activity at high temperature. To compare AidP with other AHL lactonases identified from contrasting environments, a branch-site analysis was performed to investigate evidence of cold selective pressures, using the phylogenetic tree above and the clade containing all AidP as foreground (Appendix N). The branch-site analysis indicated that 11 amino acids are under positive selection pressure in BEB analysis, even though only site 84S had posterior probability > 0.95 (Figure 4.20). This result was supported by the LRT test which had $p < 0.05$, using *aidP* genes from *Planococcus* spp. as foreground branch (Table 4.8). This strongly suggests that AidP is under cold episodic selective pressure, since these *Planococcus* spp. are isolated from Antarctica (See-Too *et al*., 2018) and, therefore, appear to have evolved adaptively in other cold environments.

Table 4.7: Amino acid sequence similarity of AidP with AHL lactonases from the metallo-*β*-lactamase superfamily.

Source	Protein	Amino acid sequence similarity with AidP (%)
Bacillus sp. strain 240B1	AiiA	26.2
Agrobacterium tumefaciens strain C58	AttM	24.9
Arthrobacter sp. strain IBN110	AhlD	27.5
Chryseobacterium sp. strain StRB126	AidC	29.0
Soil metagenome	QlcA	15.1
A. tumefaciens strain C58	AiiB	41.5

Table 4.8: Summary of CodeML results for branch-site analyses for *aidP* genes and 42 homologous genes obtained from an NCBI BLAST search. The foreground branch contains *aidP* genes for four *Planococcus* spp.

Model	**site class**	**Pro-por-tion**	**Back-ground ω**	**Fore-ground ω**	***lnL***	**LRT Statistic**	***p*-value**
Branch-site Null	0	0.89838	0.02528	0.02528	-11269.6711	4.42473	0.03542
	1	0.01171	1.00000	1.00000			
	2a	0.08875	0.02528	1.00000			
	2b	0.00116	1.00000	1.00000			
Branch-site Alterna-tive Model	0	0.91669	0.02528	0.02528	-11267.45879		
	1	0.01197	1.00000	1.00000			
	2a	0.07041	0.02528	38.61239			
	2b	0.00092	1.00000	38.61239			

**P*-value was determined using χ^2 analysis with degrees of freedom = 1.

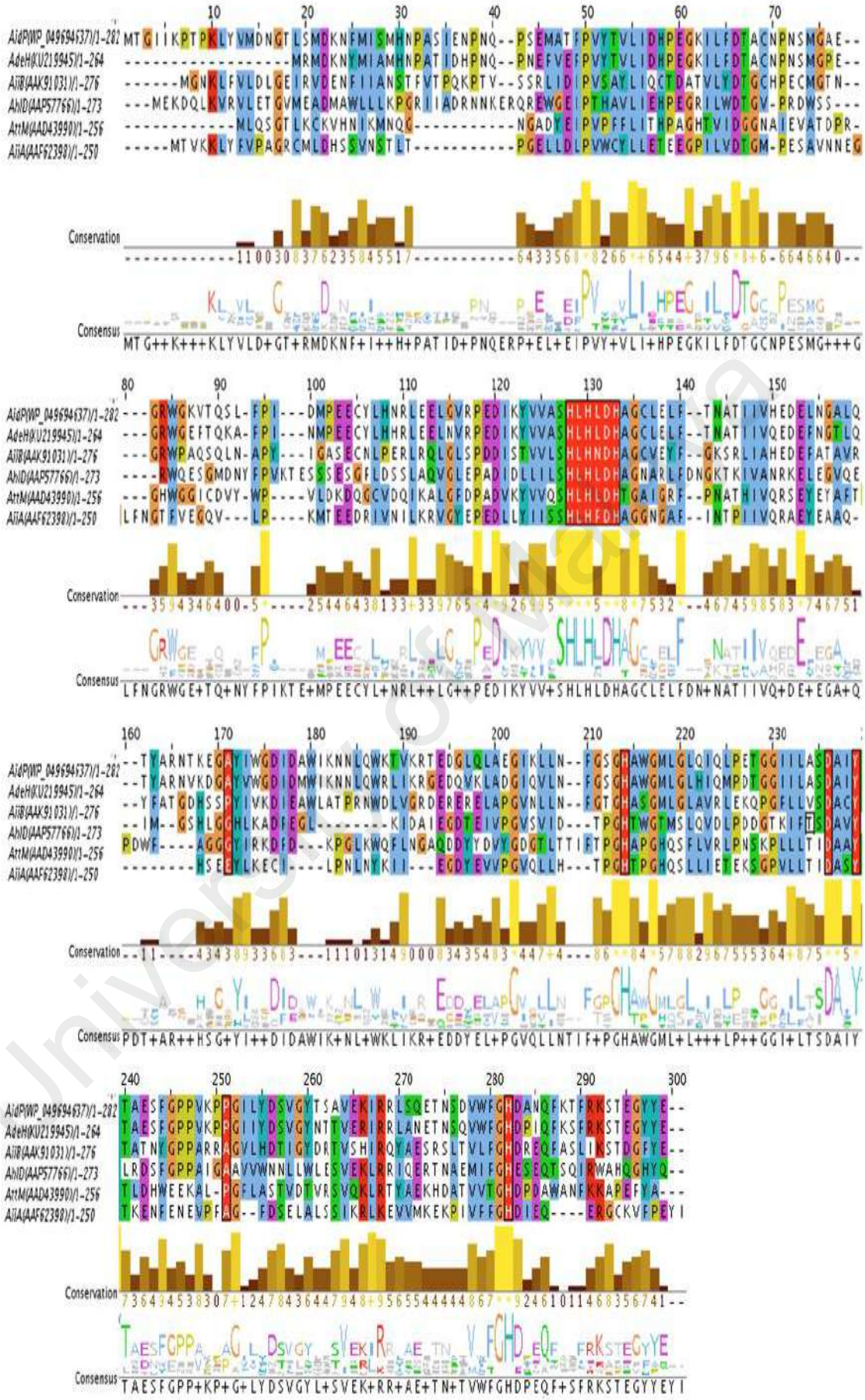


Figure 4.16: Comparison of amino acid sequences of strain L10.15^{T} AidP and five known AiiA-type lactonases.

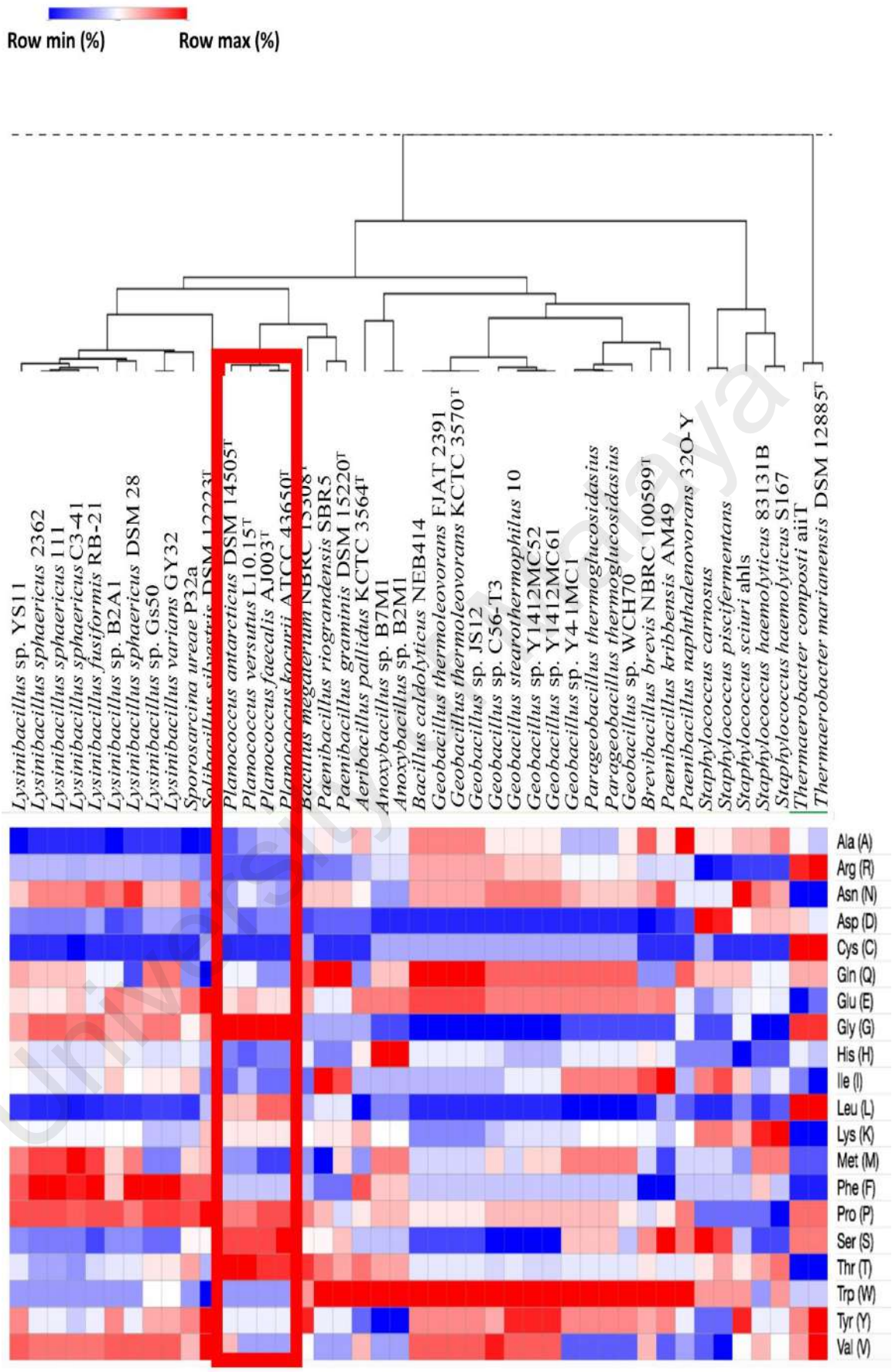


Figure 4.17: Amino acid composition analysis of AidP with other homologous genes. The red box indicates AidP and the homologous genes from *Planococcus* spp.

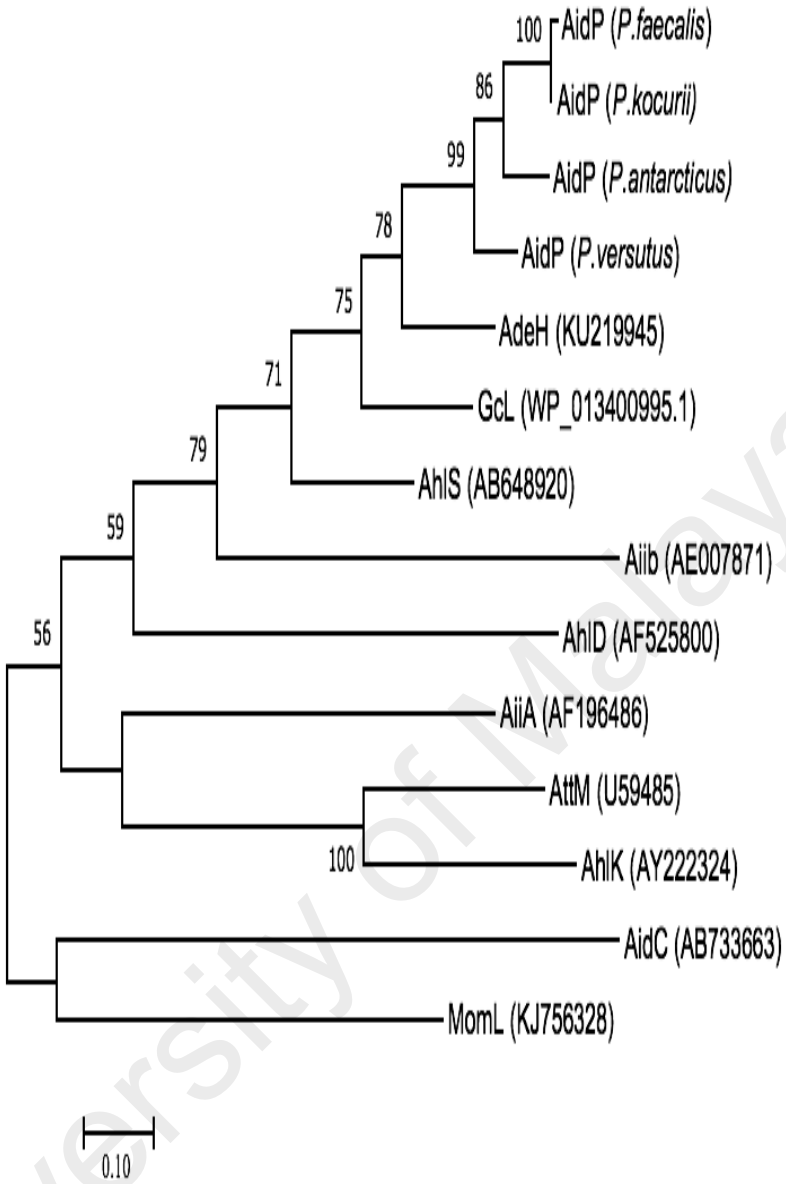


Figure 4.18: Phylogenetic tree of AidP and AHL lactonases from different families.

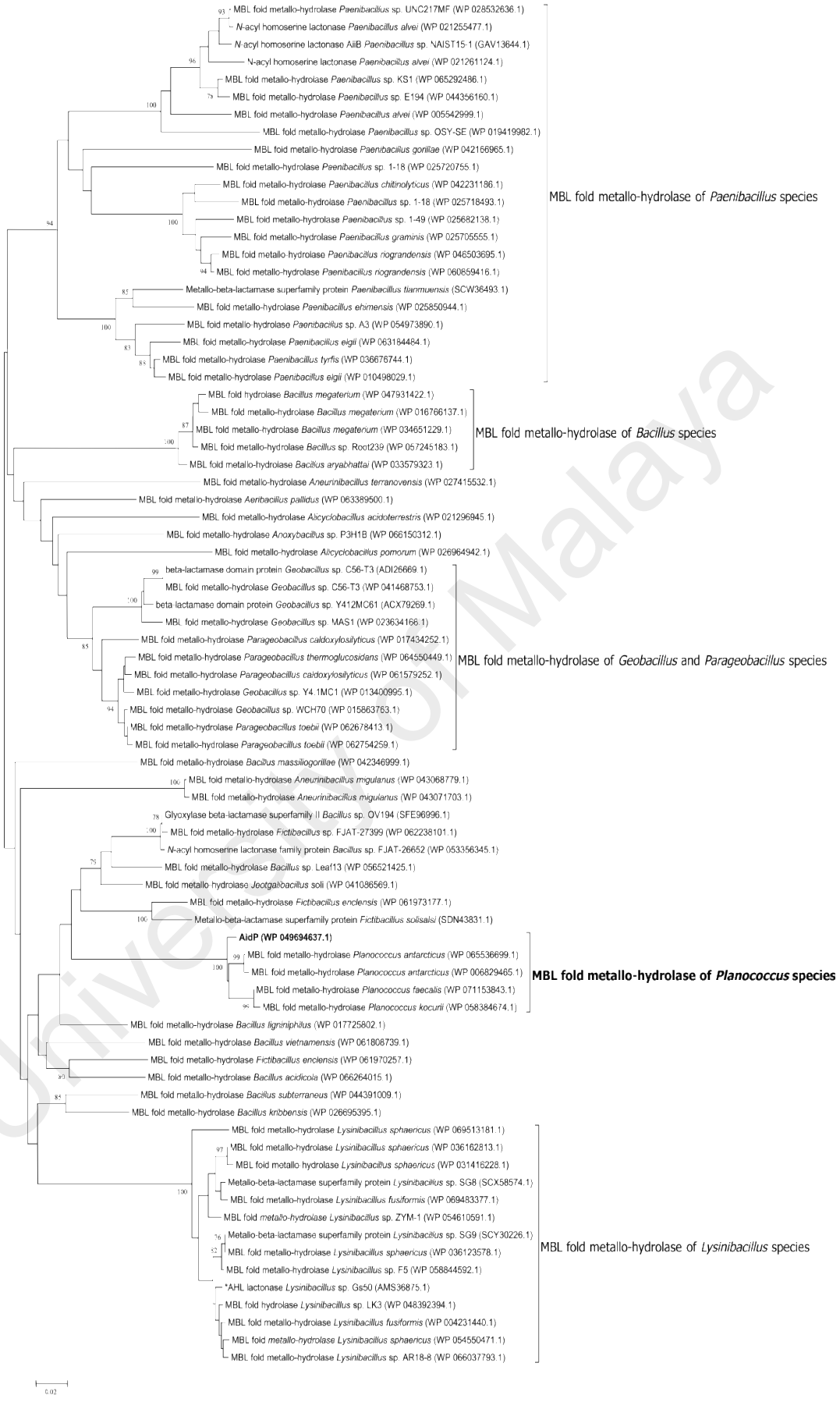


Figure 4.10: Phylogenetic tree of AidP and homologous genes with nearest identity.

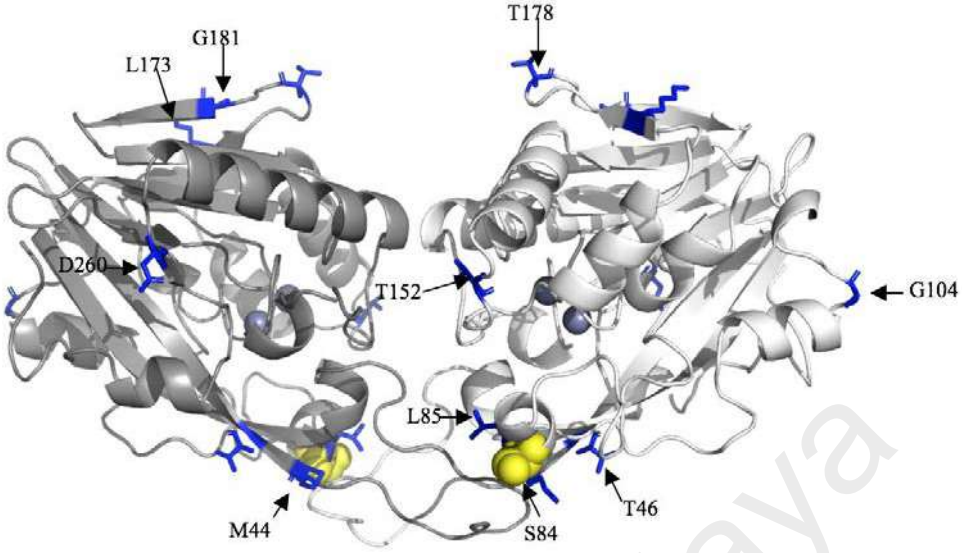


Figure 4.20: Predicted 3D structure of AidP. The yellow spheres represent sites with high probability of undergoing positive selection pressure (BEB analysis at > 95%), and the blue sticks represent positively selected sites with 50%<BEB<95%.

4.6.2 LC-MS analysis of *aidP* QQ activity in *E. coli* BL21 Star™

To verify the QQ function of *aidP*, the gene was expressed in the Champion™ pET Expression System using *E. coli* BL21 Star™. QQ activity was screened as described in Section 3.13 using RRLC. *E. coli* BL21 Star™ harbouring *aidP* degraded the synthetic C_6-HSL supplied in the assay (Figure 4.21). To further confirm that *aidP* encoded an AHL lactonase, the residual AHL molecule was analyzed using LC-MS. The result shows a shift of retention time of the residual AHL from 1.45 min (Figure 4.22a) to 0.94 min (Figure 4.22b). ESI-MS analysis of C_6-HSL with retention time 1.45 min exhibits a strong quasimolecule (M-H) ion at mass-to-charge ratio (*m/z*) of 200.0 (Figure 4.22c). However, ESI-MS analysis of the residual AHL revealed a product with *m/z* of 218.0 (Figure 4.22d). This result indicates C_6-HSL has a mass increase of 18, corresponding to a water molecule, further confirming that the *aidP* encodes an AHL lactonase.

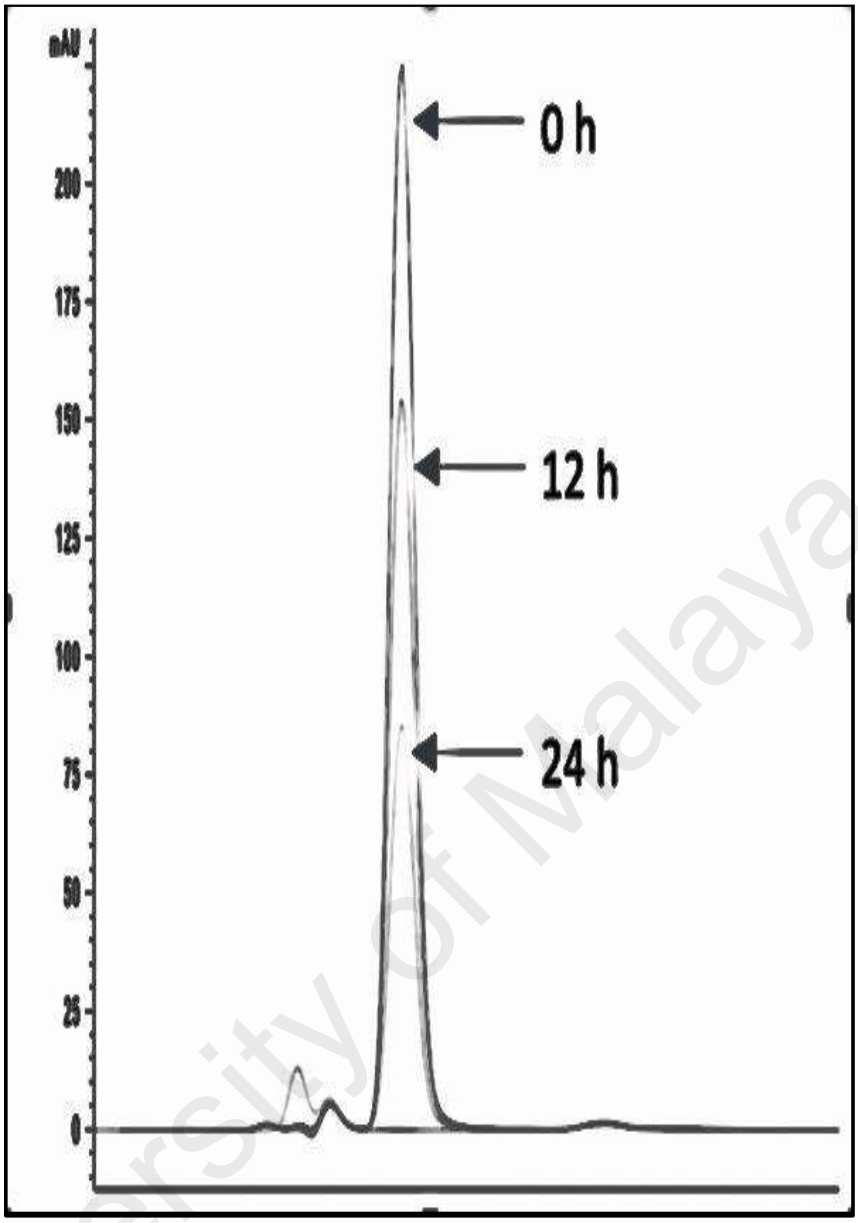


Figure 4.21: Heterologous expression study of AidP in *E. coli* BL21 Star^{TM}.

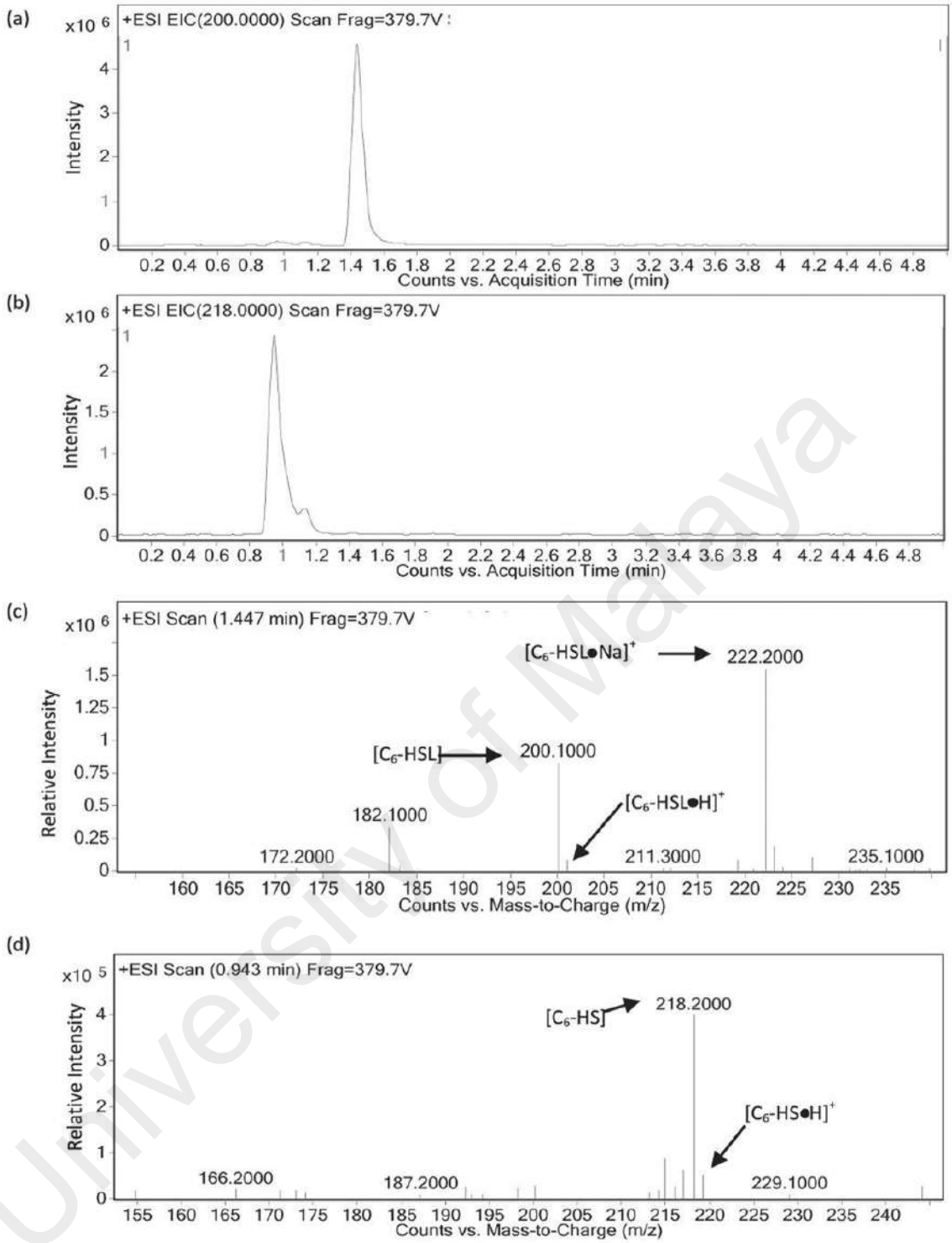


Figure 4.22: LC-MS analysis of *E. coli* BL21 Star™ habouring *aidP* hydrolyzed C_6-HSL product. (a) Chromatography profile C_6-HSL. (b) Chromatography profile of hydrolysed C_6-HSL. (c) ESI-MS analysis of LC fractions containing the 1.48 min of undigested product (d) and 0.94 min of digested C_6-HSL.

4.6.3 Purification of AidP and determination of enzyme activity

The Bioanalyzer analysis revealed that *aidP* encoded a protein with size approximately 33 kDa (Figure 4.23), as predicted using the amino acid sequence. The RRLC analysis revealed that the partially purified AidP exhibited high QQ activity, completely degrading the AHLs. The ESI-MS analysis further confirmed that AidP hydrolyzes the lactone ring of AHLs, with appearance of a product at *m/z* of 218 in addition to 200, corresponding to the incorporation of an H_2O molecule (Fig. 4.24). These results confirmed that AidP leads to the cleavage of the homoserine lactone ring on the substrate, thus producing an *N*-3-oxo-hexanoyl-L-homoserine molecule.

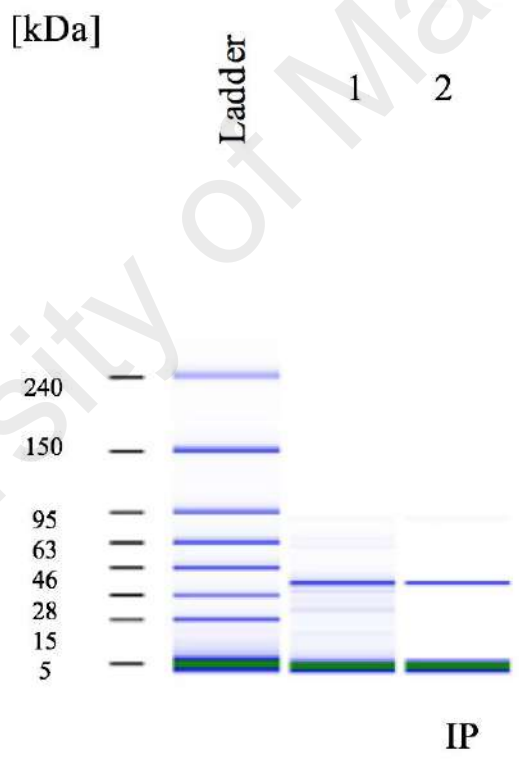
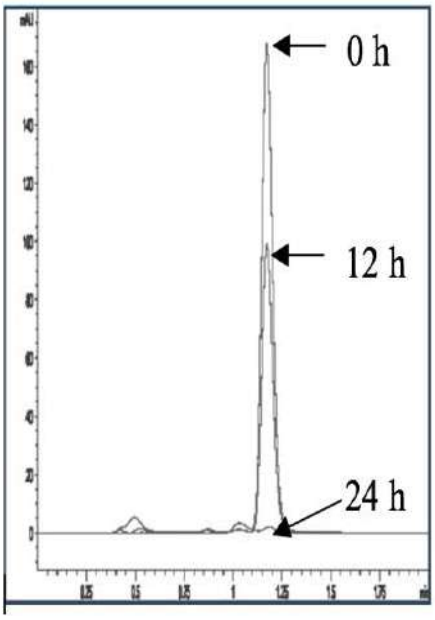


Figure 4.23: Bioanalyzer analysis of purified of AidP enzyme. Lane 1, partially purified AidP enzyme. Lane 2, partially purified AidP enzyme after IP with penta-his antibody. The protein size is approximately 33 kDa.

(a)



(b)

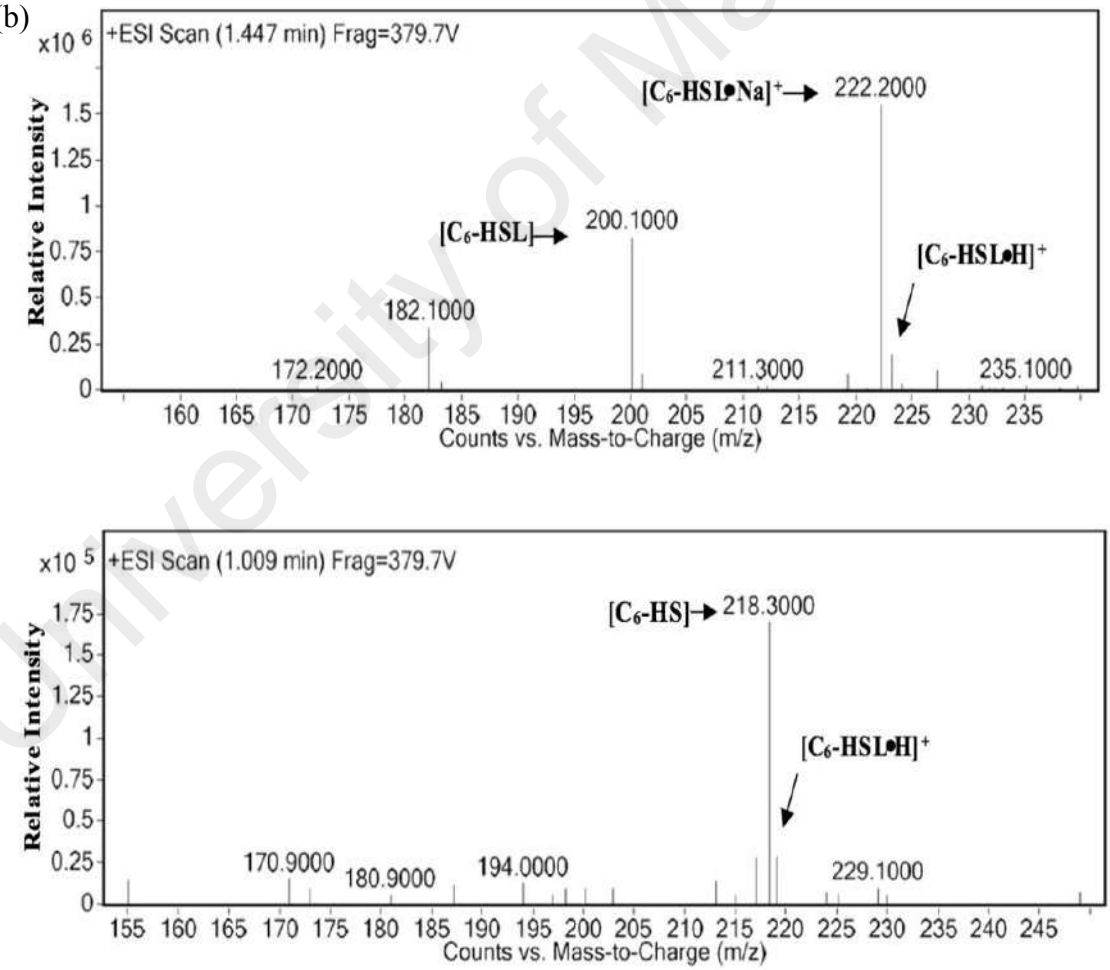


Figure 4.24: (a) RRLC of analysis of residual C_6-HSL by AidP (rt: 1.1 min). (b) ESI-MS analysis of residual C_6-HSL by AidP (rt: 4.8 min). UPLC fragment of enzyme-digested products reveals a M-H ion substrate at *m/z* of 218.

4.6.4 Substrate specificity and other properties

AidP exhibited high activities towards 3-oxo substitution of AHLs compared to un-substituted homoserine lactone. AidP exhibited high activity towards C_6-C_{10}-HSL, and weak activity against C_4-HSL and C_{12}-HSL (Figure 4.25a), which indicates that substrate specificity of AidP was significantly influenced by the length of the acyl side chain of AHLs. $3OC_6$-HSL was subsequently used to study other properties of AidP, since AidP exhibited highest activity against this compound. In the optimum pH study, AidP exhibited no activity at pH 4 or below. Increasing pH enhanced the QQ activity, reaching maximum activity at pH 8 (Figure 4.25b). The optimum temperature was approximately 27 °C, with activity significantly decreasing above 30 °C (Figure 4.25c). In terms of the thermostability of AidP, the QQ activity was markedly reduced at > 32 °C but, at 30 °C or below, AidP retained > 90% of the maximum activity (Figure 4.25d). However, at 4 °C, AidP achieved less than 10% of the maximum activity, apparently contradicting the result of the whole cell assay using strain L10.15^T. Activity was 60% of maximum at 16 °C. In the *in vitro* assay, the activity of AidP was decreased by approximately 80% by addition of EDTA (Figure 4.25e), and markedly inhibited by the addition of Mg^{2+}. However, the reported metal ion inhibitor Fe^{2+} (Cao *et al*., 2012) showed no significant inhibition. Addition of Zn^{2+} resulted in only a small impact on QQ activity, which remained at 85% of the original level. In the *in vivo* assays, the activity of AidP was again extinguished by addition of EDTA but was restored by the addition of Zn^{2+} (Figure 4.25f). The activity of AidP was slightly inhibited by the addition of Cu^{2+}. None of the other metal ion stimulated or inhibited AidP activity in these assays.

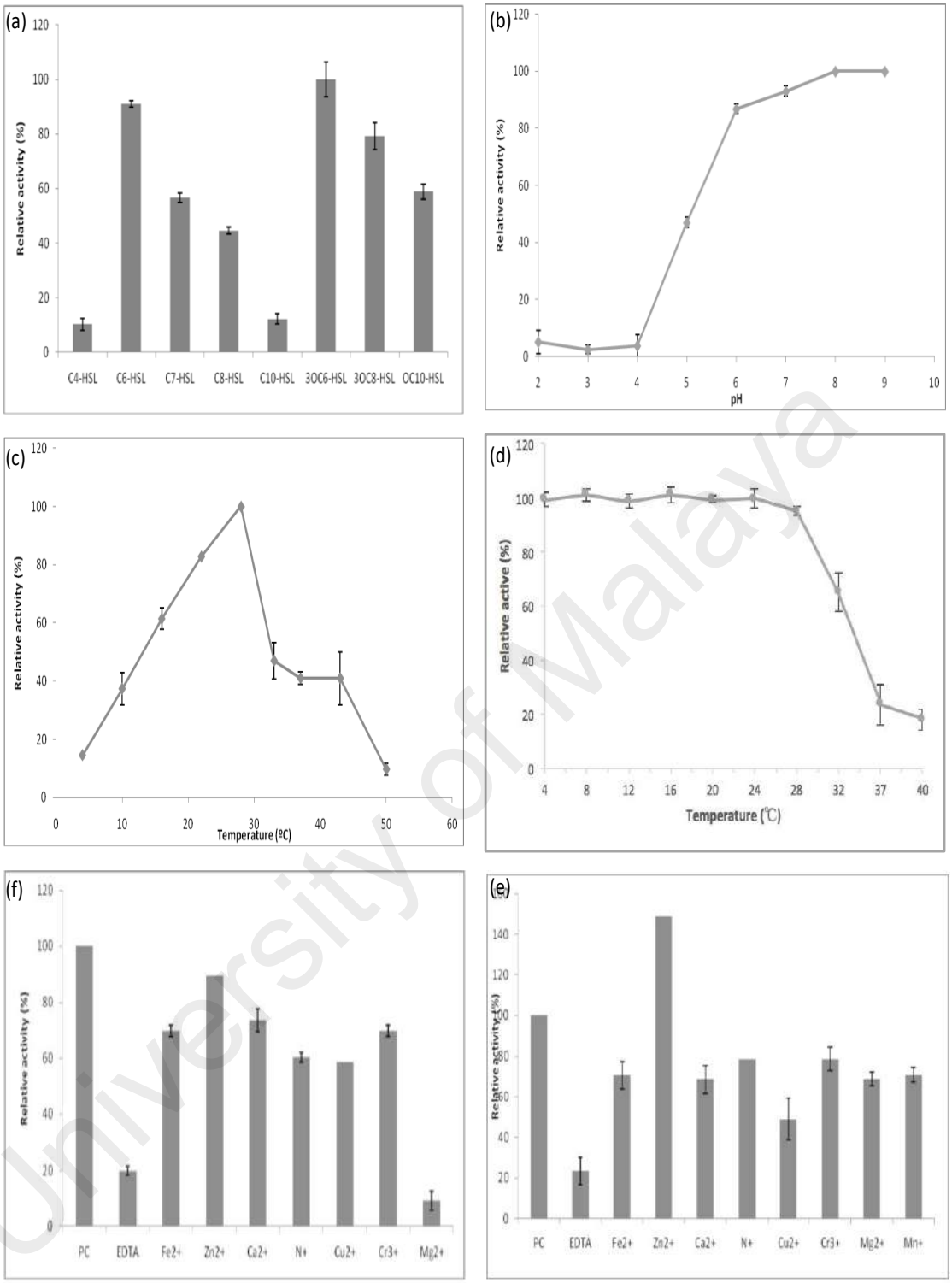


Figure 4.25: Substrate specificity and other properties of AidP. Each experiment was replicated at least 3 times, and error bars indicate standard deviation. (a). Substrate specificity of AidP. AidP was mixed with substrate solutions in buffer (pH 7). (b) Optimum pH of the AHL-degrading activity of AidP. (c) Optimum temperature of AHL-degrading activity of AidP. (d). Thermal stability of AidP. We defined 100% relative activity as the activity in the reaction buffer (pH 7) at 25°C. (e-f) The effects of EDTA and various metal ions on the AHL-degrading activity, relative to the activity in the absence of EDTA or metal ions, and including the PC (positive control).

4.6.5 AidP influence on pectinolytic activity of plant pathogenic bacteria

Chinese cabbage was used to assess the ability of AidP to attenuate the pathogenicity of the plant pathogen *Pectobacterium carotovorum*. Inoculation of wild type *P. carotovorum* strain GS101 causes maceration (browning colour) in Chinese cabbage (Figure 4.26a), however, the maceration area was significantly reduced when 1 mM AidP was co-inoculated with GS101, and the maceration of tissue was completely prevented with increase of AidP concentration to 10 mM (Figure 4.26b).

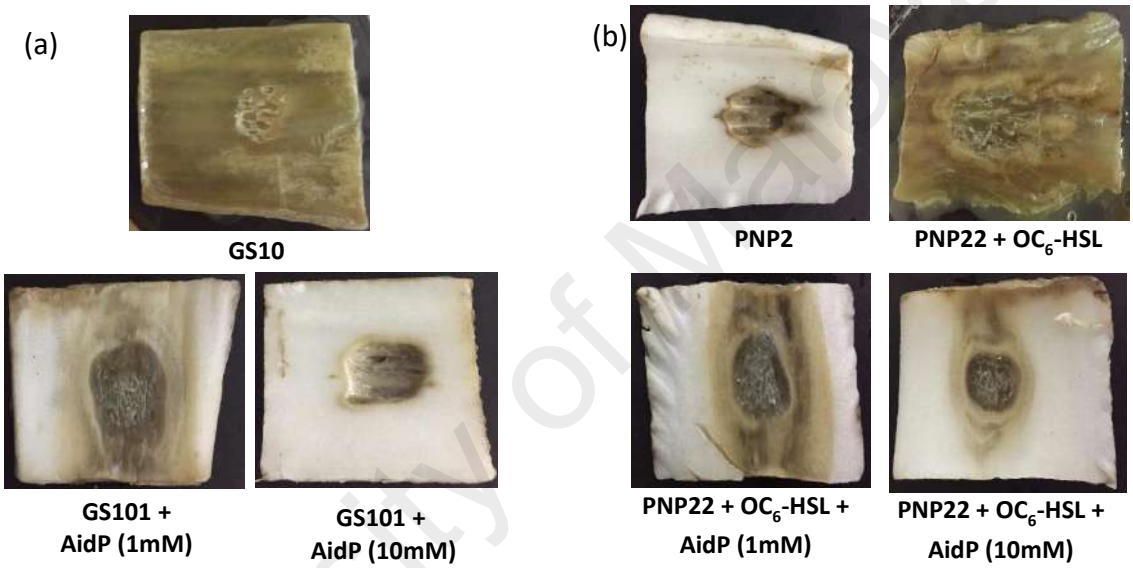


Figure 4.26: Quenching of pectinolytic activity in *P. carotovorum* strains GS101 and PNP22 by AidP. The pectinolytic inhibition assay was observed by visual inspection of maceration zones by the pathogen *P. carotovorum* strain (A) GS101 and (B) *luxI* mutant PNP22 after 5 d.

CHAPTER 5: DISCUSSION

5.1 Genome of *Planococcus versutus* strain L10.15^T

The genome of *Planococcus versutus* strain L10.15^T includes two plasmids. In pPS15-2, a putative RepB family plasmid replication initiator protein gene (WP_049694148.1) was identified, which is a common occurrence among the plasmids of cold-active bacteria (Dziewit & Bartosik, 2014). A BLASTn search using the pPS15-2 nucleotide sequence against the NCBI non-redundant nucleotide database revealed that the sequence of pPS15-2 is very similar to plasmid sequences from *P. antarcticus* DSM 14505^T (pPA05-1 and pPA05-2), *P. kocurri* ATCC 43650^T (unnamed plasmid), and *P. citreus* DSM 20549^T (pNM11). However, both the PGAP pipeline and RAST analysis indicated that pPS15-2 encoded 12 proteins, of which 11 are hypothetical proteins with unknown function. The only known protein is a phage integrase, that facilitates site-specific DNA recombination, suggesting the possibility of viral origin for pPA05-2, and the possibility of this plasmid to integrate into the chromosome. For pPA05-1, the BLASTn search against the NCBI non-redundant nucleotide database revealed that this plasmid was closely matched with the chromosome of *Planococcus* sp. PAMC 21323, *P. kocurii* ATCC 43650^T, and *P. citreus* DSM 20549^T. The annotation result revealed the presence of multiple DNA recombination proteins including resolvase, recombinase, tyrosine recombinase XerC, and mobile element protein. Even though pPA05-1 did not match any plasmid sequences from *Planococcus* sp., the BLASTn search indicated that pPA05-1 has high similarity with plasmids from *Staphylococcus aureus* strain 1128105 (p1128105) and *S. aureus* strain 1 (pSA8589). This suggests a possibility that DNA acquisition events have occurred in numerous *Planococcus* species with plasmids closely related with pPA05-1, and these have become stably integrated into the chromosome of these bacterial strains. Similar to pPA05-2, most of the genes carried by pPA05-1 are not well-

characterized, but carry the iron-regulated *sirABC* operon which facilitates iron import and confers fitness when the iron source is highly restricted. This suggests that maintaining pPA05-1 or stably integrating it into the genome confers the bacteria adaptive traits to withstand extreme conditions.

5.2 Taxonomic study of *P. versutus* L10.15^T

Even though L10.15^T shared higher than the 98% 16S rRNA gene sequence similarity for species delineation (Dighe *et al*., 2004; Keswani & Whitman, 2001) to the nearest neighbor *P. halocryophilus* Or1^T (99.3%) (Table 4.3), it appeared on a distinct branch in the 16S rRNA gene phylogenetic analysis. The pairwise comparison of 16S rRNA gene sequence similarity of all type strains of *Planococcus* spp. has also revealed higher than 98% similarity in multiple type strains which is above proposed threshold (Appendix O). This suggested that the threshold is not applicable for *Planococcus* spp. Several studies have also indicated that 16S rRNA gene sequence similarity actually has no defined threshold values unlike techniques like DNA-DNA hybridization which has a clear >70% reassociation threshold for species delineation (Janda & Abbot, 2007).

The results of the phylogenetic analysis and sequence similarity comparison of the 16S rRNA gene sequences were mostly congruent. The phylogenetic tree constructed using the NJ algorithm appeared to be more well-resolved at the node containing *P. versutus* L10.15^T, *P. halocryophilus* Orl^T, and *P. donghaensis* JH 1^T compared to the tree constructed using the ML algorithm (Figure 4.4). *P. halocryophilus* Orl^T and *P. donghaensis* JH 1^T had highest sequence similarity for the 16S rRNA gene to *P. versutus* L10.15^T. The node containing these three taxa was also low in support in both the NJ and ML phylogenetic trees, indicating that a more rigorous method should be applied to obtain a more accurate assessment of the evolutionary relationship between these taxa. However,

the phylogenetic trees generated using the 16S rRNA gene was still sufficient to show that these taxa formed a distinct branch in both trees, indicating that they are not closely related to the other members of the genus.

The ANI analysis of *P. versutus* L10.15^T with the closely related type strains revealed that *P. kocurii* ATCC 43650^T (Hao & Komagata, 1986) and *P. faecalis* CECT 8759^T (Kim *et al*., 2015) have high genome relatedness with 98.2% orthoANI value (Figure 4.4). Since the taxonomic study of *P. kocurii* was published well before that of *P. faecalis,* the latter should be reclassified to *P. kocurii.* This is perhaps not surprising as the taxonomic description of *P. faecalis* did not include ANI analysis. Even though DNA-DNA hybridization analysis indicated that *P. faecalis* does not belong to *P. kocurii*, the result of ANI analysis is known to be more consistent and less error-prone (Goris *et al*., 2007).

Flagellum was not observed in electron micrograph of strain L10.15^T, however, the cells were observed to be motile. This was also observed in one of the closely related species, *P. halocryophilus* Or1^T, even though the genome consists of flagellar sysnthesis gene (Mykytczuk *et al*., 2012). This could be due to the genes involved in flagellar sysnthesis were not expressed in the condition of sample preparation for electron micrograph.

5.3 Revisiting the taxonomy of *Planococcaceae*

16S rRNA gene sequence similarity and phylogenetic analysis have been employed as the major framework for taxonomic classification in *Planococaceae.* However, the 16S rRNA gene sequence-based phylogenetic analysis appears to have insufficient resolution to distinguish several taxa. The tree of life indicates several polyphyletic groupings were observed in *Planococcaceae* based on the 16S rRNA gene phylogenetic analysis as

mentioned in Section 2.6. This observation violates the minimum standard for bacterial genus classification, in which taxa from a genus should be monophyletic in the phylogenetic analysis, with the exception of some rare occurrences. The 16S rRNA phylogenetic analysis also clustered strain *Solibacillus silvestris* MROC3 into *Jeotgalibacillus.* As mentioned in Section 2.6, *Jeotgalibacillus* was found to be distantly related to other members of *Planococcaceae,* thus its taxonomy position in *Planococcaceae* is debatable. Therefore, the clustering of *S. silvestris* MROC3 may due to long branch attraction (LBA) artefact. However, after testing with long branch extraction method, in which the genus *Jeotgalibacillus* was removed (all/one by one), *S. silvestris* MROC3 did not cluster back to the genus *Solibacillus* group. This result rules out the possibility of the long branch attraction artefact of *S. silvestris* MROC3 to genus *Jeotgalibacillus.* Consequently, the 16S rRNA phylogenetic analysis has failed to reveal to true evolutionary relationship of *S. silvestris* MROC3.

The genome relatedness analysis has provided a threshold for species delineation within the family *Planococcaceae.* However, there remains a lack of studies conducted at genus level within *Planococcaceae* based on genomic relatedness. Methods for genus delineation based on genomic relatedness, such as Average Amino Acid Identity (AAI), appear to have flaws in several generic delineations, with extensive overlapping values with the species delineation (Qin *et al*., 2014). Methods like POCP, which were claimed to have no overlapping value in species and genus delineation, have been applied in several studies, even though the proposed threshold has been proved to be imprecise for certain bacterial genera (Pannekoek *et al*., 2016).

In this study, a phylogeny inferred from the concatenated core GCs of *Planococcaceae* is presented, and comparison with the 16S rRNA gene phylogenetic tree was conducted

to investigate the taxonomic status of the polyphyletic groups of the family *Planococcaceae* in the 16S rRNA phylogenetic tree. These polyphyletic groups include genera (1) *Planococcus* and *Planomicrobium,* (2) *Sporosarcina* and *Bhargavaea* and (3) *Kurthia* and *Rummelibacillus.* The outcome of the comparison supports removal of both genera (1) *Sporosarcina* and *Bhargavaea,* and (2) *Kurthia* and *Rummelibacillus* from the phylogenomic tree. However, *Planococcus* and *Planomicrobium* seemed to be truly polyphyletic. All other genera are resolved as monophyletic in the phylegenomic tree, although some of the genera such as *Chryseomicrobium* and *Tetzosporium* have too little sequenced genomic data to allow drawing of any conclusion (Figure 4.11). Notably, *S. silvestris* MROC3 is clustered into *Solibacillus* in the phylogenomic tree, indicating this approach has successfully placed this taxon into a more accurate taxonomic position. Therefore, the phylogenomic approach gives a clear improvement in the taxonomic classification of *Planococcaceae*.

As mentioned in Section 4.5, signature sequence 183T/C and 190A/G (following *E. coli* numbering) of the 16S rRNA gene sequence allows differentiation of strains exhibiting short rod cell morphology (*Planomicrobium*) from cocci strains (*Planococcus*). However, a few taxa, for example *Planomicrobium soli* CGMCC 1.2259^{T}, were observed to have the signature sequence of *Planococcus* while being phylogenetically close to *Planomicrobium glaciei*, and exhibited both short rod and cocci cell morphologies (Luo *et al*., 2014). Electron micrograph data is also lacking in many taxonomic studies of both genera *Planococcus* and *Planomicrobium*, which is important considering that the cell size of both genera make it difficult to differentiate coccus and short rod forms under the light microscope.

The genome relatedness analyses, however, support that genus *Planomicrobium* is a disguised *Planococcus.* Both POCP and AAI among *Planococcus* and *Planomicrobium* are considerably higher compared to other genera. In contrast with the proposed POCP threshold (50%) for genus delineation (Qin *et al*., 2014), most of the genera of *Planococcaceae* had POCP values of 50% and above (Figure 4.8a). Among taxa in the same genus there were high POCP values (Appendix I). According to the data obtained here, a 60~61% POCP value should be applied for genus delineation in *Planococcaceae.* However, there is no sequenced genome for some genera, and some of the genera only have one representative, the proposed POCP value is likely to be further amended when sequence data from more genera are available. The AAI results are in general agreement with the POCP values, and here a 70% threshold for genus delineation should apply in *Planococcaceae.*

In the scenario of *Planococcus* and *Planomicrobium,* the POCP values of both genera are generally 70% and above, except *P. maritimus* DSM 17275^{T}, *P. maritimus* Y42, and *P. flavidum* S5-TSA-19. These three strains displayed high POCP values with each other, but had lower values to other strains, although still 60% and above. The AAI analysis gave a similar result. Notably, both phylogenomic and 16S rRNA phylogenetic analyses revealed one paraphyletic group of *Planococcus* and *Planomicrobium*, consisting of *Planococcus maritimus* Y42 and *Planomicrobium flavidum* S5-TSA-19 which were clustered as distinct branch. These results indicate a possibility of these strains could be a novel genus, however, the current available data are insufficient to examine this possibility further.

In conclusion, the phylogenomic approach has proven to have good utility in resolving evolutionary relationships in *Planococcaceae.* The polyphyletic relationship of

Planococcus and *Planomicrobium* consistently occurs in both 16S rRNA phylogenetic and the phylogenomic analyses, indicating that members of both genera are indivisible. Consistent with the genome relatedness analysis, this part of the study also supports the reclassification of *Planomicrobium* into *Planococcus.*

5.4 The core-genome of *Planococcaceae*

The core-genome contains numerous genes involve in RNA metabolism, including transcription, translation and degradation. Genes encoding ribosomal proteins were particular conserved in *Planococcaceae,* which 44 (12.5%) of the total core genes encoding ribosomal protein. Other genes involve in translation or protein synthesis were also detected and were mostly highly conserved, including 13 tRNA ligases, five elongation factors, six GTPases, and other genes encoding proteins for post-translation modification. Genes important for DNA replication, including five polymerases, resolvase, DNA ligase, and DNA topoisomerase IV were also identified in the core-genome, indicate these genes are highly conserved in *Planococcaceae.*

Genes involved in energy or metabolism pathways including the pentose phosphate pathway were identified (Figure 5.1a). These genes are important as this pathway generates intermediate products for glycolysis, for example D-glyceraldehyde. The intermediate products of this pathway are also precursors for purine, pyrimidine and histidine metabolism. Genes involved in fructose and mannose metabolism were detected in the core-genome (Figure 5.1b). One of the end product of this pathway, glyceraldehyde-triphosphate, is further processed in the glycolysis pathway. Other metabolic genes of core-genome of *Planococcaceae* are shown in Appendix Q.

Genes encoding proteins involved in stress responses were also detected in the core-genome. Bacillithiol, a protein important for redox regulation, responses to oxidative stress and protecting critical Cys residues against overoxidation, seems to be very conserved in *Planococcaceae.* The molecular chaperone DnaJ (heatshock protein), peroxide-responsive transcriptional repressor PerR (functions as peroxide responsive repressor during oxidative stress), redox-regulated ATPase YchF (negative regulators of the oxidative stress response), GTPase ObgE (ribosome assembly and stringent response/stress response), RNA polymerase sporulation sigma factor SigH (displacement of normal RNA polymeras during sporulation), and redox-sensing transcriptional repressor Rex (transcriptional repressors that regulate the expression of respiratory genes by sensing the redox state according to the intra-cellular NAD+/NADH balance), were identified in the core-genome.

QS regulates the expression of essential genes to alter the phenotype according to the bacterial population density. The core-genome identified included the *Sec* gene that encodes Sec machinery that is important for transportation of signaling peptides (Lazazzera, 2001), *eep* gene that encodes metalloprotease for signaling peptide processing, and *Hfq* gene that encodes a protein involved in small RNA (sRNA)-mediated riboregulation. The core-genome also includes a fatty ligase gene *rpfB,* that is involved in the fatty acid type Diffusible Signal Factor (DSF) system. However, the synthase gene, *rpfF*, is absent.

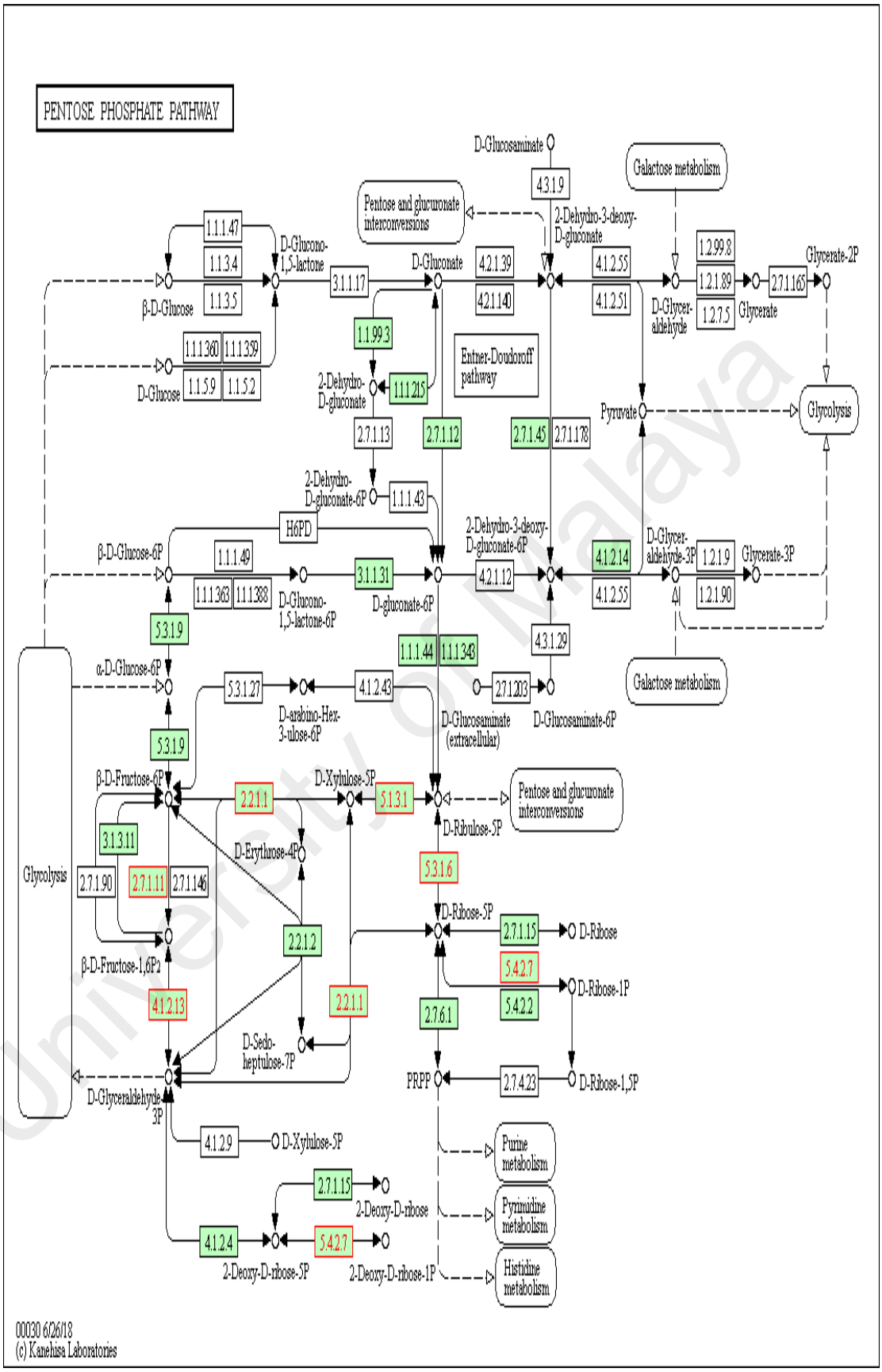


Figure 5.1: The genes involved in (a) Pentose Phosphate Pathway and (b) Fructose and mannose metabolism, catabolic pathways that provide energy in the core-genome of *Planococcaceae.*

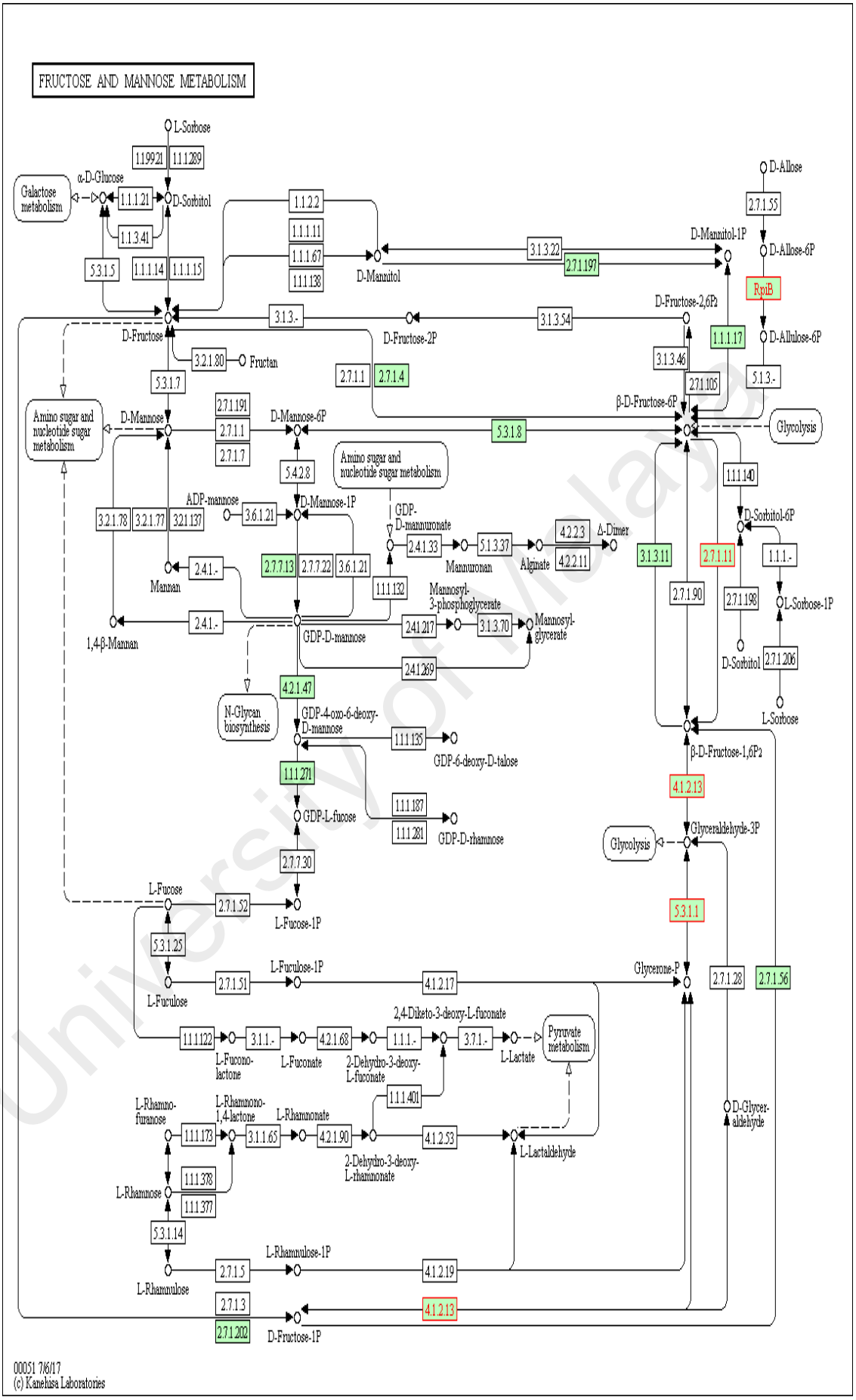


Figure 5.1, continued.

5.5 Positive selection of the core gene

Since transcriptomic and proteomic studies of responses to cold-stress in strains of *P. halocryophilus* have revealed that cold-stress leads to up-regulation of the genes and increased production of the proteins in the core-genome, particularly those responsible for energy production and conversion, translation and carbohydrate metabolism (Raymond-Bouchard *et al*., 2017; Mykytczuk *et al*., 2013). This suggests that some amino acid sequences of the core-genome might undergo selection pressure to act more efficiently in cold temperatures. The analysis revealed that 50 genes have at least one site with high posterior probability to be positively selected in cold-related *Planococcus* (Table 4.8). Notably, some crucial functional proteins like DNA polymerase I, the key protein in DNA replication, has at least five sites under high selection pressure. A few tRNA-ligases including threonine--tRNA-ligase, histidine--tRNA-ligase, alanine--tRNA-ligase, phenylalanine--tRNA-ligase, valine--tRNA-ligase, aspartate--tRNA-ligase and isoleucine--tRNA-ligase have at least one positive selected site. These tRNA-ligases are important for translation or protein synthesis. Other proteins involve in protein synthesis, including 30S ribosomal protein, 50S ribosomal protein, Elongation factor G, GTPase Era and etc. GTPase Era are also known to play important roles in 16S rRNA processing and 30S ribosomal subunit biogenesis, and possibly also in cell cycle regulation and energy metabolism. This suggests that maintaining protein synthesis in low temperature conditions is the main challenge to these bacteria, and substitution with amino acids that increase efficiency at low temperature is critical. Enzymes involve in carbon metabolism, including fructose-bisphosphate aldolase of the TCA cycle, were identified to have sites under high selection pressure.

5.6 Analysis of trait evolution

Carotenoid pigments are important in modulation of the membrane fluidity and sustaining homeoviscosity durating low temperature in cold-adapted bacteria (De Maayer *et al.,* 2014; Dieser *et al.*, 2010). In addition, carotenoid pigments are important in protection from harmful radiation (Krinsky, 1978). Therefore, it is not surprising that many members of *Planococcaceae* harbour genes encoding carotenoid biosynthesis proteins. Carotenoids can be converted into a wide range of products. Therefore, many bacteria produced oxygenase enzymes to oxidize carotenoids (Sui *et al*., 2013). However, in some strains capable of carotenoid biosynthesis in *Planococcaceae,* the gene encoding carotenoid oxygenase has not been identified from the analysis, including *Jeotgalibacillus* and some strains from *Planococcus*. Interestingly, some *Planococcus* do harbour the oxygenase gene, and it was only absent in strains with cold origin including *P. versutus* L10.15^{T}, *P. antarcticus* DSM 14505^{T}, *P. faecalis* AJ003^{T}, *P. kocurii* ATCC 43650^{T} and *Planococcus* sp. PAMC 21323. *Bhargavaea* and some strains in *Planococcus* share a most recent ancestor with strains harbouring the carotenoid oxygenase gene, strongly suggested that a gene loss event has occurred in these strains. Therefore, instead of converting carotenoids into another functional form, retaining carotenoids in the cell seems to be more advantageous to these cells.

Several transcriptomic study on the effects of cold temperature treatment in some bacteria have induced the up-regulation of the lipopolysaccharide biosynthesis gene (De Maayer *et al*., 2014), even though there is a lack of study on the role of lipopolysaccharides in cold-adaptation in Gram-positive bacteria. In Gram-negative bacteria, lipopolysaccharide is the major component of the outer membrane, and serves as primary sensor of cold (Corsaro *et al*., 2017). As mentioned in Section 4.6, the lipopolysaccharide biosynthesis gene has been identified in a few genera of

Planococcaceae, however, only strains isolated from cold environments have gene duplication for this gene (Figure 4.14).

Accumulation of cryoprotectants and antifreeze proteins in bacterial cells reduces the risk of cellular freezing for bacteria in cold environments. Cryoprotectants prevent protein aggregation by stabilizing cytoplasmic macromolecules. Even though *Planococcaceae* lack biosynthesis genes for cryoprotectants, numerous transporter genes were detected. These indicate the cells undergo a high uptake and accumulation of the cryoprotectants in the cells. A few strains even harbour five copies of the gene encoding the glycine/betaine ABC transporter protein in the genome, for example *P. antarticus* DSM 14505^T and *P. halocryophilus* DSM 24743^T, that were isolated from Antarctica and Artic, respectively. Raymond-Bouchard *et al.* (2017) and Mykytczuk *et al.* (2013) reported inconsistent upregulation of the glycine/betaine ABC transporter gene/protein in transcriptomic and proteomic studies, but did not conduct transcriptome or proteome profile comparison studies with other strains of *Planococcus* which harbour fewer copies of the gene to investigate the effect of gene duplication in this strain. Interestingly, among all the strains, only *Sporosarcina* isolated from sea ice harbours a gene encoding antifreeze protein, even though the cryoprotectant transporter gene is not present (Table 5.1). This suggests that these strains employ different strategies to accumulate cryoprotectants in the cell.

Table 5.1: Gene relating to cold-adaptation in *Planococcaceae*.

Strain	Counts:			
	Glycine/ betaine ABC transporter	**PTS mannitol transporter**	**short-chain fatty acid transporter**	**Antifreeze protein**
B. cecembensis T14	1	0	1	0
B. cecembensis DSE10^T	1	0	1	0
B. cecembensis B-C2	1	0	1	0

Table 5.1, continued

Strain	Counts:			
	Glycine/ betaine ABC transporter	PTS mannitol transporter	short-chain fatty acid transporter	Antifreeze protein
Jeotgalibacillus sp. 22-7	2	2	1	0
J. campisalis SF-57^T	2	2	1	0
J. soli P9^T	3	2	0	0
J. malaysiensis D5^T	1	2	0	0
J. alimentarius YKJ-13^T	1	2	0	0
P. antarcticus DSM 14505^T	5	1	1	0
P. donghaensis DSM 22276^T	3	3	2	0
P. donghaensis MPA1U2	3	3	1	0
P. halocryophilus DSM 24743^T	5	3	1	0
P. faecalis AJ003^T	3	1	0	0
P. kocurii ATCC 43650^T	2	1	0	0
P. maritimus DSM 17275^T	3	0	1	0
P. maritimus Y42	2	2	1	0
P. maritimus mku009	3	0	1	0
P. maritimus SAMP	3	0	1	0
P. versutus L10.15^T	4	1	1	0
Planococcus sp. PAMC 21323	2	1	2	0
P. halotolerans SCU63^T	4	1	0	0
P. salinarum ISL 16^T	3	1	0	0
P. maitriensis S1^T	1	1	1	0
P. plakortidis DSM 23997^T	1	1	1	0
P. citreus DSM 20549^T	1	1	1	0
P. rifietoensis M8^T	1	1	1	0
Planococcus sp. MB-3u-09	1	1	1	0
Planococcus sp. Urea-3u-39	1	1	1	0
Planococcus sp. Urea-trap-24	1	1	1	0
Planococcus sp. CAU13	1	0	1	0

Table 5.1, continued

Strain	Counts:			
	Glycine/ betaine ABC transporter	**PTS mannitol transporter**	**short-chain fatty acid transporter**	**Antifreeze protein**
P. massiliensis ES2^T	1	3	1	0
P. flavidum S5-TSA-19	0	0	2	0
P. okeanokoites IFO 12536^T	2	0	1	0
Planomicrobium sp. MB-3u-38	2	0	1	0
P. okeanokoites Marseille P2029	2	0	1	0
P. soli CGMCC 1.12259^T	1	3	0	0
P. glaciei UCD HAM	0	3	0	0
P. glaciei CHR43	0	3	0	0
S. silvestris MROC3	5	0	0	0
S. kalamii ISSFR-015^T	5	0	0	0
S. silvestris DSM 12223^T	5	0	0	0
S. isronensis B3W22^T	5	0	0	0
S. silvestris StLB046	5	0	0	0
S. isronensis Marseille P3605	5	0	0	0
Solibacillus sp. R5-41	1	0	1	0
S. pasteurii NCTC 4822^T	4	0	1	1
S. psychrophila DSM 6497^T	4	2	0	1
S. newyorkensis 2681	1	0	0	0
S. koreensis S-K12	1	0	1	0
Sporosarcina sp. P1	0	0	1	1
Sporosarcina sp. P3	2	0	1	1
Sporosarcina sp. P8	0	0	1	1
Sporosarcina sp. P10	0	0	1	1
Sporosarcina sp. P12	0	0	1	1
Sporosarcina sp. P16a	0	0	1	1
Sporosarcina sp. P17a	1	0	1	1

Table 5.1, continued

Strain	Counts:			
	Glycine/ betaine ABC transporter	PTS mannitol transporter	short-chain fatty acid transporter	Antifreeze protein
Sporosarcina sp. P18a	1	0	1	1
Sporosarcina sp. P19	0	0	1	1
Sporosarcina sp. P20a	0	0	1	1
Sporosarcina sp. P21c	0	0	1	1
Sporosarcina sp. P25	0	0	1	1
Sporosarcina sp. P30	0	0	1	1
Sporosarcina sp. P31	0	0	1	1
Sporosarcina sp. P32a	0	0	1	1
Sporosarcina sp. P33	0	0	1	1
Sporosarcina sp. P35	0	0	1	1
Sporosarcina sp. P37	0	0	1	1
Sporosarcina sp. S204	0	0	1	1
Sporosarcina sp. D27	2	0	1	1
Sporosarcina sp. EUR3	2	0	1	0
Paenisporosarcina sp. TG20	2	0	1	0
Paenisporosarcina sp. TG-14	3	0	1	0
P. indica PN2^{T}	2	0	0	0
V. arvi DSM 16317^{T}	2	0	0	0
Viridibacillus sp. FSL-H7-0596	2	0	0	0
V. arenosi FSL-R5-0213	2	0	0	0
Viridibacillus sp. FSL-H8-0123	2	0	0	0
Viridibacillus sp. OK051	2	0	0	0
R. stabekisii PP9	1	0	0	0
R. pycus DSM 15030^{T}	0	0	1	0

Table 5.1, continued

Strain	Counts:			
	Glycine/ betaine ABC transporter	**PTS mannitol transporter**	**short-chain fatty acid transporter**	**Antifreeze protein**
Rummeliibacillus sp. POC4	0	0	1	0
K. sibirica ATCC 49154^T	1	0	0	0
K. zopfii NCTC 1059^T	2	0	0	0
Kurthia sp. 11kri321	2	0	0	0
K. huakuii LAM0618^T	1	0	0	0
K. senegalensis JC8E^T	1	0	0	0
C. excrementi ET03^T	1	0	1	0
T. hominis VT-49^T	1	0	1	0
U. thermosphaericus A1	2	0	1	0

5.7 AidP as a Novel Class of AHL-lactonase Enzyme

The enrichment of *P. versutus* L10.15^T was achieved using the protocol described by Chan *et al.* (2009), that previously targeted mesophilic QQ bacteria. With modification to a lower growth temperature at 4 °C, and reduced strength of the QQ bacterial enrichment medium, an uncharacterized psychrotolerant QQ bacterium was successfully isolated. This suggests that the enrichment medium could be used to isolate other extremophiles with modification of the growth condition, pH or even ingredients of the medium.

P. versutus L10.15^T not only degraded a broad range of AHLs with carbon side chain lengths between C_4 and C_{12}, but it also had high QQ activity at 4 °C, the lowest temperature for bacterial QQ activity that has yet been reported. To rule out the possibility of the degradation of AHLs being through lactonolysis under alkaline conditions (Yates *et al.,* 2002), the pH of every reaction for the assay was observed, with

the results showing that the resting cells of *P. versutus* L10.15^T do not change the pH of the reaction assay. Therefore, QQ activity by strain L10.15^T was inferred to be achieved *via* an enzymatic reaction, supported by the result of the acidification assay (Appendix P). *P. versutus* L10.15^T was therefore inferred to produce an AHL lactonase, a class of QQ enzyme that hydrolyzes the homoserine lactone ring by cleaving its ester bond.

Even though PGAP failed to annotate the gene encoding the enzyme responsible for the QQ activity, another annotation pipeline, the RAST server, identified the QQ gene and revealed it to be an *N*-acyl homoserine lactonase. The BLASTp search against the NCBI protein database indicated PGAP annotated the QQ gene as an MBL fold hydrolase, and this gene was also detected in the genomes of several *Planococcus* sp. with amino acid sequence similarity of 98%. MBL fold metallo-hydrolases with amino acid sequence similarity 85% or below were identified in bacteria strains from the order Bacillales.

The BLASTp search also identified several genes annotated as AHL lactonase (Figure 4.10). However, only one AHL lactonase, namely AdeH from *Lysinibacillus* sp. Gs50, has been confirmed as functional QQ enzyme (Garge & Nerurkar, 2016). Therefore, homologous AdeH genes from *Lysinibacillus* spp. with high sequence similarity were used for the amino acid usage study (Figure 4.8).

BLASTp search against the NCBI Conserved Domain database (CDD) also detected a AHL-lactonase MBL fold domain (Appendix L) similar to the AHL lactonase (AiiA) identified in *Bacillus thuringiensis*, with e-value 3.82×10^{-78}, which indicating the presence of a predicted protein domain of the query sequence that has a high confidence

level of the inferred function with the domain model. This provides further support that AidP is an AHL lactonase enzyme from the metallo-*β*-lactamase family, and could be novel class of AHL lactonase from this family that is yet to be characterized. CDD also classified that this domain belongs to the metallo-hydrolase-like MBL fold superfamily.

The Zn^{2+}-binding motif, HXHXDH, is the signature of the MBL superfamily AHL lactonases. Crystallographic studies of AHL lactonases have revealed the role of Zn^{2+} in the enzymatic reaction (Liu *et al*., 2007, 2008; Mascarenhas *et al*., 2015; Momb *et al*., 2008). These studies have also revealed the functionally crucial amino acid residues of AHL lactonase that play critical roles in the catalytic reaction. Therefore, a multiple gene sequence alignment study using other AHL lactonases from the MBL superfamily with AidP was conducted (Figure 4.16). This revealed the presence of numerous of functionally-crucial amino acid residues in AidP, based on the crystallographic studies of AHL lactonases from the MBL superfamily. A previous studies conducted on AiiA by Liu *et al*. (2008) also revealed the loss of function in AiiA through the substitution of tyrosine (Tyr^{194}) and aspartic acid (Asp^{108}) residues.

The multiple alignment study showed that both functional crucial amino acids residues are present in AidP as Tyr^{222} and Asp^{121}. The tyrosine residue (Tyr^{222}) renders a stable condition for the enzyme to interact with the substrate, and aspartic acid (Asp^{121}) is a proton-donor that interacts with the hydroxyl leaving group of the final product to maintain the active site structure (Liu *et al*., 2005; Liu *et al.*, 2007). The aspartic acid residue (Asp^{219}), corresponding to Asp^{191} of AiiA enzyme or D_{213} in AiiB enzyme, facilitates proper positioning by forming a zinc bridge to the substrate during the catalysis. The glycine residue located in the *N*-acyl binding region (Gly^{207}) in AiiA, was identified in AidP as Gly^{235} in the same region. Even though the function of this glycine residue

remains unclear, a mutagenesis study of this amino acid residue causes the QQ activity to be significantly reduced.

Homologous genes of *aidP* (with sequence similarity of 98%) were identified in the genomes of three closely related taxa of *P. versutus* L10.15^{T}. including *P. antarcticus, P. faecalis* and *P. kocurii.* As mentioned in Section 5.2, *P. faecalis* and *P. kocurii* belong to same taxon inferred from the OrthoANI analysis (Figure 4.4). This suggests that bacterial strains from this taxon (*P. kocurii*) are present in the Antarctica, since *P. faecalis* which is a later heterotypic synonym of *P. kocurii* was isolated there. On the other hand, this gene or any close analogues was not detected in the genomes of other *Planococcus* spp. With the evidence of the presence of a QQ gene in the genome of *Planococcus* spp. originating from Antarctica, QQ activity may confer advantages in competition with other bacteria that utilize AHLs as signaling molecules in the complex soil environments of the continent.

Notably, the phylogenetic study showed that AHL lactonases from *Lysinibacillus* sp. strain Gs50 (AdeH) and *Geobacillus caldoxylosilyticus* strain YS-8 (GcL) are closely related to AidP. AdeH was reported to have a similar thermostability profile to AidP, with the thermostability significantly reduced above 30 °C. However, AdeH still exhibits high enzyme activity at 37 °C. GcL, nonetheless, exhibits both high thermostability and high optimum temperature. Even though these enzymes are phylogenetically closely related, they exhibit different temperature-activity profiles, suggesting the enzyme activity is affected by amino acid usage or substitution that affects the structure of the protein. Even a single amino acid substitution can drastically affect the enzyme structure by changing the polarity or charge of the functional group of the amino acid (Feller, 2010; Tattersall *et al*., 2012). Saavedra *et al*. (2018) demonstrated how a single sequence change in a cold-

adapted enzyme can change the thermodynamic characteristics even if the change of sequence is distant from the active site.

Numerous studies have identified several prominent amino acid composition characteristics in cold-adapted enzymes, congruent with the amino acid composition profile of AidP. AidP is particularly high in glycine and low in arginine content (Figure 4.17). Cold-adapted enzymes are generally low in arginine, probably as the functional group of arginine facilitates the ion pairs bonding and confers a high thermostability to the protein (Davail *et al*., 1994; Feller *et al*., 1994). Another QQ enzyme family, phosphotriesterase-like AHL lactonases, which is known to have high thermostability, has also been observed to have reduced number of glycine residues (Hawwa *et al*., 2009).

When branch-site analysis was conducted to investigate average ω ratio over all sites in the enzyme, the results showed that several amino acids in AidP are under high positive selection with high posterior probability. This indicates that AidP has undergone a substitution of amino acid conferring advantages in the adaptation of catalytic activity to low temperature. Notably, the analysis revealed that a glycine site (G104) has undergone an episodic selection pressure with BEB posterior probability almost 0.95 (Figure 4.20). Glycine substitution in a protein has been shown to greatly affect the flexibility of the protein when the temperature is low (Saavedra *et al*., 2018).

Most of the known AHLs lactonases such as AiiA from *Bacillus* sp. 240B1 (Wang *et al*., 2004) have relatively high activity toward unsubstituted AHLs. Therefore, the characteristic of AidP of exhibiting high relative activities toward 3-oxo substitution of AHLs compared to the unsubstituted AHLs contrasts with the characteristics previously reported in AHL lactonases. Furthermore, AidP exhibited high activities towards most

AHLs tested. However, similar to AiiA, AidP did not show any degrading activity against L-homoserine lactone or γ-butyrolactone.

Antimicrobial resistance developed by bacteria has become a global concern. Alternative strategies such as QQ have been investigated by many research groups, as QQ does not affect the viability of the bacterial cell, and thus minimizes the selection pressure and reduces the risk of resistance development towards the targeted pathogen. As the number of effective antibiotics is reducing, the study of QQ agents as an alternative biocontrol agent becomes more critical (Rasmussen & Givskov, 2006). Extreme environments including the polar regions are known tobe important sources of novel industrial enzymes with distinct properties (Nichols *et al*., 1999). In this context, a QQ enzyme that has activity at low temperature may confer advantage.

AidP is the first reported QQ enzyme with low temperature activity. This study also provides the first report of a bacterium with QQ activity from Antarctica. AidP is classified as a novel class of AHL lactonase enzyme because of the low amino acid sequence similarity compared with other known AHL lactonases. Even though AidP does not display as strong activity as in the cell of *P. versutus* L10.15^{T}, the results indicate that AidP may undergo structural modifications during the catalysis reaction in bacterial cell, permitting high activity at lower temperature. High enzyme activity at low temperature is always associated with low thermostability (Feller & Gerday, 2003). However, AidP exhibits even higher thermal instability compared to other AHL lactonases resulting and the enzyme is only active in a short temperature range, unlike other AHL lactonases that exhibit broad active temperature range.

Cold-adapted enzymes are known to have discrete structural modifications enabling them to remain active at low temperature. The sequence of the active site is known to be conserved, and therefore the active site is usually the most thermolabile structural element in a cold-adapted enzyme. The ion Zn^{2+} is known to be a crucial element of AHL lactonase, and the restoration of enzyme activity by the addition of Zn^{2+} demonstrated that the zinc-binding motif at the active site of the AidP may play role in the structural modification at low temperature. This is not surprising as zinc-binding motifs have high histidine contents (HXHXDH ~ H) and, at low temperature, the charge of histidine residues in a protein is greatly affected by any decrease in pH (Yancey & Somero, 1978).

P. carotovorum is a plant pathogen that causes soft rot disease on a wide variety of plants. Since the pathogenicity of *P. carotovorum* is QS-regulated, AHL lactonases have been studied in efforts to control the pathogenicity of *P. carotovorum*. Heterogenous expression of AHL lactonases in *P. carotovorum* greatly weakens the production of AHL molecules, thus inhibiting the pectinolytic enzyme production (Dong *et al*., 2000; Mei *et al*., 2010; Torres *et al*., 2017). Genetically modified plants expressing the AHL lactonase gene showed substantially enhanced resistance against *P. carotovorum* (Dong *et al*., 2001). All these findings support the potential of AHL lactonases to be used as a microbial antagonist.

However, concerns have also been raised about disease-resistant genetically engineered crops. Essentially, QQ enzymes induce a selection pressure on the targeted bacteria, even though the imposed selection pressure is generally lower compared to that of other antimicrobial reagents (Chen *et al*., 2013). Moreover, the effect of AHL lactonase is specific and potent (Schikora *et al*., 2011), and continuous expression of this enzyme will stimulate the emergence of QQ-resistant bacteria. Furthermore, AHLs have essential

functions in plant immunity, growth and development (Ali, 2013; Bai *et al*., 2012; Veliz-Vallejos *et al*., 2014). Therefore, disease-resistant genetically engineered crops with AHL lactonase may impact positive effects derived from bacteria-plant interactions. Finally, beneficial endophytes with AHL-mediated QS systems may be affected by the expression of AHL lactonase from the host plant. These beneficial endophytes play crucial roles in chlorophyll production, plant rooting and nutrient uptake in the host plant (Sessitsch *et al.*, 2005), and some of them produce AHLs to enhance their colonization of host plants and to counter phytopathogens (Liu *et al*., 2011). Therefore, direct application of AHL lactonases to treat the infected plants, by targeting the specific plant pathogens which are known to have AHL-mediated QS systems, is recommended. This strategy both mitigates the dependency on antibiotics that accelerate the development of antibiotic-resistant pathogens, and minimises the risk of developing QQ-resistant pathogens.

In addition to enhancing competitive ability in the Antarctic environment, the cold-active QQ enzymes of *P. versutus* L10.15^{T} could give additional advantages to this strain. Previously, *P. rifientoensis* has been reported to promote the growth of plants (Rajput *et al*., 2013), even though a complete genome analysis of *P. rifientoensis* identified no gene sequence coding for *N*-acyl homoserine lactonase (See-Too *et al*., 2016a). As QQ enzymes produced by bacteria have been shown to effectively prevent the plant pathogen, *Pectobacterium* sp., from causing soft root disease on plant tubers (Pearce *et al.,* 2012), we hypothesize a capability of *P. versutus* L10.15^{T} to promote plant growth, and a potential for it to act as a biocontrol agent in horticulture. The development of psychrotolerant QQ bacteria with potential for use as biocontrol, remediation or growth promoting agents would be advantageous. In combination with the possible positive influence of nitrogen-fixing ability on plant growth, we suggest that *P.*

versutus L10.15^{T} could be a beneficial bacterium to explore for applications in agriculture in cold environments.

Chapter 6: CONCLUSION

In this study, a novel species of the genus *Planococcus, Planococcus versutus* was described. The phylogenetic analysis of the 16S rRNA gene sequence of the type strain of *Planococcus versutus,* strain L10.15^T indicated that this strain formed a distinct branch in phylogenetic tree from type strains of *Planococcus* with the nearest 16S rRNA gene sequence similarity. The OrthoANI analysis also supported that strain L10.15^T does not belong to any of these taxa. Together with the chemotaxonomic and phenotypic analyses, strain L10.15^T was confirmed as a novel species that belongs to genus *Planococcus,* of the family *Planococcaceae.*

On the other hand, phylogenomic, phylogenetic and genome relatedness studies all supported the close relationship between *Planococcus* and *Planomicrobium*. However, a polyphasic approach is now required to further confirm the reclassification of genus *Planomicrobium* to genus *Planococcus*, which includes the FAME analysis. The phylogenomic approach showed high resolution in elucidating evolutionary relationships within *Planococcaceae.* Similar approach also provided insight into the study of adaptive traits and in this study, traits related to cold-adaptation in *Planococcus, Planomicrobium, Kurthia, Sporosarcina* and other strains were focused on. This is important to increase the understanding of mechanisms and pathways crucial for cold-adaption, especially in terms of overcoming the biochemical and physiological challenge for survival at low temperature.

An extensive characterization has been conducted on AidP, a thermolabile QQ enzyme from the metallo-β-lactamase superfamily. This study has demonstrated that AidP attenuates pathogenicity of *P. carotovorum,* and inhibits its pectolytic activity against

Chinese cabbage. This indicates that AidP is a promising anti-QS reagent and should now be investigated in field tests. Since AidP is thermolabile, the risk of developing resistance or affecting the microbiome of the human body is considerably reduced. From the amino acid usage study of this enzyme, a number of characteristics of cold-adapted enzymes were identified. The CodeML test also identified a number of amino acids under high selection pressure. Further study, particularly employing crystallography, may increase understanding of the mechanism of action of AidP at low temperature and of the enzyme's thermostability.

6.1 Future Work

Moving from the bench to field application is the major challenge for the study of QS inhibition to control diseases. Piewngam *et al*. (2018) demonstrated the use of probiotic *Bacillus* strains to eliminate a threatening pathogen, *Staphylococcus aureus*, from the human intestine via QS inhibition. This indicates QS inhibition is a promising method to be applied in real situations. As AidP has been proven able to reduce pathogenicity of *P. carotovorum* in plants and it is not active at human body temperature, field tests should be further carried out to further investigate whether AidP is able to control outbreaks of *P. carotovorum* in agriculture.

The pan-genomic study identified several possible strategies for cold adaptation in family *Planococcaceae.* Further rigorous experiments should be conducted to verify these findings. For example, a comparative transcriptomic/proteomic study on strains with gene duplication for cold-adapted traits with other related strains. Lastly, further comparative analysis for genera *Planococcus* and *Planomicrobium* is required to elucidate the taxonomic relationship of these two genera.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS

1. **See-Too, W. S.**, Convey, P., Pearce, D. A., & Chan, K. G. (2019). Characterization of a novel N-acylhomoserine lactonase, AidP, from Antarctic *Planococcus* sp. *Microbial Cell Factories, 17,* Article#179.

2. **See-Too, W. S.**, Chua, K. O., Lim, Y. L., Chen, J. W., Convey, P., Mohidin T., … & Chan, K. G. (2019). Complete genome sequence of *Planococcus donghaensis* JH1^{T}, a pectin-degrading bacterium. *Journal of Biotechnology, 20*(252), 11–14.

3. **See-Too, W. S**., Ee, R., Lim, Y. L., Convey, P., Pearce, D. A., Yin, W. F., & Chan, K. G. (2017). *AidP*, a novel *N*-Acyl homoserine lactonase gene from Antarctic *Planococcus* sp. *Scientific Reports, 7,* Article#42968.

4. **See-Too, W. S**., Ee, R., Madhaiyan, M., Kwon, S. W., Tan, J. Y., Lim, Y. L., ... Chan, K. G. (2017). *Planococcus versutus* sp. nov., isolated from soil. *International Journal of Systematic and Evolutionary Microbiology, 67*(4), 944–950.

5. **See-Too, W. S**., Tan, J. Y., Ee, R., Lim, Y. L., Convey, P., Pearce, D. A., … Chan, K. G. (2016). De novo assembly of complete genome sequence of *Planococcus kocurii* ATCC 43650(T), a potential plant growth promoting bacterium. *Marine Genomics, 28,* 33–35.

6. **See-Too, W. S**., Convey, P., Pearce, D. A., Ee, R., Lim, Y. L., Yin, W. F., & Chan, K. G. (2016). Complete genome of *Planococcus rifietoensis* M8(T), a halotolerant and potentially plant growth promoting bacterium. *Journal of Biotechnology, 2218,* 114–115.

PAPER PRESENTED

1. **See-Too, W. S.**, Convey, P., & Chan, K. G. (2017). Characterization of a novel *N*-acylhomoserine lactonase, AidP, from Antarctic *Planococcus* sp. Paper presented at the 22nd Biological Sciences Graduate Congress (BSGC) 2017, 19th-21st December 2017, Singapore.

2. **See-Too, W. S.**, Convey, P., & Chan, K. G. (2017). Characterization of a novel *N*-acylhomoserine lactonase, AidP, from Antarctic *Planococcus* sp. Paper presented at the 2nd International Conference for Molecular Biology and Biotechnology (ICMBB) 2017, 1st-2nd November 2017, Kuala Lumpur, Malaysia.