POLYPHASIC STUDY, SPECIES DESCRIPTION AND SIGNIFICANCE OF NOVEL *Chryseobacterium* SPECIES ISOLATED FROM POULTRY SOURCES

By

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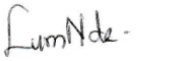
DECLARATION

I, **Adeline Lum Nde**, declare that the PhD Degree research dissertation that I herewith submit for the PhD Degree qualification at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.



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LIST OF ABBREVIATIONS

A	Absorbance
A	Entropy constant
AAI	Amino Acid Identity
AL	Aminolipids
ANI	Average Nucleotide Identity
API	Analytical Profile Index
ATCC	American Type Culture Collection, Manassas, Virginia
a_w	Water activity
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
C.	*Chryseobacterium*
°C	Degrees Celsius
CDS	Coding sequences
CFA	Cellular fatty acid
CFU	Colony Forming Units
DDBJ	DNA Data Bank of Japan
dDDH	digital DNA-DNA hybridization
DDH	DNA-DNA hybridization
DMC	Direct Microscopic Counts
DNA	Deoxyribonucleic acid
DSM	Deutsche Sammlung von Mikro-organismen

DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E	Activation energy/ Temperature coefficient
E.	*Elizabethkingia*
e.g.	For example
ECL	Equivalent Chain Length
Ed(s)	Editor(s)
Eh	Oxidation-reduction potential
EMBL-EBI	European Bioinformatics Institute
ENA	European Nucleotide Archive
***et al*.,**	(et alii) and others
etc.	Et cetera
F.	*Flavobacterium*
FAME	Fatty acid methyl esters
g	Gram
G+C	Guanine and Cytosine
GC	Gas chromatography
GGDC	Genome-Genome Distance Calculator
GL	Glycolipids
h	Hour(s)
h^{-1}	Per hour
H_2S	Hydrogen sulphide
HACCP	Hazard Analysis Critical Control Point
HCl	Hydrochloric acid
HPLC	High-Performance Liquid Chromatography

kg	Kilogram
IF	Inoculating fluid
KCTC	Korean Collection of Type Cultures
KOH	Potassium hydroxide
KP2	Kimura two
L	Lipids
LMG	Laboratory of Microbiology, University of Ghent, Belgium
LPSN	List of Prokaryotic Names with Standing in Nomenclature
M	Molar
Mb	Megabases
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram
MIDI	Microbial Identification System
min	Minute
mg	Milligram
ml	Millilitre
mm	Millimetre
Mol	Mole
Mol%	Mole percentage
mRNA	Messenger RNA
NA	Nutrient agar
NB	Nutrient broth
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information

NCTC	National Collection of Type Cultures
ND	Not detected/determined
NGS	Next-Generation Sequencing
nm	Nanometer
OD	Optical Density
OGRI	Overall Genome Related Index
ONPG	O-nitrophenyl-beta-D-galactopyranoside
PCR	Polymerase Chain Reaction
PE	Phoshatidylethanolamine
PM	Phenotype microarray
pp	Page(s)
R	Universal gas constant
RAST	Rapid Annotation with Subsystems Technology
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
sec	Second(s)
SEM	Scanning Electron Microscope
spp.	Several identified species
sp.	Species or unknown/unidentified/unspecified species
^{T}	Type strain
T	Temperature measured in Kelvin
TEM	Transmission Electron Microscope
TGI	Temperature Gradient Incubator
TLC	Thin Layer Chromatography

Tr	Trace
TSBA	Trypticase Soy Broth Agar
UFSBC	University of the Free State Bacterial Culture Collection
T_m	Melting temperature
™	Trade mark
UK	United Kingdom
USA	United States of America
µl	Microlitre
µm	Micrometer
μ_{max}	Maxium growth rate
v/v	Volume per volume
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background to the study

Bacterial taxonomy was initiated in the late 19th century during which bacteria were classified based on phenotypic markers like morphology, growth requirements or pathogenic potential (Lehmann & Neumann, 1896). They were later classified based on their physiological and biochemical properties (Orla-Jensen, 1909; Buchanan, 1955). Between the 1960s and the 1980s, chemotaxonomy (Minnikin *et al*., 1975), numerical taxonomy and DNA–DNA hybridization techniques (Brenner *et al*., 1969; Johnson, 1991) were used.

Since the 1970's, polyphasic taxonomy has been used since it integrates genotypic, chemotypic and phenotypic characteristics in order to classify organisms into their natural groups. Polyphasic taxonomy was introduced by Colwell (1970) to encompass successive or simultaneous studies on groups of prokaryotes using methods chosen to yield high quality genotypic and phenotypic data. Their introduction has led to improvements in the classification of prokaryotes that in turn has provided a sound basis for stable nomenclature and improved identification (Zhi *et al*., 2012). Both the classical and new methods are now used in determining whether a strain belongs to a known taxon or constitutes a novel one (Tindall *et al*., 2010).

In 1923, the genus *Flavobacterium* consisted of 46 yellow-pigmented mainly Gram-negative, rod-shaped, non-endospore forming, chemoorganotrophic bacteria. It was far from homogeneous since all yellow-pigmented poorly described taxa were placed in this genus (Weeks, 1981). Jooste (1985) suggested that the genus *Flavobacterium* be accommodated in a new family, *Flavobacteriaceae*, together with the genera *Sphingobacterium* and *Weeksella* (Holmes, 1992). As of the time of writing, the family comprises of 113 genera, only ten of which are currently associated with food: *Bergeyella,*

Chryseobacterium, Empedobacter, Flagellimonas, Flavobacterium, Myroides, Salegentibacter, Tenacibaculum, Vitellibacter and *Weeksella* (Hugo & Jooste, 2012; Parte, 2018).

The genus *Chryseobacterium* was proposed by Vandamme *et al*. (1994) to accommodate six renamed and regrouped flavobacterial strains following the thorough emendation of the genus *Flavobacterium*. The renamed species were *Chryseobacterium* [*F*.] *indologenes*, *C*. [*F*.] gleum, *C*. [*F*.] *indoltheticum*, *C*. [F.] *balustinum*, [*F*.] *breve* and *C*. [*F*.] *meningosepticum* (Bernardet *et al*., 2006). The genus *Chryseobacterium* was formerly a member of the family *Flavobacteriaceae* (Bernardet *et al*., 2011). In 2019, however, García-López and co-workers reclassified it into a new family, *Weeksellaceae*, phylum *Bacteroidetes*.

Chryseobacterium is ubiquitous in nature and have been isolated from clinical, environmental, industrial and food sources (Hugo & Jooste, 2012). *Chryseobacterium* species have been found to cause spoilage in a variety of food products: dairy, fish, meat and poultry (Hugo & Jooste, 2012). Temperature is one of the intrinsic factors which play a role in food spoilage. Microorganisms will only grow over certain temperature ranges and secrete byproducts which cause food spoilage.

Members of this genus show strong proteolytic (Vandamme *et al*., 1994) and lipolytic (Hantsis-Zacharov *et al*., 2008a) activities which lead to rendering food products undesirable for consumption. Apart from the spoilage activities of these microorganisms, they have found application in the poultry industry where they produce keratinolytic enzymes that degrade chicken feathers (Charimba, 2012). Despite the industrial importance of keratinases, the development of commercially viable decomposition of keratinaceous materials such as feather, hair and hoofs has been slow and difficult (Lange *et al*., 2016). *Chryseobacterium* may have great commercial value in the detergent and leather industries (Brandelli *et al*., 2010; Gupta *et al*., 2013). All these benefits enable them to possess potential in biotechnological, non-polluting processes (Riffel *et al*., 2007).

1.2 **Purpose, hypothesis and objectives of the study**

1.2.1 *Purpose*

i. To investigate two unidentified strains isolated from poultry feather waste and chicken portions in order to obtain more knowledge and better understanding of their characteristics and correct taxonomic status.
ii. To subject the unidentified strains to the latest taxonomic techniques to more accurately characterize and classify them.
iii. To describe and name any new species that might emerge from the comprehensively characterized strains.
iv. To investigate the growth characteristics of the new species.
v. To determine the phenotypic differentiation and potential application in the food, agriculture or medical industries, between the novel strain and its closest relatives.

1.2.2 *Hypotheses*

i. *Chryseobacterium* will occur in the chicken portion and poultry feather waste since they have been isolated from raw chicken.
ii. The examination of the two unidentified strains isolated from poultry feather waste and chicken portions using phenotypic and molecular techniques will reveal their exact taxonomic identities.
iii. The examination of the growth characteristics of the unidentified strains will give an indication of its spoilage potential in food.
iv. The phenotypic microarray characteristics of the novel species will not only be able to differentiate the novel species from its nearest neighbours, but also give insight in its potential application in the food, agriculture and medical industries.

Hypothesis i) and ii) will be tested in Chapter 3; hypothesis iii) will be tested in Chapter 4, while hypothesis iv) will be evaluated in Chapter 5.

1.2.3 *Objectives*

i. To subject two unidentified strains obtained from poultry feather waste and chicken portions to the latest polyphasic taxonomic techniques to more accurately characterize and classify them.
ii. To describe and name the new species.
iii. To determine the growth characteristics of the unidentified strains in comparison with the growth characteristics of members of the same genus isolated from similar sources.
iv. To use phenotype microarray technology to characterise the unidentified strains and determine the potential applications of these strains.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Chryseobacterium has recently been reclassified into the family *Weeksellaceae* by García-López and co-workers (2019) from its former family, *Flavobacteriaceae*. This reclassification was based on its overall genomic divergence as it appeared as paraphyletic in a GBDP (Genome BLAST Distance Phylogeny) tree, ULT (unconstrained 23S (i.e. large subunit) rRNA) gene trees and URT (unconstrained 16S rRNA) gene tree reduced to genome-sequenced strains. The new family, *Weeksellaceae* was proposed to accommodate those *Flavobacteriaceae* that did not form a clade together with the type genus, *Flavobacterium* (García-López *et al*., 2019).

Flavobacteria species have been isolated from a variety of clinical and environmental sources (Jooste & Hugo, 1999). Spoilage defects due to flavobacteria have been reported in various products including butter (Wolochow *et al*., 1942; Jooste *et al*., 1986a), creamed rice (Everton *et al*., 1968) and canned vegetables (Bean & Everton, 1969). Other food sources that have been reported to contain *Chryseobacterium* spp. include fish (*C. piscium*), meat and meat products, raw chicken (*C. vrystaatense* and *C. carnipullorum*) and dairy products (de Beer *et al*., 2005; Bernardet *et al*., 2006; de Beer *et al*., 2006; Charimba *et al*., 2013).

Food spoilage can be considered as any change in a product that makes it unacceptable for human consumption (Hayes, 1985). Spoilage can be due to physical damage (caused by bruising, pressure, freezing, drying and radiation), chemical damage (oxidation and colour changes), insect damage or the appearance of off-flavours and off-odours from growth and metabolism of microorganisms in the product (Gram *et al*., 2002). Gram *et al*. (2002) defined the spoilage potential of a microorganism as the ability of a pure culture

to produce the metabolites that are associated with the spoilage of a particular product. Psychrotolerant flavobacteria produce lipolytic and proteolytic enzymes that are the main cause of spoilage in dairy products (Sørhaug & Stepaniak, 1997). The major cause of bitterness in milk is the formation of bitter peptides due to the action of proteolytic enzymes (Springett, 1996).

In the first description of *Chryseobacterium* given by Vandamme *et al*. (1994) it was stated that members of the genus show strong proteolytic activity. Roussis *et al*. (1999) found that *Flavobacterium* MTR3 proteinases were active at 32 – 45 °C, and exhibited considerable activity at 7 °C. The enzyme was active at pH 6.0 – 8.0, and exhibited considerable activity at pH 6.0 in the presence of 4% NaCl. Hantsis-Zacharov and co-workers (2008a) showed that two *Chryseobacterium* strains isolated from raw milk showed both proteolytic and lipolytic activity, which makes them likely candidates as spoilage organisms in the milk.

The aims of this literature review were firstly to understand the taxonomy of the genus *Chryseobacterium*. Secondly, to illustrate the ecology of *Chryseobacterium* in food sources. Thirdly, to discuss some of the available polyphasic taxonomic techniques. The fourth aim was to investigate the determination of food spoilage characteristics by microbial growth kinetics, predictive microbiology, production of enzymatic activities and the use of phenotype microarray analysis. Lastly, the applications of *Chryseobacterium* will be highlighted.

2.2 The genus *Chryseobacterium*

2.2.1 History

The genus *Chryseobacterium* was built on the ruin of the former genus *Flavobacterium* (Bernardet *et al*., 2002; Bernardet, 2011). Throughout its history, the genus *Flavobacterium* has undergone many changes with some species being reclassified and redefined. Difficulties were encountered in separating *Flavobacterium* from similar genera like *Cytophaga* and *Flexibacter*. This was based on the fact that differentiation of these

genera were based on ultrastructural features like cellular morphology (Reichenbach, 1989; Holmes, 1992) which tend to have limited taxonomic value (Reichenbach, 1989). The introduction of phylogenetic analysis has provided new insights concerning the taxonomic relationships within this cluster of organisms, which was referred to as the *Flavobacterium-Cytophaga* rRNA cluster (Gherna & Woese, 1992; Nakagawa & Yamasato, 1993; Segers *et al*., 1993).

The first description of *Flavobacterium* included 46 yellow pigmented mainly Gram-negative, rod shaped, non-endospore forming, and chemoorganotrophic species (Bergey *et al*., 1923). In 1939, the polar flagellates were removed from the genus in the fifth edition of *Bergey's Manual of Determinative Bacteriology* (Bergey *et al*., 1939). The genus was further restricted to only Gram-negative species in the seventh edition (Weeks & Breed, 1957). In 1984, *Flavobacterium* was restricted to non-motile and non-gliding species and described as Gram-negative, yellow, aerobic rods usually growing at 5–30 °C (Holmes *et al*., 1984a). Restriction of the genus continued after it was recognized that the type species, *F. aquatile*, did not represent the genus (Holmes, 1993). *Flavobacterium aquatile* was subsequently set aside in Holmes's taxonomic review in the second edition of *The Prokaryotes* (Holmes, 1992) but after a decision by the Judicial Commission of the International Committee of Systematic Bacteriology, *F. aquatile* was required to remain the type species (Bernardet *et al*., 1996) and the other bacterial species of the genus *Flavobacterium* had to be relocated to other or new genera.

In the second edition of *The Prokaryotes*, *Flavobacterium* species were divided by Holmes (1992) into four natural groups. The first group (group A) included [*F*] *balustinum*, [*F*.] *breve*, [*F*.] *gleum*, [*F*.] *indologenes*, [*F*.] *indoltheticum* and [*F*.] *meningosepticum*. Phylogenetic studies done by Vandamme *et al*. (1994) indicated that these species formed a tight cluster, and therefore, *Chryseobacterium* (*C*.) was proposed as a new generic epithet for these organisms.

Vandamme *et al*. (1994) described *Chryseobacterium* to accommodate six species formerly classified within the genus *Flavobacterium*, namely *Chryseobacterium balustinum, Chryseobacterium gleum, Chryseobacterium indologenes,*

Chryseobacterium indoltheticum, [*Chryseobacterium*] *meningosepticum* and *Chryseobacterium scophthalmum*. Although *C. balustinum* and *C. indoltheticum* were the two oldest species, they were not chosen as the type species since they were inadequately characterized with each only represented by a single strain. Since *C. gleum* was well characterized with both its genotypic and phenotypic structures properly studied, it was chosen as the type species of the genus *Chryseobacterium* (Holmes *et al*., 1984b; Vandamme *et al*., 1994).

Kim *et al*. (2005a) later allocated [*Chryseobacterium*] *meningosepticum* and [*Chryseobacterium*] *miricola* to a new genus *Elizabethkingia* under the epithets *Elizabethkingia meningoseptica* and *Elizabethkingia miricola*.

2.2.2 Current Taxonomy

More species of *Chryseobacterium* have been added since its description by Vandamme and co-workers (1994). According to the List of Prokaryotic Names with Standing in Nomenclature (LPSN), there are currently 113 validly named *Chryseobacterium* species (Parte, 2018; Annexure). This list almost doubles that previously reported in 2011 with 61 *Chryseobacterium* species (Bernardet *et al*., 2011). They currently fall under the kingdom *Bacteria*, phylum *Bacteroidetes* (which was previously known by the names '*Cytophaga-Flavobacterium-Bacteroides* group, *Flavobacterium-Bacteroides* phylum and rRNA superfamily V [Bernardet *et al*., 2002]), class *Flavobacteriia*, order *Flavobacteriales* and family *Weeksellaceae* (García-López *et al*., 2019).

2.2.3 Description of *Chryseobacterium*

The genus was described by Vandamme *et al*. (1994) as:

Chry.se.o.bac.te´ri.um. Gr. adj. *chryseos* golden; L. neut. n. *bacterium* a small rod; N.L. neut. n. *Chryseobacterium* a yellow rod.

The cells of this organism are Gram-staining-negative, non-motile, non-spore-forming rods with parallel sides and rounded ends, typically being 0.5 µm wide and 1 – 3 µm long. Intracellular granules of poly-β-hydroxybutyrate are absent. The organisms are aerobic and chemoorganotrophic. All strains grow at 30°C while most strains grow at 37°C. Growth on solid media is typically pigmented (yellow to orange), but non-pigmented strains do occur. Colonies are translucent (occasionally opaque), circular, convex or low convex, smooth, and shiny, with entire edges. In terms of enzyme activity, all species are positive for catalase, oxidase, and phosphatase and strong proteolytic activity occurs. Several carbohydrates, including glycerol and trehalose, are oxidized. Esculin is hydrolyzed while agar is not digested. Chryseobacteria are resistant to a wide range of antimicrobial agents.

The major branched-chain fatty acids are iso-$C_{15:0}$, iso-$C_{17:1}\omega 7c$ (which may have been incorrectly annotated in previous work as iso-$C_{17:1}\omega 9c$), iso-$C_{17:0}$ 3-OH and iso-$C_{15:0}$ 2-OH (annotated as part of summed feature 3, but may also be annotated as summed feature 4, depending on the MIDI system and the peak naming tables used) (Montero-Calasanz *et al*., 2014). The major polyamine is *sym*-homospermidine (Kämpfer *et al*., 2009b). Phosphatidylethanolamine is the major polar lipid and the polar lipid profile contains three common unidentified lipids and two common unidentified aminolipids (Wu *et al*., 2013).

The type species of the genus is *C. gleum*. The DNA base compositions of *Chryseobacterium* species range from 28.8 – 49.3 mol% guanine plus cytosine (G+C) (Annexure; Hugo *et al*., 2019).

The *Sejongia* strains were found to have 16S rRNA gene sequences highly similar to those of *Chryseobacterium haifense* and *Chryseobacterium hominis* and were transferred to the genus *Chryseobacterium* (Kämpfer *et al*., 2009a). Later that year, *Kaistella koreensis* which was the only species belonging to the *Kaistella* genus was reclassified as *Chryseobacterium* (Kämpfer *et al*., 2009b).

2.4 *Chryseobacterium* in food

Chryseobacterium species inhabit a wide range of sources like food (dairy products, meat and poultry, freshwater and marine fish, molluscs and crustaceans); edible plants; soil *per se*, as well as that in contact with root crops. They have also been isolated from other sources e.g., freshwater environments and drinking water; marine environments (including those in the polar regions); clinical sources (e.g. patients and the hospital environment); plants, cats, dogs, birds, amphibians and other reptiles, sea urchins; the eggs and digestive tracts of insects; and also from the vacuoles or cytoplasm of amoebae (Bernardet & Nakagawa, 2006).

Members of *Weeksellaceae* are often regarded as spoilage bacteria in perishable food products. *Chryseobacterium* has been reported to be found in many food sources (Table 2.1).

2.4.1 Fish

Chryseobacterium spp. was found in the mucus of healthy fish which implies that they may be commensals (Lijnen *et al*., 2000; Bernardet *et al*., 2006). *C. balustinum* was isolated from the scales of halibut (*Hippoglossus hippoglossus*) freshly obtained from the Pacific Ocean and was considered as a spoilage organism (Harrison, 1929). Multiple species of *Chryseobacterium* were isolated from marine fish by Engelbrecht and co-workers (1996). These species produced H_2S and hydrolysed substrates like gelatine, casein, to name a few. Pungent and stale odours were reported in the muscle extracts which suggested involvement in fish spoilage. de Beer and co-workers (2006) described *C. piscium* from the South Atlantic Ocean of South Africa. The production of urea and phenylalanine deaminase suggested that *C. piscium* may be involved in spoilage (de Beer *et al*., 2006). González *et al*. (2000) suggested that *Chryseobacterium* spp. were not an important cause of spoilage in fish because they comprised less than 1% of the bacterial communities of the fish that they sampled.

Mudarris & Austin (1989) reported the presence of *C. scophthalmum* in a farmed turbot (*Scophthalmus maximus*) in Scotland. The bacterium was recovered from the gills and

viscera of a fish that exhibited hyperplasia of the gills, hemorrhage of the eyes, skin, and jaw, necrosis and hemorrhage of the brain, stomach, intestine, liver, and kidney, and ascites within the peritoneum. They also recovered *C. scophthalmum* from healthy adult and juvenile wild turbot. Beside the *Chryseobacterium* species that are considered fish spoilers, several other species (not only *C*. *scophthalmum*) are *bona fide* fish pathogens.

2.4.2 Meat and Poultry

Meat and poultry can be contaminated by a variety of microorganisms, some of which are food-borne pathogens, and others can cause spoilage when stored under chilled conditions. Flavobacteria and pseudomonads are known to cause spoilage in food and food products (Forsythe, 2000). 'Flavobacteria' is usually used as a generic name for yellow-pigmented rods when meat spoilage is discussed in literature (Hendrie *et al*., 1969). Flavobacteria, which are responsible for spoilage, may originate from the poultry itself or from the abattoir environment (Hang'ombe *et al*., 1999).

Formerly, chryseobacteria were referred to as CDC Group IIb isolates and were isolated from poultry and meat products (Hayes, 1977; García-López *et al*., 1998). In a study done by Olofsson *et al*. (2007), *Chryseobacterium* was reported as the second most abundant organism in the microbial flora of freshly cut meat. *Bacillus* was the most dominant with *Staphylococcus* being the least dominant. *Pseudomonas* spp. became the dominating bacterial type when the meat was stored at 4°C. *Chryseobacterium gleum* and *C. indologenes* often form part of the initial bacterial flora of raw meat (Bernardet *et al*., 2005).

Chryseobacterium vrystaatense was isolated from raw chicken at different stages of processing, at a plant in South Africa. Although the spoilage capacity of the strains was not tested, they are generally regarded as potential spoilage organisms in meat and poultry (de Beer *et al*., 2005). Charimba *et al*. (2013) isolated *C. carnipullorum* from raw chicken carcasses at a broiler processing plant in Bloemfontein, South Africa.

2.4.3 Dairy products

The flavobacterial/chryseobacterial species are believed to have the potential to cause spoilage defects in dairy products because they are able to utilise a wide range of compounds. Contamination during milking comes from the teat surface, the udder, milking equipment, and the milking parlour environment. After the collection of milk, it is usually stored in a cooling tank. It is during cold storage where psychrotolerant bacteria dominate the flora and produce extracellular enzymes like proteases and lipases, which contribute to the spoilage of dairy products (Hantsis-Zacharov & Halpern, 2007).

Chryseobacterium bovis (Hantsis-Zacharov *et al*., 2008a) and *C. haifense* (Hantsis-Zacharov & Halpern, 2007b) were both isolated from raw cows' milk when a study was done on the diversity of psychrotolerant bacteria in raw milk in Israel. *Chryseobacterium* was isolated together with five other bacteria from 8-day-old cheeses in France (Saubusse *et al*., 2007). In a study carried out on the microbial communities in goats' milk during a lactation year, *C. indologenes* was among the bacterial types isolated from milk during autumn (Callon *et al*., 2007). Sharma and Anand (2002) isolated *Chryseobacterium* isolates from the biofilms of dairy plants. They were isolated from the post-chiller during packaging and the buffer tank outlet during pre-packaging (Sharma & Anand, 2002).

Holmes *et al*. (1984a) first isolated the type species of the genus, *C. gleum* from a hospital environment. It was later isolated by other researchers from the dairy environment (Jooste *et al*., 1985; Welthagen, 1991; Hugo & Jooste, 1997; Hugo *et al*., 1999). Jooste (1985) was the first to isolate chryseobacterial isolates from raw milk in South Africa. *Chryseobacterium joostei* was one of the species that was described from these isolates (Hugo *et al*., 2003).

2.4.4 Other food sources

Shimomura *et al*. (2005) isolated *C. shigense* which was considered to be part of the normal flora from a lactic acid beverage in Japan. More so, in a recent study done by Lin *et al*. (2017), they reported the isolation of *C. endophyticum* from a maize leaf. Four

strains of *C. balustinum* were found on potatoes where they played an antagonistic role against plant-pathogenic fungi and a plant parasitic nematode (Krechel *et al*., 2002). In butter (Wolochow *et al*., 1942; Jooste *et al*., 1986a), creamed rice (Everton *et al*., 1968), and canned vegetables (Bean & Everton, 1969) *Chryseobacterium/Flavobacterium* were reported to result in spoilage.

Table 2.1. *Chryseobacterium* isolated from food sources (Parte, 2018).

Species	G+C (mol%)	Food source	References
C. aahli	34.1	Lake trout (*Salvelinus namaycush*)	Loch & Faisal, 2014
C. arothri	36.5	Pufferfish (*Arothron hispidus*)	Campbell *et al*., 2008
C. bovis	38.6	Raw cow's milk	Hantsis-Zacharov *et al*., 2008b
C. camelliae	41.7	Green tea leaves	Kook *et al*., 2014
C. carnipullorum	36.6	Raw chicken portion	Charimba *et al*., 2013
C. carnis	34.0	Beef	Holmes *et al*., 2013
C. chaponense	Not determined	Atlantic salmon (*Salmo salar*)	Kämpfer *et al*., 2011
C. echinoideorum	36.4	Edible sea urchin	Lin *et al*., 2015
C. endophyticum	37.2	Maize leaf	Lin *et al*., 2017
C. gallinarum	Not determined	Raw chicken portion	Kämpfer *et al*., 2014b
C. joostei	37.0	Raw cow’s milk	Hugo *et al*., 2003
C. piscium	33.6	Fish	de Beer *et al*., 2006
C. oranimense	Not determined	Raw cow’s milk	Hantsis-Zacharov *et al*., 2008a

Species	G+C (mol%)	Food source	References
C. oncorhynchi, C. tructae C. viscerum	36.3 33.6 – 36.1 38.6	Rainbow trout	Zamora *et al*., 2012a, b & c
C. vrystaatense	37.1	Chicken-processing plant	de Beer *et al*., 2005

2.5 Polyphasic taxonomy techniques

Currently, taxonomy of prokaryotes relies on polyphasic combinations of phenotypic, chemotaxonomic and genotypic characteristics (Vandamme *et al*., 1996; Stackebrandt *et al*., 2002; Tindall *et al*., 2010) (Fig. 2.1). A number of researchers are currently making use of this approach (Bernardet *et al*., 2005; Ramasamy *et al*., 2014; Kämpfer *et al*., 2016; Wang *et al*., 2016b; Lin *et al*., 2017).

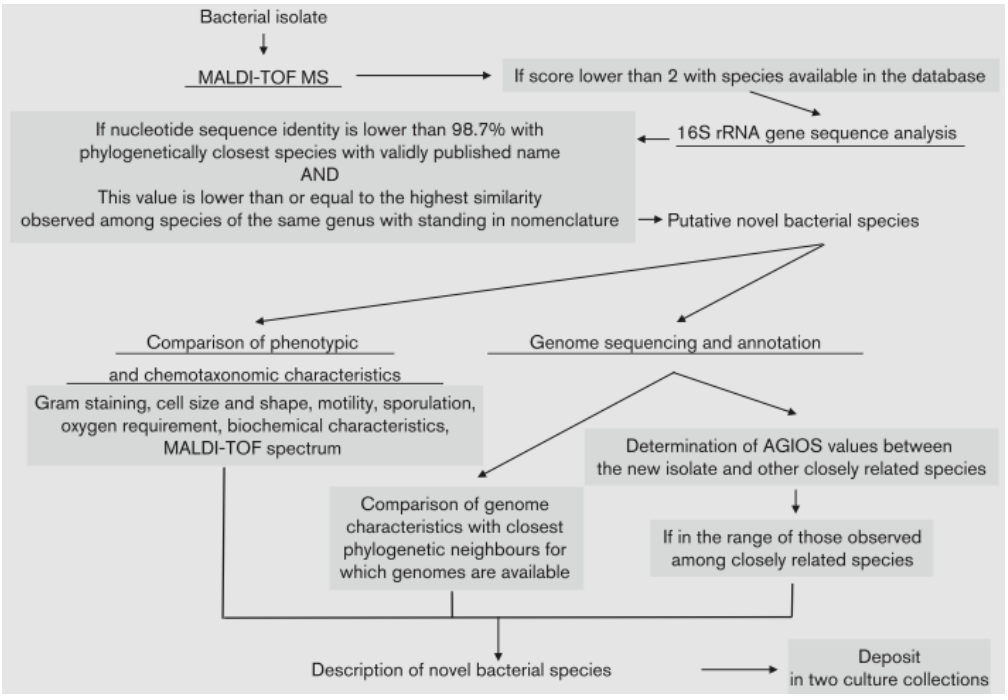


Fig. 2.1 Polyphasic strategy used in taxonomic laboratories for the taxonomic classification of bacterial isolates (Ramasamy *et al*., 2014).

2.5.1 Genotypic methods

Classical genotypic methods initially used for bacterial identification included pulsed field gel electrophoresis (PFGE), restriction fragment length polymorphism (RFLP), plasmid DNA profiling and RFLP derivative methods (Vandamme *et al*., 1996). With the introduction of more advanced molecular techniques like 16S rRNA gene sequencing, DNA-DNA hybridization (DDH), guanine and cytosine ratio (G+C Ratio) and whole genome sequencing, identification has become easier.

2.5.1.1 16S rRNA gene sequencing

rRNA is very important in the study of phylogenetic relationships because it is present in all bacteria, is functionally constant and is composed of highly conserved as well as more variable domains (Woese, 1987; Schleifer & Ludwig, 1989; Stackebrandt & Goebel 1994; Ramasamy *et al*., 2014). Moreover, rRNA can be sequenced directly with the aid of the reverse transcriptase enzyme (Qu *et al*., 1983; Lane *et al*., 1985). This property distinguishes them from other cell features except for a few of the smaller RNA species (Woese, 1987).

There are three kinds of rRNA molecules (5S, 16S, 23S), all of which can be used for phylogenetic analysis. The 5S rRNA gene (120 bp) is small while the 23S rRNA gene (3300 bp) is large. The 16S rRNA gene (1650 bp) is mainly used due to its appropriate intermediate size (Amann *et al*., 1995; Mora & Amann, 2001). A cut-off value of 97% has long been used as the 16S rRNA gene sequence identity value to classify bacterial isolates as novel taxa at the species level while 95% is used as the cut-offs to classify bacterial isolates as novel taxa at the genus level (Stackebrandt *et al*., 2002). However, 98.7% was proposed by Stackebrandt & Ebers (2006) for the delineation of novel taxa at the species level.

Despite the numerous advantages of the 16S rRNA gene, it also exhibits some limitations as a taxonomic marker including: (i) its high degree of conservation in some genera, as is the case for species of the genus *Brucella*, which do not differ by more than 1%

(Gándara *et al*., 2001); (ii) the presence of nucleotide variations among multiple rRNA operons in a single genome (Rainey *et al*., 1996; Acinas *et al*., 2004) and (iii) the possibility of 16S rRNA genes being acquired by HGT that may distort relationships between taxa in phylogenetic trees (Jain *et al*., 1999).

However, the 16S rRNA gene sequence alone cannot be used to delineate species within certain groups and additional genotypic characteristics are often required (Stackebrandt & Goebel, 1994; Gillis *et al*., 2001).

2.5.1.2 Whole genome sequencing (WGS)

In 1999, Fitz, Gibbon & House (1999) proposed that the presence or absence of genes within genomes might be used to assess taxonomic relationships among prokaryotes. More studies suggested genomic sequences to represent a source of taxonomic parameters including chromosomal gene order and metabolic pathways (Snel *et al*., 1999; Huson & Steel, 2004), comparison of orthologous genes (Coenye & Vandamme, 2003) and the presence of indels or single nucleotide polymorphisms (SNPs) in conserved genes (Gupta, 2001). Phylogenetic studies based on the comparison of orthologous genes and the presence or absence of genes further showed good similarity with studies built by comparison of 16S rRNA gene sequences (Zhi *et al*., 2012). The use of whole genome sequencing has made gene content based approaches promising in the field of bacterial taxonomy. In addition, it reveals a substantial number of unique genes present only in a particular genome which can be used for its taxonomic classification (Gupta & Sharma, 2015). Whole genome sequencing has been used to sequence some chryseobacterial strains like *C. indologenes* and *C. oranimense* (Sharma *et al*., 2015; Cimmino & Rolain, 2016; Wang *et al*., 2016a).

Since the introduction of genome sequencing technologies, the number of sequenced prokaryotic genomes has rapidly increased. Genomic sequences are often recommended for taxonomic studies since they use reliable and reproducible data (Ramasamy *et al*., 2014). Initially, genome sequencing was labour intensive and money-consuming and thus poorly adapted to routine use. With the decreasing cost and high throughput of next-

generation sequencing methods, they are now commonly being used and have enabled thousands of genomes to be sequenced (Soon *et al*., 2013).

Overall genome-related indices (OGRIs) are values identified as analogous values to DDH values (Chun & Rainey, 2014). Examples of OGRIs are digital DNA-DNA hybridization (dDDH), average nucleotide identity (ANI), amino acid identity (AAI) and mol% G+C.

DNA-DNA Hybridization (DDH) versus Digital DNA-DNA Hybridization (dDDH)

Before the advent of whole genome sequencing, DNA-DNA hybridization (DDH) or DNA-DNA reassociation was a widely used technique to estimate the genetic relatedness between micro-organisms and is still considered as the 'gold standard' criterion for species delineation of prokaryotes (Wayne *et al*., 1987). The development of DDH has allowed the indirect comparison of gene sequences. DNA–DNA hybridization is performed in cases where the new taxon contains more than a single strain, in order to show that all members of the taxon have a high degree of hybridization among each other. DDH is necessary when strains share more than 97% 16S rRNA gene sequence similarity. If the new taxon shows this high degree of similarity to more than one species, DDH is performed with all relevant type strains to ensure that there is sufficient dissimilarity to support the classification of the strain(s) as a new taxon (Tindall *et al*., 2010). A DDH value ≤ 70% indicates that the tested bacteria belong to distinct species.

The technique is based on the fact that at high temperatures, DNA can be denatured, but the molecule can be brought back to its native state by lowering the temperature (reassociation). It is based on three parameters i.e., i) G + C mol%, ii) the ionic strength of the solution and iii) the melting temperature of the DNA hybrid (T_m). T_m is the only variable parameter out of the three (as ionic strength can be kept constant). Therefore, the more the similarity between the heteroduplex molecule, the higher the temperature that will be required to separate it (high T_m value) (Prakash *et al*., 2007).

This method is disadvantageous because of: i) the cut-off values are not applicable to all prokaryote genera in particular, determining the taxonomic status of an isolate is impossible when the phylogenetically closest species have DDH values of 70% and more, as is the case for most species of the genus *Rickettsia* (Fournier & Raoult, 2009); ii) determining DDH requires special facilities available in a limited number of laboratories; and iii) it is a labour-intensive and expensive method that lacks reproducibility (diverse methods can yield different results) and cannot be used to establish a comparative reference database incrementally (Stackebrandt, 2003; Tindall *et al*., 2010).

Eversince WGS has been used, the digital DNA-DNA hybridization (dDDH) data can now be calculated directly from the WGS data. Various software tools are available to calculate dDDH (Chun *et al*., 2018) e.g., the Genome-Genome Distance Calculator (http://ggdc.dsmz.de/).

Average Nucleotide Identity (ANI)

It is only recently that genome-derived measurements of genetic relatedness based on methods like ANI and average amino acid identity (AAI) have been used for species descriptions (Thompson *et al*., 2015). The ANI is gradually replacing DDH values especially for strains whose genome sequences are known. The ANI of conserved genes present in two sequenced strains represents a robust measure of the genetic and evolutionary distance between them, because it shows a strong correlation with 16S rRNA gene sequence similarity and the mutation rate of the genome, it is not affected by lateral transfer or variable recombination rates of single (or a few) genes and it offers resolution at the subspecies level (Konstantinidis & Tiedje, 2005a).

ANI can be calculated using Kostas lab ANI calculator (http://enve-omics.ce.gatech.edu/ani/). Values of ANI that are 95 – 96% can be regarded equal to DDH values of 70% and can be used as a boundary to delineate species (Goris *et al.*, 2007; Richter & Rosselló-Móra, 2009).

Amino Acid Identity (AAI)

With the introduction of genotypic methods for prokaryotic delineation, the amino acid identity (AAI) of a bacterium in comparison with its closest relatives are used in the delineation of novel species. This is usually calculated with the use of computer-based calculation tools like Kostas lab AAI calculator (Rodriguez-R & Konstantinidis, 2014) which estimates the average AAI using both best hits (one-way AAI) and reciprocal best hits (two-way AAI) between two genomic datasets of proteins. It estimates genome-wide identity between distant organisms and is recommended for more distantly related populations as resolution is progressively lost at the nucleotide level, as nucleotide sequences change more rapidly than amino acid sequences, which are complex and more sensitive over greater evolutionary distances (Konstantinidis & Tiedje, 2005b; Rodriguez-R & Konstantinidis, 2014). The Newman lab AAI calculator is also used to calculate AAI (Newman *et al*., 2019). The cut-off value for strains belonging to the same species is 95%.

Guanine and Cytosine Ratio (G+C Ratio)

DNA base composition is used as a classical genotypic method for the standard description of bacterial taxa (Vandamme *et al*., 1996). DNA is double-stranded with both strands being complementary to one another. These strands are linked by base pairs; G-C (Guanine-Cytosine) and A-T (Adenine-Thymine) with the ratios of G/C and A/T usually constant at 1. The relative ratio [G+C]/[A+T] varies from genome to genome (Mora & Amann, 2001). The variation in the percent G+C content is not more than 3% within a well-defined species and not more than 10% within a well-defined genus and it varies from 24 – 76% in the bacterial world (Prakash *et al*., 2007).

The base ratio of a DNA molecule is generally described as the relative abundance of the pair G+C, and is commonly called G+C content. The DNA base ratio is calculated in percentage of G+C: [G+C]/[A+T+C+G] X 100. The greater the difference between two organisms, the less closely related they are (Mora & Amann, 2001). Theoretically, DNA molecules with differences of greater than 20 – 30 mol % can have virtually no sequences

in common (Logan, 1994). Empirically, it has been shown that organisms that differ by more than 10 mol % do not belong to the same genus and that 5 mol % is the common range found within a species (Mora & Amann, 2001). This method is used to distinguish between phenotypically similar and genomically different strains (Goodfellow & O'Donnell, 1993) and is used for the description of species and genera. Bernardet *et al*. (2011) reported the G+C ratio of *Chryseobacterium* to range between 29 and 39 mol %. However, *Chryseobacterium frigidum* was reported with a G + C content of 49.3 (Kim *et al*., 2016) thus suggesting that this range needs to be amended.

Although this method is taxonomically useful in separating groups, it is limited in that base compositions do not necessarily indicate close relationships because the determinations do not take the linear sequences of bases in the DNA molecules into account (Mora & Amann, 2001). Moreover, this method is advantageous because it directly measures deoxyribonucleotide content and may detect methylated or unusual nucleotides (Lévy-Frébault & Portaels, 1992).

When the WGS data of an organism is, however, available, the G+C content can be calculated from a high-quality genome sequence, therefore, replacing the traditional methods mentioned above (Hahnke *et al*., 2016).

2.5.2 Chemotaxonomic methods

The term chemotaxonomy refers to the application of analytical methods for collecting information on different chemical constituents or chemotaxonomic markers of bacterial cells in order to group or organize them into different taxonomic ranks (Vandamme *et al*., 1996; Mora & Amann, 2001).

Chemotaxonomic methods are used to compare members of closely related taxa especially where novel genera are being proposed. The principle of chemotaxonomy is based on uneven distribution of these markers among different microbial groups (Goodfellow & O'Donnell, 1993). Chemotaxonomy deals with various structural elements of the cell including the outer cell layers (peptidoglycan, techoic acids, mycolic acids, etc.), the cell membrane (fatty acids, polar lipids, respiratory lipoquinones, pigments, etc.) or

constituents of the cytoplasm (polyamines) (Tindall *et al*., 2010). These features are a direct reflection of the expression of the genetic information of an organism (Mora & Amann, 2001). Specific chemicals like amino acids, proteins, lipids and sugars provide good characters for classification and identification (Goodfellow & O'Donnell, 1993). Due to the fact that there is variation in chemical composition due to genetic differences and not due to cultivation conditions, cultures must be grown under standardised conditions prior to comparative chemotaxonomic work (Mora & Amann, 2001).

2.5.2.1. Fatty acid methyl esters

In fatty acid analysis, lipids which are present in bacterial cells are analysed and used to delineate clusters (Welch, 1991). Fatty acids are the major constituents of lipids and polysaccharides and have been used extensively for taxonomic purposes. The variability in chain length, double-bond position, and substituent groups has proven to be very useful for the characterisation of bacterial taxa (Suzuki *et al*., 1993). Bernardet *et al*. (2002) indicated that the presence or amount of some fatty acids could be of value to differentiate a new taxon from existing taxa of the genus. Fatty acid composition is one of the most used chemotaxonomic markers for taxonomic purposes, and its use is highly recommended (Tindall *et al*., 2010).

However, one of the major drawbacks of the technique, like most phenotyping methods, is that the composition may vary depending on the cultivation conditions. For this reason, either an accurate reproduction of the culture conditions reported for closely related taxa must be performed, or simultaneous experimentation with the reference material is necessary (Mora, 2012). Hugo *et al*. (1999) noted that *Chryseobacterium* species could not be differentiated on the basis of fatty acid profiles, whilst those of the related genera *Elizabethkingia meningoseptica*, *Bergeyella zoohelcum* and *Empedobacter brevis* are distinct. Fatty acids that are common to *Chryseobacterium* are the branched-chain fatty acids (iso-$C_{15:0}$, iso-$C_{17:1}$ *ω*7*c*, iso-$C_{17:0}$ 3-OH, and summed feature 4 [iso-$C_{15:0}$ 2-OH or $C_{16:1}$ *ω*7*t* or both]) (Montero-Calasanz *et al*., 2014).

2.5.2.2. Polar lipids

The biosynthesis of polar lipids is not fully understood. Their diversity is associated with the cell membrane(s) and is not limited to just phospholipids (Tindall *et al*., 2010). They are major constituents of the lipid bilayer of bacterial membranes and have been studied frequently for classification and identification purposes. Other types of lipids, such as sphingophospholipids, occur in only a restricted number of taxa and were shown to be valuable within these groups (Jones & Krieg, 1984; Vandamme *et al*., 1996). Phosphatidylethanolamine seems to be common to most chryseobacterial species (Hantsis-Zacharov *et al*., 2008b; Kirk *et al*., 2013; Kämpfer *et al*., 2014a; Kämpfer *et al*., 2015a; Guo *et al*., 2016; Joeng *et al*., 2017; Lin *et al*., 2017).

2.5.2.3. Respiratory quinones

Respiratory lipoquinones are widely distributed in both anaerobic and aerobic organisms within the *Bacteria* and *Archaea.* They are divided into two basic structural classes, naphthoquinones and benzoquinones, with a third class being the benzothiophene derivatives (Tindall *et al*., 2010). The naphthoquinones are subdivided into the phylloquinones and the menaquinones, with the former occurring less commonly in bacteria. Respiratory menaquinones are found in the cytoplasmic membrane of most prokaryotes and play important roles in electron transport, oxidative phosphorylation and active transport (Collins & Jones, 1981; Collins, 1994). Menaquinone 6 is the only respiratory quinone or the major respiratory quinone in *Chryseobacterium* (Bernardet *et al*., 1996; Hugo *et al*., 2019).

2.5.2.4. Pigments

Natural pigments like flexirubins and carotenoids have been extracted from bacteria. Flexirubins are found in the outer membrane of Gram-negative bacteria, and were first extracted from *Flexibacter elegans* now called *Chitinophaga filiformis* (Reichenbach *et al*., 1974; Kämpfer *et al*., 2006). They possess a conserved structural feature [ω-(4-

hydroxyphenyl)-polyene carboxylic acid chromophore, esterified with a 2,5-dialkylresorcinol] which has enabled them to be used as chemotaxonomic markers for bacteria in the phylum *Bacteroidetes* (Schöner *et al*., 2014). This chemotaxonomic feature is used for species delineation. A number of chryseobacterial species produce flexirubin-type pigments (de Beer *et al*., 2005; de Beer *et al*., 2006; Quan *et al*., 2007; Charimba *et al*., 2013; Chaudhary & Kim, 2017; Divyasree *et al*., 2018). A few species produce carotenoid pigments after induction by light (Hantsis-Zacharov & Halpern, 2007b; Joung & Joh, 2011).

2.5.3 Phenotypic methods

Phenotypic methods include all methods that are not directed towards the DNA and RNA, including the chemotaxonomic techniques.

2.5.3.1 Conventional phenotypic analysis

The classical or traditional phenotypic tests are used in identification schemes in the majority of microbiology laboratories. They constitute the basis for the formal description of taxa, from species and subspecies up to genus and family. While genotypic data are used to allocate taxa on a phylogenetic tree and to draw the major borderlines in classification systems, phenotypic consistency is required to generate useful classification systems and may therefore influence the depth of a hierarchical line (Vandamme *et al*., 1996). The classical phenotypic characteristics of bacteria comprise morphological, physiological, and biochemical features. Individually, many of these characteristics have been shown to be irrelevant as parameters for genetic relatedness, yet as a whole, they provide descriptive information enabling us to recognize taxa.

The morphology of a bacterium includes both cellular (shape, endospore, flagella, inclusion bodies, Gram staining) and colonial (colour, dimensions, form) characteristics. The physiological features include data on growth at different temperatures, pH values, salt concentrations, or atmospheric conditions, while the biochemical features include

growth in the presence of various substances such as antimicrobial agents (e.g. zones of inhibition), and data on the presence or activity of various enzymes and metabolization of compounds (glucose test, carbohydrate utilisation, etc.) (Vandamme *et al*., 1996).

One of the major disadvantages with phenotypic methods is the conditional nature of gene expression wherein the same organism might show different phenotypic characters in different environmental conditions. One must note that phenotypic data must be compared with a similar set of data from the type strain(s) of closely related organism(s). Reproducibility of results between different laboratories is another problem, therefore, only the standardized procedure should be used during execution of the experiment (Bernardet *et al.*, 2002; Prakash *et al*., 2007).

2.5.3.2 Automated systems

Miniaturized phenotypic fingerprinting systems have been introduced and may in the future replace classical phenotypic analyses. These systems mostly contain a battery of dehydrated reagents, and addition of a standardized inoculum initiates the reaction (growth, production of enzymatic activity, etc.). The results are interpreted as recommended by the manufacturer and are readily available with a minimal input of time. A number of systems are commercially available like API and the outcome of a particular test with a commercial system is sometimes different from that with a classical procedure, but the same is often true for two classical procedures in the same test. Clearly, phenotypic tests must be performed under well-standardized conditions to obtain reproducible results (Vandamme *et al*., 1996).

The API system

The API test system can contain up to 20 different biochemical tests that consist of microtube/cupsules with substances that are dehydrated but changes colour when an enzymatic reaction takes place. The substrate can either be assimilated or fermented by

the organism (The Global Health Network, 2013). Examples of commercial API systems are API® ZYM, API® 50CH, API® 20E, API® ID 32E and API® 20NE.

The Biolog system

Biolog Inc. is the company responsible for the production of the OmniLog™ identification and phenotype MicroArray systems. The principle of this technology relies on a tetrazolium dye that changes colour when microbial (or mammalian) cells metabolise substances that promote respiration (Fox, 2008). This dye is incorporated in a 96-well microplate (BIOLOG, 2008). The 96-wells contain different carbon sources (sugars, hexose phosphates, amino acids, hexose acids, carboxylic acids, esters, fatty acids, etc.) and other chemical sensitivity tests (NaCl, acidic pH, lactic acid, reducing power, etc.). If the cells are metabolically active, they reduce the redox dye and a purple colour is formed in all “positive” wells.

In the identification system, environmental and pathogenic micro-organisms produce a characteristic pattern or “metabolic fingerprint” from discrete test reactions within the 96-well microplate. These patterns are then analyzed with sophisticated interpretation software and compared to extensive organism and pathogen databases. In the manual systems (MicroLog 1 and MicroLog 2) visual readings are taken for both Gram-positive and Gram-negative microplates and the reaction entries are done manually while in the semi-automated systems (Microstation), the microplates are read by a microstation coupled to a database. In a fully automated system (OmniLog™), the microplates are read by an OmniLog™ incubator. In this system metabolic fingerprints develop rapidly often providing results in four hours or less. These results can be automatically read and recorded in seconds. Intelligent software compensates automatically for different colour/turbidity intensities, eliminating the subjectivity of visual interpretation. The patterns are then compared to an extensive database for final identification. It can identify over 1,900 species of aerobic bacteria, anaerobic bacteria, yeast and filamentous fungi (Hugo & Pohl-Albertyn, 2011).

Biolog Phenotype MicroArrays™ (PM) are a breakthrough platform technology for measuring cell pathway activities and phenotypes by analysing cells under thousands of culture conditions. Through amplification and precise quantification of phenotypes, researchers are able to obtain an unbiased perspective of the effect on cells of genetic differences, environmental change, and exposure to chemicals or drugs. Panels of up to 1920 phenotypic tests have been developed for bacterial and fungal cells, as well as mammalian cells (BIOLOG, 2008). The Phenotype MicroArray (PM) OmniLog™ technology provides a set of nearly 2000 culture conditions in which one can test the ability of a microorganism to respire and grow. The set includes about 200 carbon sources, 400 nitrogen sources, 100 phosphor and sulphur sources, 100 nutrient supplements, and a range of conditions varying the pH, ion and osmotic status of the culture environment. This enables scientists to see what stimulates growth and, equally important, what inhibits growth (Bochner *et al*., 2008).

2.6 Determination of food spoilage characteristics

Food spoilage can be defined as “any sensory change (tactile, visual, olfactory or flavour) which the consumer considers to be unacceptable.” Spoilage may result from physical damage, insect damage, indigenous enzyme activity in the animal or plant tissue or by microbial infections (Rawat, 2015). Microbial growth is one of the main causes of food spoilage.

2.6.1 Microbial growth kinetics

Microbial growth kinetics is the relationship between the specific growth rate (μ) of a microbial population and the substrate concentration (s) (Kovárová-Kovar & Egli, 1998). The specific growth rate is the fraction of the original amount of biomass (or number of cells) by which the population increases per unit time. Growth in this case is regarded as an increase in cell numbers or biomass, and is characterised by four main phases: lag phase, exponential phase, stationary phase and death phase (Fig. 2.2) (Maier & Pepper,

2015). Cells experience distinct physiological changes in these different phases of growth.

In the lag phase cells physiologically adapt to the new culture conditions. This involves specific messenger RNA (mRNA) induction and protein synthesis to meet new culture requirements. In this phase, the growth rate is zero, as little, or no cell division occurs, hence there is no increase in population size. There is intense metabolic activity in which the microorganisms grow in size. This usually lasts for an hour to several days. The lag phase can be influenced by initial inoculum size and the type of medium. A large inoculum will result in a short lag phase (depending on the growth phase of the inoculum). Short lag phases are also obtained when cells are grown in rich media (Madigan *et al*., 2015; Maier & Pepper, 2015).

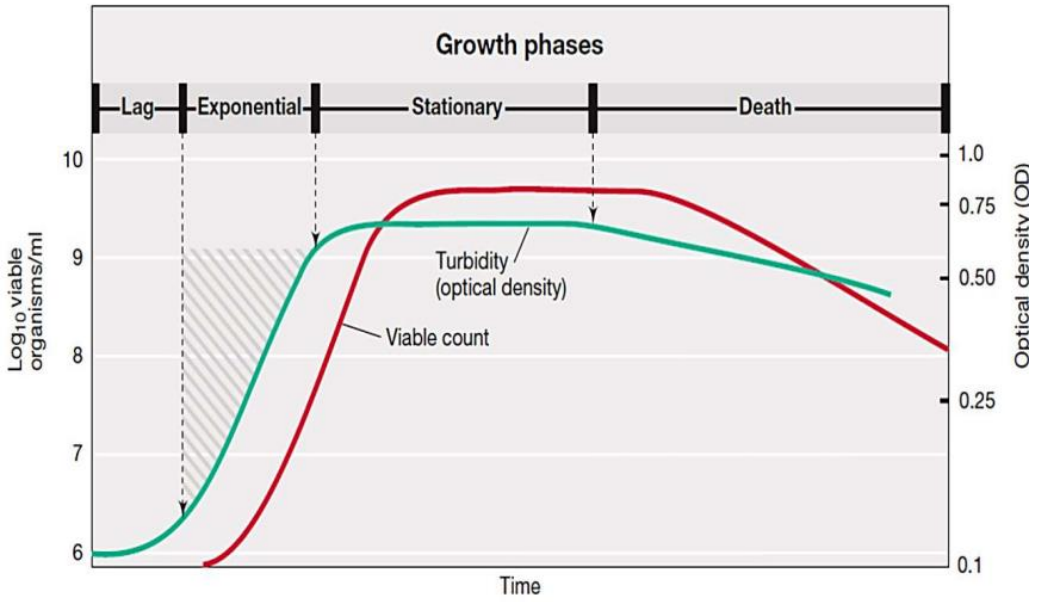


Fig. 2.2. Bacterial growth curve indicating the different phases of growth (Madigan *et al*., 2015).

The exponential growth phase is experienced when there is a maximum, constant growth rate when the limiting substrate concentration is in excess. In this phase, the rate of increase of cells in the culture is proportional to the number of cells present at any particular time (Madigan & Martinko, 2006). It is the period with the most rapid growth where cells begin to divide and generation time reaches a constant minimum. Cells are

at their highest metabolic activity as genetic material (DNA and RNA), cell wall components and other growth-related substances are produced. During this phase the cells are most susceptible to adverse environmental factors, like nutrients and temperature. Many microorganisms excrete by-products during this phase while other bacterial species incorporate intracellular carbonaceous storage compounds or polyphosphates, especially when grown on glucose (Buchanan, 1918; Wanner & Egli, 1990), hence spoilage in food products is likely to occur at this stage. The maximum specific growth rate of a microorganism is determined from the exponential phase of a growth curve (Egli, 2016).

During the stationary phase, the available nutrients become completely used up and waste products build up and become toxic to cells or result in cell growth inhibition. These adverse conditions result in an increase in the competition for nutrients, hence cells become less metabolically active. In this phase the number of dividing cells equal the number of dying cells hence resulting in no increase in population growth (cryptic growth). Due to nutrient stress, stationary phase cells are generally smaller and rounder than cells in the exponential phase (Madigan *et al*., 2015; Maier & Pepper, 2015).

The death phase is characterised by a decrease in population size, where the number of dying cells exceed the number of cells produced. The cell number decreases at a logarithmic rate although the rate of cell death is usually slower than the rate of growth during the exponential phase. A continuous decrease in nutrient availability and an increase in the production of waste products contribute to the increase in cell deaths (Maier & Pepper, 2015).

2.6.2 Predictive Microbiology

Predictive microbiology is the integration of traditional microbiology knowledge with those found in the disciplines of mathematics, statistics and information systems and technology to describe microbial behaviour in order to prevent food spoilage as well as food-borne illnesses (Fakruddin *et al*., 2011). It is based upon the idea that the responses of populations of microorganisms to environmental factors are reproducible, and that by

considering environments in terms of identifiable dominating constraints, it is possible, from past observations, to predict the responses of those microorganisms (Ross & McMeekin, 1994). Predictive microbiology has been applied in numerous fields of microbiology (Table 2.2) (Jankovic *et al*., 2016).

Table 2.2 Applications of predictive microbiology (adapted from Jankovic *et al*., 2016).

Area of application	**Example**
Hazard Analysis Critical Control Point (HACCP)	Preliminary hazard analysis identification and establishment of critical control point (s) Corrective actions Assessment of importance of interaction between variables Risk assessment
Risk assessment	Estimation of changes in microbial numbers in a production chain Assessment of exposure to a particular pathogen
Microbial shelf life studies	Prediction of the growth of specific food spoilage microorganisms Prediction of growth of specific foodborne pathogens
Product research and development	Effect of altering product composition on food safety and spoilage Effect of processing on food safety and spoilage Evaluation of effect of out-of-specification circumstances
Temperature function integration and hygiene regulatory activity	Consequence of temperature in the cold chain for safety and spoilage
Education	Education on safety, especially non-technical people
Design of experiments	Number of samples to be prepared Defining suitable intervals between sampling

The use of mathematic and computer modeling (Table 2.3) to describe and predict the behaviour of foodborne microorganisms is playing a huge role in food microbiology. It can be used to predict microbial safety or shelf life of products, to find critical points in the process, and to optimize production and distribution chains (Zwietering *et al*., 1991).

Predictive microbiology is divided into two main headings: Kinetic models and probability models. Kinetic models involves modelling the extent and rate of growth of microorganisms of concern, while probability modelling involves the construction of models to predict the likelihood of some event (Ross & McMeekin, 1994). Two approaches are used in kinetic modelling. Firstly, the growth rate is modelled and then used to make predictions based on exponential population growth. In the second approach, a sigmoid function is fitted to the observed population growth curve and the effect of environmental factors on the values of parameters of that fitted sigmoid curve are modelled. Predictions are made by evaluating the fitted function (Gibson *et al*., 1988; Fakruddin *et al*., 2011).

There are two main types of models, mechanistic and empirical models (Table 2.3). The former are based on an underlying mechanism within the cells that control its metabolism while the later are generally more statistical in nature. Experimental data is acquired relating to biological responses (such as growth, survival or inactivation), and fitted to an appropriate mathematical relationship with no attempt to infer a mechanistic rationale (Buchanan & Whiting, 1997). Empirical and semi-mechanistic (which uses a combination of biological mechanisms and empirical formulations) models are the most used models in food microbiology. Many other models like Arrhenius formulations, Hinshelwood and Schoolfield models were created based on the mechanistic approach (Hinshelwood, 1946; Mohr & Kraweic, 1980; Esener *et al*., 1981; Schoolfield *et al*., 1981). Mechanistic models, though not widely used, are advantageous in allowing greater latitude in extrapolating beyond the limits of available experimental data (Buchanan & Whiting, 1997).

Five models (Arrhenius, Ratkowsky, Hinshelwood, Zwietering and cardinal temperature model with inflection), have been used to describe the change in maximum specific growth rate (μ_{max}) as a function of temperature T (°C) (Rosso *et al*., 1993).

Table 2.3. Predictive models (adapted from Grimaud *et al*., 2017). CTMI, Cardinal Temperature Model with Inflection; DEB, Dynamic Energy budget.

Models			
Empirical models	**Semi-empirical models**	**General mechanistic models**	**Protein-stability based models**
Square-root CTMI Blanchard Bernard and Rémond Eppley-Norberg	Arrhenius equation	Master reaction Hinshelwood DEB theory	Modified Master reaction Proteome based Heat capacity based

Arrhenius models, being a semi-empirical model, also falls under the secondary kinetic models (models used for describing the effect of environmental conditions on microbial growth) category (Jankovic *et al*., 2016). This model was created by Svaute Arrhenius in 1889 who correlated temperature and the rate constant through the equation, $\mu_{max} = Ae^{-(E/RT)}$. The specific growth rate is represented by μ; A is an entropy constant; E is the activation energy (temperature coefficient); R is the universal gas constant (8.314 $J.mol^{-1}.K^{-1}$) and T is the absolute temperature which is measured in Kelvin. The Arrhenius-type and Bēlehradék-type models are used in interpreting the effects of temperature in microbial ecology in foods. They show minimal differences in the goodness-of-fit hence are very effective in homogenising variance (McMeekin *et al.*, 2013).

Predictive microbiology has been applied in numerous fields of microbiology (Jankovic *et al*., 2016). Bekker and co-workers (2015) used an Arrhenius equation to determine the relationship between the specific growth rate and temperature of *Chryseobacterium joostei*. *Chryseobacterium joostei* grew over a wider temperature range and had the highest maximum specific growth rate and growth temperature (36.9°C) when compared to the other microorganisms (*Chryseobacterium bovis* and *Pseudomonas fluorescens*).

The three strains had low activation energies, hence indicating that they were least sensitive to temperature changes in their respective temperature ranges.

Despite the use and applications of predictive microbiology, it also has some limitations. Firstly, any predictions made outside the experimental ranges are usually not accurate, and in some cases are nonsensical. Secondly, models usually predict faster growth rates than are observed which make them fail-safe but they may be overly conservative. Thirdly, models derived in static conditions may not be applicable to fluctuating conditions, for example those in which environmental conditions (temperature, pH, gaseous atmosphere and a_w) change during the life of the product. Lastly, the microorganisms' previous incubation conditions can affect the subsequent growth rate of the microorganism. Therefore, care is required when applying microbial models to experimental data (Fakruddin *et al*., 2011).

2.6.3 Enzymatic activities of *Chryseobacterium*

Microbial damage by moulds, yeasts and bacteria seems to be the most common form of spoilage in perishable foods like meat, fish and poultry meat. Bacterial contamination is more dangerous because very often food does not look bad even though severely infected, it may appear quite normal. Enzymes are responsible for the destruction of polymers in some foods while chemical reactions like oxidation and rancidity spoil other foods (Rawat, 2015). Proteases and lipases are responsible for spoilage in many food products.

2.6.3.1 Proteolytic activity

Industrially, proteases fall under the most important category of enzymes due to their wide range of applications. They have biotechnological applications in food processing, leather processing and production of protein hydrolysates (Banik & Prakash, 2004). The microbial enzyme, keratinase which is a particular class of proteolytic enzymes, is gaining much attention in the poultry industry. They have been isolated from *Chryseobacterium* spp.

and have been found to be responsible for degrading the insoluble protein keratin which is found in chicken feathers (Brandelli *et al*., 2010). Brandelli & Riffel (2005) reported an extracellular keratinase from a *Chryseobacterium* sp. kr6 growing on raw feathers. The enzyme was produced between 25 °C and 37 °C and showed maximum activity and yield at 30 °C. The enzyme activity was higher when feather or feather meal was used as growth substrates. Keratinase produced by *Chryseobacterium* sp. kr6 was able to completely degrade feather waste (Riffel *et al*., 2003; Riffel *et al*., 2007). Venter *et al*. (1999) also reported on and characterised a heat-stable metalloprotease produced by *Chryseobacterium indologenes* Ix9a. The enzyme had an optimum pH of 6.5 and an optimum temperature of 50 °C. The psychrotolerant bacterium, *C. oranimense* was isolated by Hantsis-Zacharov *et al*. (2008a) from raw cow's milk in Israel. This bacterium possessed both proteolytic and lipolytic activities. *Chryseobacterium carnipullorum* isolated from raw chicken portions was also reported by Charimba (2012) to possess both proteolytic and keratinolytic activities.

2.6.3.2 Lipolytic activity

Lipases are triacylglycerol acylhydrolases (EC 3.1.1.3) that catalyze the hydrolysis of triacylglycerol to glycerol and fatty acids (Verma *et al*., 2012). Rancid off-flavours in food happen as a result of the hydrolysis of these triacylglycerols (Huis in't Veld, 1996). Lipases are unique as they hydrolyse fats into fatty acids and glycerol at the water-lipid interface and can reverse the reaction in non-aqueous media (Ghosh *et al*., 1996). They often express other activities such as phospholipase, isophospholipase, cholesterol esterase, cutinase, amidase and other esterase type of activities (Svendsen, 2000).

Microbial lipases have gained special industrial attention due to their ability to resist extremes of temperature, pH, and organic solvents, and chemo-, region-, and enantio-selectivity (Verma *et al*., 2012). Lipases are considered as fundamental part of numerous industries including pharmaceuticals, tea, dairy, cosmetics, food, leather, detergents, oleo-chemicals, agrochemicals and of many bioremediation processes (Kiran *et al*., 2016). There is limited information on the lipolytic activity of chryseobacteria in literature.

In a study done by Hantsis-Zacharov *et al*. (2008a), they showed lipolytic activity in a *C. bovis* strain isolated from milk.

2.6.4. Phenotype Microarray (OmniLog™) analysis

Phenotype Microarray (PM) technology is a high-throughput method that provides over 1920 set of culture conditions under which one can test the ability of a microorganism to respire and grow. This includes hundreds of C-sources, N-sources, P- and S-sources, nutrient supplements, and various conditions varying the pH, ion, and osmotic status of the culture environment. This technology can be used to determine nutrients that stimulate and inhibit growth. Phenotypes are the expression of genotypes and reveal gene functions, hence PMs allow microbiologists to observe genetically inherited traits and aid in genetic manipulations (Bochner *et al*., 2008). The utilisation of a wide range of carbon substrates by microorganisms can be used to assess the spoilage potential of a given microorganism.

Phenotype MicroArrays have been used in understanding phenotypic properties of diverse microorganisms and also to improve taxonomy and phenotypic description of species (Bochner *et al*., 2008).

2.7 Applications of *Chryseobacterium*

Chryseobacterium indologenes isolated from soil by a sequential enrichment strategy was able to degrade toxic compounds like furan and phenolics, which were obtained as products of acid pre-treatment of lignocellulosic biomass (López *et al*., 2004). A bacterial consortium comprising *Chryseobacterium indologenes, Comamonas testosterone* SB2, *Pseudomonas corrugate* SB4 and *Stenotrophomonas maltophilia* SB5 were isolated from agricultural soil in Indonesia and were used in a growth study. The study showed that the bacteria were capable of degrading the toxic environmental pollutant, 4-chloroaniline (4CA). Growth studies were done with aniline and 4CA as single and mixed substrates.

However, the bacteria preferred to grow on and utilise aniline rather than 4CA, although they were both eventually depleted from the supernatant (Radianingtyas *et al*., 2003).

An application of *Chryseobacterium* was seen in a new *Chryseobacterium* sp. strain SSJ1 which was isolated from agricultural soil with biodegradable potential. The bacterium was able to degrade flubendiamide, which is a non-environmental friendly pesticide. Among five other flubendiamide-resistant bacterial strains used for the study, *Chryseobacterium* sp. strain SSJ1 was the most efficient flubendiamide-resistant organism with a maximum resistance of up to 1000 $mg.l^{-1}$ flubendiamide and degraded about 89.06% of the initial pesticide. The homology of the partial sequence with that from the NCBI database was compared and confirmed the identity of the strain as *Chryseobacterium indologenes* (Jadhav & David, 2016).

Another *Chryseobacterium* sp. was found in the rhizospheres of a wide variety of plants like tomatoes, sweet potatoes, wheat and cucumber where it was seen to protect plants against pathogens and it also possessed plant growth-promoting properties, which makes them beneficial in both the agricultural and industrial sectors (Nishioka *et al*., 2016).

A 24 kDa endopeptidase was purified from a *Chryseobacterium* sp. which was isolated from fish. The enzyme was capable of degrading human plasminogen and yielding an angiostatin-like fragment, hence resulting in the reduction of cell-associated plasmin activity (Lijnen *et al*., 2000).

Another potential application was demonstrated by *C. proteolyticum* which produced an extracellular protein-glutaminase that catalyzes the deamidation of glutaminyl residues in intact proteins without transglutaminase and protease activities. Protein alteration resulted in a protein with an improved amphiphilic character that could be used as an emulsifier or foaming agent in the food industry (Yamaguchi *et al*., 2001).

Pentachlorophenol (PCP) is used as an insecticide, fungicide, algicide, herbicide and disinfectant and as an ingredient in anti-fouling paint. Its accumulation through the food chain is dangerous since it is mutagenic and comutagenic and poses significant health hazards to humans. PCP degradation was investigated by using mixed cultures of *Chryseobacterium gleum*, *Agrobacterium radiobacter* and *Pseudomonas* sp.

Chryseobacterium gleum demonstrated the highest PCP degradation ability (Yu & Ward, 1996).

An application of *Chryseobacterium* was noticed in the production of a cold-active serine protease from a novel *Chryseobacterium* sp. The strain showed highest protease production at 5 °C. An increase in the fragmentation of myofibrils was observed when freshly cut lean cow meat was treated with the enzyme. An increase in tenderisation was also observed hence making this enzyme a candidate to develop potential applications in the food processing industry (Mageswari *et al*., 2017).

Many other applications of *Chryseobacterium* lay in the applications of its keratinase enzymes. Keratinases are microbial enzymes capable of degrading keratin. Keratin is a fibrous and recalcitrant structural protein and is the third most abundant polymer in nature after cellulose and chitin. It is a structural component of skin, hair, feather, horns, bones, fur, claws, hides, wool, scales, bristles, hooves, cloves, nails, beaks, reptilian osteoderm, and fish teeth and slime (McKittrick *et al*., 2012; Gopinath *et al*., 2015).

Metalloproteases which degrade keratin have been obtained from *Streptomyces* sp. 594 (De Azeredo *et al*., 2006), *Lysobacter* NCIMB 9497 (Allpress *et al*., 2002; Wang *et al*., 2008), *Chryseobacterium* sp. (Silveira *et al*., 2012; Wang *et al*., 2008), *B. subtilis* MTCC 9102) (Balaji *et al*., 2008), *Microbacterium* sp. strain kr10 (Thys & Brandelli 2006), and *Pseudomonas aeruginosa* (Lin *et al*., 2009). These enzymes are capable of degrading keratin and show importance in the hydrolysis of feather, hair and wool to clear obstructions in the sewage system during wastewater treatment. Moreover, some keratinases degrade α-keratin and thus have been used to dehair hides in the leather industry. This is advantageous in that it does not affect the tensile strength of the leather and also reduces pollution caused by their chemical counterparts (Chaturvedi *et al*., 2014).

Better dehairing potential of these enzymes has led to the development of greener hair-shaving technology and personal care products. Furthermore, they show great potential in the new area of prion degradation for treatment of the “mad cow” disease (Langeveld *et al*., 2003; Gupta & Ramnani, 2006). In addition, keratinases can be added to detergents for wool cleaning and used for degumming of silk and finishing textile fibers, which

increases its smoothening, shining, dyeing and shrink-proof capabilities (Souza *et al*., 2007; Lv *et al*., 2010). Some keratinolytic proteases are commercially available and are marketed using different names (Lange *et al*., 2016)

A number of *Chryseobacterium* sp. have been reported to produce keratinolytic enzymes with feather-degrading potential (Riffel *et al*., 2003; Riffel *et al*., 2007; Casarin *et al*., 2008; Wang *et al*., 2008; Lv *et al*., 2010; Silveira *et al*., 2012; Bach *et al*., 2015). These microbial keratinases therefore pose a better, cost-effective and environmentally friendly method to address the feather disposal problem in the poultry industry.

The applications of organisms in specific industries, as well as the spoilage potential of an organism, can be studied using the OmniLog Phenotype Microarray™ system of Biolog™. See section 2.5.3.2 for a discussion on this technology.

2.8 Conclusions

The genus *Chryseobacterium* was born from a series of changes made to the family *Flavobacteriaceae*. Vandamme *et al*. (1994) described the genus to accommodate six former *Flavobacterium* species with *C. gleum* being the type strain. The genus *Chryseobacterium* later gave birth to the new genus *Elizabethkingia* (Kim *et al*., 2005a). As of date 113 species (Parte, 2018) of *Chryseobacterium* are validly published as compared to the six which were available in 1994 (Vandamme *et al*., 1994).

Initially, only phenotypic tests were used to delineate prokaryotes. This was later changed due to the shortcomings of the lone method. Physiological, biochemical and chemotaxonomic methods were subsequently introduced. The aforementioned methods did not address the genotypic properties of prokaryotes hence the introduction of a polyphasic approach which encompassed both phenotypic and genotypic methods. This method is now used as the standard method for prokaryotic delineation in addition to the classical methods.

Chryseobacterium strains are ubiquitous, occupying a wide range of different sources ranging from dairy, poultry, food, clinical and soil environments. Some strains are

beneficial to certain environments like the soil while others are harmful to others (dairy, poultry, food and clinical). The enzymatic activities of *Chryseobacterium* strains have been associated with their spoilage potential especially in dairy, poultry and food products.

The main cause of spoilage in food comes from microbial activities within the food product. Microbial growth kinetics is used to investigate the growth profiles of bacteria under different environmental conditions. The introduction of predictive microbiology allows for predictions of microorganisms under different conditions to be made and this can be linked to real food and environments. Understanding microbial behaviour will therefore add value to understanding food spoilage potential.

Phenotype microarray technology provides a wide range of substrates whose utilisation patterns can be used to predict food spoilage potential. This allows for an easier, quick and less time consuming way to assess microbial spoilage using over 1920 substrates or chemicals. This technology may also be used to investigate the potential applications of an organism. *Chryseobacterium* species have application in several industries. It is therefore important that more research be done on these applications to explore its benefits in order to provide solutions where necessary.

CHAPTER 3

POLYPHASIC TAXONOMIC STUDY OF *Chryseobacterium* ISOLATES FROM CHICKEN AND THE DESCRIPTION OF *Chryseobacterium pennae* SP. NOV.

Abstract

A polyphasic approach was used to investigate two aerobic, yellow-pigmented Gram-negative, flexirubin-producing, non-spore-forming, bacterial strains (1_F178^T and 5_R23647) isolated from a chicken broiler plant in Bloemfontein, South Africa. A comparison of the 16S rRNA gene sequences of the two organisms with the sequences of all the type strains of the most closely related species of the genus *Chryseobacterium* showed the highest sequence similarities of strains 1_F178^T to *Chryseobacterium jejuense* (99.1%) and *Chryseobacterium nakagawai* (98.7%), and that of strain 5_R23647 to *Chryseobacterium piscium* (98.8%) and *Chryseobacterium balustinum* (98.6%). Strain 1_F178^T contained menaquinone MK-6 as the predominant respiratory quinone. The major fatty acids of the isolates were iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and iso-$C_{17:1}$ *ω*9*c* which confirmed their affiliation to the genus *Chryseobacterium*. The polar lipid profile comprised of phosphatidylethanolamine and four aminolipids, two glycolipids and one lipid, which are presently uncharacterized. The genome of both strains were sequenced and assembled with strain 1_F178^T and 5_R23647 having genome sizes of 6.2 Mbp and 4.7 Mbp and G+C contents of 35.6 and 33.5 mol%, respectively. Overall genome similarity metrics (Average Nucleotide Identity (ANI), Amino Acid Identity (AAI) and digital DNA-DNA Hybridization (dDDH)) confirmed greatest similarity of strain 1_F178^T to *Chryseobacterium jejuense* (86.4%, 89.3% 31.4%) and *Chryseobacterium nakagawai* (86.6%, 89.6%, 32.7%), and that of strain 5_R23647 to *Chryseobacterium piscium* (96.3%, 96.7%, 68.3%) and *Chryseobacterium balustinum* (93.0%, 92.9%, 51.7%), hence disqualifying strain 5_R23647 as a new species since its ANI and AAI values were above the threshold value (95%). Based on evidence presented in this study, strain 1_F178^T is

considered to represent a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium pennae* sp. nov. is proposed. The type strain is 1_F178^T = KCTC 62759^T = LMG 30779^T.

3.1. Introduction

With the introduction of the polyphasic approach, a number of chryseobacterial species like *C*. *jejuense*, *C*. *nakagawai*, *C*. *piscium*, *C*. *balustinum* and *C*. *carnipullorum* have been described using this approach (Vandamme *et al*., 1994; de Beer *et al*., 2006; Weon *et al*., 2008; Charimba *et al*., 2013; Holmes *et al*., 2013). However, there is still a shortage in genotypic data as not all chryseobacterial strains have had their whole genome sequenced.

The aim of this study was to carry out polyphasic studies on two novel bacterial strains (1_F178^T and 5_R23647) isolated from poultry portions and feather waste. Whole genome sequencing was done as part of the genotypic studies, with PATRIC (Pathosystems Resource Integration Center) and RAST (Rapid Annotation with Subsystem Technology) algorithms used to assemble and annotate the genomes of the aforementioned strains. The second aim was to describe and name the new species based on bacterial taxonomy.

3.2. Materials and methods

3.2.1. Cultures used and their maintenance

The details of the isolates and reference strains used in this study are given in Tables 3.1 and 3.2, respectively. All reference strains were obtained from international culture collections: National Collection of Type Cultures (NCTC) in England; Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Germany; Laboratorium voor Microbiologie, Ghent (LMG) in Belgium, in a freeze-dried state in ampoules. For shorter-term maintenance, the isolates were freeze-dried on 5 mm diameter filter paper discs in sealed Petri dishes and stored at -20 °C. Before use the strains were reactivated

in 10 ml nutrient broth (NB; Oxoid CM67). Purity was checked by streaking on nutrient agar (NA; Oxoid CM003) and Gram-staining. Incubation was at 25 °C for 48 h. The pure cultures on nutrient agar slants were stored at 4 °C for short-term maintenance and re-streaked every 4 to 6 weeks.

Table 3.1 Unidentified isolates used for the polyphasic study.

Isolate	Source	Place of isolation	Date of isolation
1_F178^T	Chicken feather waste	Bloemfontein (Abattoir)	2010
5_R23647	Chicken portion	Bloemfontein (Abattoir)	2002/3

Table 3.2 Reference strains used for the polyphasic study. NCTC, National Collection of Type Cultures; DSM, Deutsche Sammlung von Mikroorganismen; LMG, Laboratorium voor Microbiologie, Ghent.

Genus and species	Culture collection	Source of isolation	Reference
Chryseobacterium jejuense	DSM 19299^T	Soil	Weon *et al*., 2008
Chryseobacterium nakagawai	NCTC 13529^T	Kidney abscess	Holmes *et al*., 2013
Chryseobacterium piscium	LMG 23089^T	Fresh marine fish	de Beer *et al*., 2006
Chryseobacterium balustinum	NCTC 11212^T	Heart blood of fresh water fish	Holmes *et al*., 1984a
Chryseobacterium gleum	NCTC 11432^T	Vaginal swab	Holmes *et al*., 1984b; Vandamme *et al*., 1994)

3.2.2. Genotypic methods

3.2.2.1. DNA Extraction

Freeze-dried filter paper discs containing strains 1_F178^T or 5_R23647 were revived in 10 ml of NB for 48 h at 25 °C after which the cultures were each transferred to 90 ml of fresh NB in 500 ml Erlenmeyer flasks. They were cultivated for 48 h on an orbital shaker at 70 rpm for 48 h at 25 °C. After cultivation, the cells were harvested by centrifugation at 3000 x *g* for 5 min and the subsequent pellet was used for DNA extraction. DNA was extracted by using the NucleoSpin® Microbial DNA (Macherey-Nagel) kit, according to the manufacturer's instructions. The extracted gDNA quantity and quality was assessed using the Nanodrop ND-1000 (v3.3.0) spectrophotometer (Thermo Scientific). The extracted DNA was stored at -20 °C until use.

3.2.2.2. 16S rRNA gene sequencing

The DNA of the isolates in Table 3.1 were subjected to whole-cell polymerase chain reaction (PCR) amplification of the 16S rRNA gene according to the manufacturer's instructions (Applied Biosystems); dNTPs (10 mM) were added to 0.2 ml PCR tubes, followed by the forward and reverse primers (10 µM). The forward primer used was 27F (5"-AGAGTTTGATCCTGGCTCAG-3", Integrated DNA Technologies) and the reverse primer was 1492R (5"-GGTTACCTTGTTACGACTT-3", Integrated DNA Technologies). The buffer (ThermoPol®; 5 µl of 10x concentration) and 1 µl of the DNA template sample were added to the PCR tubes. In addition, 0.3 µl of 5000 U/ml Taq DNA polymerase (New England BioLabs Inc.) was added. The reaction volume was adjusted to 50 µl by addition of nuclease-free water and a quick spin of 1 s was done to collect all the liquid at the bottom.

Thermal cycling was conducted using a 2720 Thermocycler (Applied Biosystems) that was programmed as follows: initial denaturation at 94 °C for 5 min, 35 amplification cycles; denaturing at 94 °C for 30 sec, annealing at 50 °C for 30 seconds and elongation at 72 °C for 115 s. Final elongation was allowed at 72 °C for 5 min and the reaction was kept at 4 °C until further processing.

3.2.2.3. Visualisation of PCR amplicons

The PCR products were visualized on a 1% w/v agarose (Seakem® LE Agarose, Lonza) gel to which ethidium bromide stain was added. The agarose gel was prepared using 1x TAE buffer (containing 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0).

The gel wells were loaded with 1 µl of 6x loading buffer mixed with 5 µl of the PCR product. The DNA marker Thermo-Scientific O'gene Ruler™ (3 µl) was loaded alongside the samples. Electrophoresis was performed for 34 min at 9 $mV.cm^{-1}$.

The PCR amplicons were viewed using a Gel Doc™ EZ Imager (Bio-Rad) and photographed using ImageLab™ software (version 5.0, Bio-Rad).

3.2.2.4. Amplicon clean-up and sequence reactions

Amplicon lengths of approximately 1500 bp corresponding to the expected amplicon bands were excised and purified with the Wizard® SV Gel and PCR Clean-Up system (Promega) per manufacturer's instructions. These were used as templates for the subsequent sequencing reactions using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The forward primer used was 27F (5"-AGAGTTTGATCCTGGCTCAG-3", Integrated DNA Technologies) and the reverse primer was 1492R (5"-GGTTACCTTGTTACGACTT-3", Integrated DNA Technologies). The primers were used in separate reactions during sequencing. For each reaction, two separate mixtures were prepared respectively for the 27F primer and the 1492R primer. The ddNTP chain termination method (Sanger sequencing) was used followed by sequencing clean-up reactions using the EDTA/ethanol precipitation protocol in the BigDye manual. The samples were submitted for Sanger sequencing at the University of the Free State, Bloemfontein, South Africa.

Sequence data was analysed and aligned using Geneious Pro R9 software (http://www.geneious.com, Kearse *et al*., 2012) and compared with sequences on the EzBioCloud (https://www.ezbiocloud.net/) database (Yoon *et al*., 2017) to identify closely related validly published species.

Phylogenetic and molecular evolutionary analyses were conducted with MEGA (Molecular Evolutionary Genetics Analysis) software version 7 (Tamura *et al*., 2016) using neighbour-joining and maximum likelihood methods with Kimura two (KP2) parameter distance measure to determine the relationship of the unidentified isolates to that of the type strains of the already identified 113 *Chryseobacterium* species (http://www.bacterio.net/chryseobacterium.html). Confidence values were estimated from bootstrap analysis of 1000 replicates.

3.2.2.5. Whole Genome Sequencing

Genomic DNA of strains 1_F178 ^{T} and 5_R23647 were extracted as described in 3.2.2 with the DNA quality checked with a Nanodrop ND-1000 (v3.3.0) spectrophotometer. The samples were then sent to the Next Generation Sequencing unit at the University of the Free State, Bloemfontein, South Africa where additional quality control measures were performed before sequencing. The Nextera® XT DNA Library Prep kit was used to sequence the gDNA according to manufacturer's instructions. An Illumina MiSeq sequencer was used to sequence the genome and the assembly was performed on the PATRIC platform (https://www.patricbrc.org/), with SPAdes 3.10.0 as the assembly method.

The sequenced genomes were uploaded to RAST (Rapid Annotation with Subsystems Technology) database (http://rast.nmpdr.org) for annotation. Genome related data including gene number, genome size, G+C content, coverage, N50 value, number of contigs and full 16S rRNA sequence were obtained through RAST. The whole-genome shotgun project was deposited in DDBJ/ENA/GenBank (https://www.ncbi.nlm.nih.gov/) under the identification numbers as indicated in Table 3.3.

Table 3.3 GenBank identification information for *Chryseobacterium* sp. 1_F178^T and 5_R23647.

Identification information	*Chryseobacterium* sp. 1_F178^T	*Chryseobacterium* sp. 5_R23647
Accession	QNVT00000000	QNVW00000000
BioProject	PRJNA478523	PRJNA478529
BioSample	SAMN09519169	SAMN09519216
SUB ID	SUB4224542	SUB4224706
GenBank assembly accession	GCA_003385515.1	GCA_003391035.1
Assembly name	ASM338551v1	ASM339103v1
WGS project	QNVT01	QNVW01
Taxid	2258962	2258964

Average Nucleotide Identity (ANI), Amino Acid Identity (AAI), digital DNA-DNA Hybridisation (dDDH), DNA G+C content data and the Venn diagram with shared and unique genes were all obtained from whole genome sequencing (WGS) data using the algorithms as shown in Table 3.4.

3.2.2.6. Average Nucleotide Identity (ANI)

The ANI values were obtained by using three different ANI calculators; Kostas Lab ANI calculator, JSpecies ANIb calculator and ChunLab's OAT (Orthologous Average Nucleotide Identity Tool) which measures the overall similarity between two genome sequences. Values between 95~96% are proposed as cut-off for species demarcation (Lee *et al*., 2016).

Table 3.4 Algorithms and tools used for the calculation of the overall genome relatedness indices (OGRI) and Venn diagram creation.

OGRI	Tool	URL link	Reference
ANI	Kostas lab ANI calculator	http://enve-omics.ce.gatech.edu/ani/	Rodriguez-R & Konstantinidis, 2016
ANI	Orthologous Average Nucleotide Identity Tool (OAT)	https://www.ezbiocloud.net/tools/orthoani	Lee *et al*., 2015
ANI	JSpecies (ANIb)	http://jspecies.ribohost.com/jspeciesws/	Richter *et al*., 2015
AAI	Newman lab ROSA calculator	http://lycofs01.lycoming.edu/~newman/ROSA.html	Newman *et al.*, 2019
dDDH	Genome-Genome Distance Calculator (GGDC 2.0, formula 2)	http://ggdc.dsmz.de/	Meier-Kolthoff *et al*., 2014
Venn diagram	Venn Diagram Data Generator V 2.1	http://lycofs01.lycoming.edu/~newman/CurrentResearch.html	Gale *et al*., 2014

3.2.2.7. Amino Acid Identity (AAI)

Amino Acid Identity was determined by using the Newman Lab AAI calculator (Bortniak *et al*., 2019). This tool constructs matrices of AAI and BBH (Bidirectional Best Hit) values

from a set of files derived from the RAST Sequence Based Comparison Tool after comparing up to 11 genomes.

3.2.2.8. Digital DNA- DNA Hybridization (dDDH)

The GGDC (Genome-Genome Distance Calculator) web service used for dDDH provides a method for inferring whole-genome distances, which mimics classic DDH without inheriting its pitfalls. Once the assembled genome sequences were obtained, they were uploaded to the distance calculation form of the GGDC website for the calculation of the intergenomic distances. This was done together with the reference genomes.

3.2.2.9. Venn diagram

The exported data from comparison of annotated genome sequences by the RAST SeedViewer Sequence Based Comparison tool were copied to the Venn diagram data generator tool created by the Newman Lab (Bortniak *et al*., 2019). This extracted the desired information (e.g. number of genes present in every genome as well as the number and annotated function of unique and shared genes), which was used to construct the Venn diagram.

3.2.3. Conventional phenotypic tests

The strains used for the entire polyphasic studies are shown in Tables 3.1 and 3.2. The reference strains shown in Table 3.2 were chosen based on their phylogenetic relatedness to the unidentified strains, taking into consideration their similarity values in EzBioCloud.

3.2.3.1. Morphological tests

In order to observe colony morphology, cell cultures were streaked on nutrient agar (NA) plates and incubated for 48 h at 25 °C. The presence or absence of fruity odour as well

as the colour of the colonies were noted. Gram-staining, the production of oxidase, catalase and phosphatase enzymes were determined according to MacFaddin (1980). Motility was determined by phase-contrast examination of wet mounts from NB (CM67; Oxoid). Gliding motility was determined according to the protocol of Jooste (1985).

Bacterial cells were prepared for scanning electron microscopy (SEM) visualisation by first growing the cells on NA for 48 h at 25 °C after which the cells were scraped off and put into a 15 ml conical centrifuge tube containing 0.1 M (pH 7.0) sodium phosphate-buffered glutaraldehyde (3%) for at least 3 h to fix the cells. A second fixation step was done for 1 h in the same buffer with the addition of osmium tetroxide (1%). The material was collected on 0.2 µm polycarbonate membrane filters and dehydrated in a graded ethanol series (50%, 70% and 95% for 20 min in each phase followed by two changes in 100% for 1 h in each phase). The material was dried using a critical point dryer (Tousimis, Maryland, U.S.A.). After drying, material was mounted on stubs (Cambridge pin type, 10 mm) using double sided carbon tape and gold coated (±60 nm) with a Bio-Rad sputter coater (United Kingdom). Specimens were examined and analysed with a JSM-7800F extreme-resolution analytical field emission SEM (FE-SEM).

For transmission electron microscopy, the cell material was obtained as described for SEM above. The cell material was fixed in 0.1 M (pH 7.0) sodium phosphate-buffered glutardialdehyde (3%) for at least 3 h. After fixation, the samples were gently pelleted, washed and re-suspended in buffer. A drop of the suspension was placed onto a formvar grid and air-dried. A 2% solution of uranyl acetate dissolved in distilled water was used to negatively stain the sample. The specimens were examined with a Philips CM100 transmission electron microscope (FEI, The Netherlands).

3.2.3.2. Biochemical tests

Strains were cultivated in 10 ml nutrient broth (NB), after which the cell culture was transferred and cultivated in fresh 100 ml of NB in 500 ml Erlenmeyer flasks at 25 °C for 48 h. The cells were then centrifuged at 3000 x *g* for 10 min with an Eppendorf 5430 R centrifuge (Eppendorf AG, Hamburg, Germany). The supernatant was discarded and the

cell pellets were washed with phosphate buffer (0.1 M, pH 7). Centrifugation was repeated with the same conditions as before. Cell pellets were then re-suspended in 10 ml of fresh phosphate buffer and standardized in comparison with a McFarland number 2 density (6 x 10^8 CFU/ml) standard (Difco 0691326). This served as the inoculum for the different biochemical tests.

The following range of phenotypic tests were carried out according to Cowan (1974) and MacFaddin (1980) unless otherwise indicated: oxidative or fermentative metabolism of glucose; methyl red and Voges Proskauer reactions; gluconate oxidation; potassium cyanide tolerance; malonate utilization; growth in 0 – 5% (w/v) sodium chloride; growth at 4, 20, 32, 35, 37 and 42 °C; growth on cetrimide agar (Merck 5284), MacConkey agar (Oxoid CM0007), Simmon's citrate agar (Oxoid CM155); growth in 0 – 6% (w/v) sodium chloride; reduction of 0.4% selenite (Holmes *et al*., 1975); nitrate and nitrite reduction; production of acid from 10% (w/v) glucose and lactose; alkaline reaction on Christensen's citrate agar (Holmes *et al*., 1975); production of ammonia from arginine; lysine decarboxylase, ornithine decarboxylase, deoxyribonuclease (Oxoid CM321+ 0.01% toluidine blue), β-galactosidase (ONPG), hydrogen sulphide (TSI method), indole (Kovac's reagent), 3-ketolactose, phenylalanine deaminase, urease on Christensen's urea agar (Richard & Kiredjian, 1995; Hugo, 1997); hydrolysis of esculin (Yabuuchi *et al*., 1990), casein, gelatine (plate method), starch (West & Colwell, 1984), Tween 20, Tween 80 (West & Colwell, 1984), tyrosine (Barrow & Feltham, 1993); acid production in D-mannitol, L-arabinose, trehalose, ethanol and D-xylose. The sugars were incorporated at a final concentration of 1% (w/v).

3.2.3.3. Automated phenotypic tests

3.2.3.3.1. BIOLOG Omnilog Gen III Identification System

The unidentified strains and reference strains were cultivated on NA plates for 24 h at 25 °C and their profiles determined using the BIOLOG Omnilog Gen III identification system (BIOLOG Inc., Hayward, CA, USA) according to the manufacturer's instructions. Cells were cultivated on NA plates for 24 h after which a sterile swab was used to pick up a cell

colony. This was suspended in an inoculating fluid (IF-A) at the recommended cell density (90 – 98% T). A 100 µl of the cell suspension was then inoculated into each of the GEN III MicroPlate wells. The inoculated microtitre plates were incubated at 25 °C for 24 h in the OmniLog incubator/reader to allow the phenotypic fingerprint to form. The RetroSpect 2.0 software was used to view the data.

3.2.3.3.2. API identification system

Analytical profile index (API) was done based on the manufacturer's instructions. Both API® 20 NE (bioMérieux® SA, France, Ref 20050) and API® ZYM (bioMérieux® SA, France, Ref 25200) were used.

For API® 20 NE and API® ZYM, cells were grown on NA for 24 h after which single colonies were suspended in 2 ml of sterile 0.85% NaCl. The suspension was then brought to a turbidity equivalent to 0.5 McFarland (for API® 20 NE) and 5 McFarland (for API® ZYM). This was used to inoculate the API® 20 NE and API® ZYM strips according to the manufacturer's instructions. The strips were incubated at 25 °C for 24 h (for API® 20 NE) and 4 h (for API® ZYM).

3.2.3.4. Chemotaxonomic Methods

3.2.3.4.1. Fatty acid methyl ester analysis

Analysis of cellular fatty acids was done at the Lycoming College, U.S.A. according to the method of Sasser (1990). Cells were cultivated on tryptic soy broth agar (TSBA) at 25 °C for 24 h after which approximately 5 mg of single colonies were placed in a clean vial. The cells were saponified with NaOH, methanol and distilled water (45:150:150, w/v/v), and then methylated with 325 ml of 6 N HCl and 275 ml of methanol. The fatty acids were then extracted with hexane and methyl tert-butyl ether (200:200, v/v). The samples were cleaned by using NaOH and distilled water (10.8:900; w/v) in order to reduce contamination of the column, detector and injection port liner. The fatty acid methyl esters (FAME) were separated with the Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.), consisting of an Agilent model 6890N gas

chromatograph fitted with a 5% phenyl-methyl silicon capillary column (0.2 mm X 25 m), a flame ionization detector, Agilent model 7683A automatic sampler, and a HP-computer with MIDI data base (Hewlett-Packard Co., Palo Alto, California, U.S.A.). The following gas chromatographic parameters were used: 2 µl injection volume; column head pressure was 60 kPa; ultra-high-purity hydrogen was used as the carrier gas; column temperature ranged from 170 to 270 °C at 5 °C.min^{-1}; temperature of the injection port was 240 °C; detector temperature was 300 °C; and the septum purge was 5 ml.min^{-1}. The peaks were automatically integrated and the fatty acid percentages calculated using Excel.

3.2.3.4.2. Polar lipids

Polar lipid extraction was done according to the method by Nguyen & Kim (2017) with some modifications. Cells were cultivated on tryptic soy agar (TSA) at 25 °C for 24 h after which the cells were scraped and placed in clean glass vials. Chloroform and methanol (2:1, v/v) were added and stirred overnight at 180 rpm, at 25 °C. Cell debris was removed by centrifugation at 3,200 x *g* for 15 min at room temperature. Polar lipids were then extracted by evaporating the solvent at 35 °C in a rotary vacuum evaporator (Labotec, Heidolph VV2011) attached to a peristaltic tube pump (Verder Deutschland GmbH – Dusseldorf). The precipitate was resuspended in 6.5 ml of chloroform:methanol:0.3% aqueous NaCl (6:10:3, v/v/v) after which 2 ml of chloroform and 2 ml of 0.3% aqueous NaCl were added, mixed for 15 min and centrifuged at 4,400 x *g* for 15 min. The lower layer was then evaporated as discussed above. The lipid precipitate was resuspended in 300 µl of chloroform:methanol (2:1, v/v), filtered using a 0.2 µm syringe filter, and then analysed by two-dimensional thin-layer chromatography (TLC).

Each sample (15 µl) was spotted on a silica gel 60 (Merck Millipore) plate. Chloroform:methanol:water (65:25:4, v/v/v) were used as mobile phase for the first chromatography dimension and once the run was complete, the plates were air-dried before placing them in the second mobile phase consisting of chloroform:glacial acetic acid:methanol:water (40:7.5:6:2, v/v/v/v). The plates were air-dried before they were analysed. Three TLC plates were prepared per sample for different characterization purposes. The first TLC plate was used to detect the total lipid profile and was immersed

in phosphomolybdic acid (12 g phosphomolybdic acid in 250 ml of ethanol). Plate 2 was used for amino lipid and phospholipid detection and was immersed in ninhydrin reagent (ninhydrin:butanol:10% acetic acid (200:95:5, w/v/v)). The last plate was used for the detection of glycolipids and was immersed in α-naphthol:sulphuric acid:methanol reagent (2.4:10:80, w/v/v). Excess stain was removed by resting the edge of the plates on a paper towel to absorb the stain after which the spots were developed by baking the plates in an oven (Plate 1: 160 °C for 15 – 30 min; Plate 2: at 110 °C for 15 min and Plate 3: 120 °C for 3 – 5 min).

3.2.3.4.3. Respiratory lipoquinones

The presence of respiratory lipoquinones was determined according to the method by Reddy *et al*. (2007). Cells were cultivated on TSA at 25 °C for 24 h after which approximately 100 mg of cells were obtained and smeared into screwed cap glass tubes. Three millilitres of hexane:methanol (1:2, v/v) was added and the mixture was placed in an orbital shaker at room temperature for at least 30 min. Then, 1 ml of ice-cold hexane was added, vortexed briefly, and incubated on ice for 15 min. Centrifugation then followed at 3000 x *g* for 3 min to separate the methanol and cell debris from the hexane phase. The upper hexane phase was transferred to a large glass vial labelled with quinone and the strain designation. Then, 2 ml of aqueous 0.3% NaCl and 2 ml of hexane were added to the remaining methanol/cell debris fraction and mixed with a Pasteur pipette. This was centrifuged at 3000 x *g* for 3 min to separate the methanol and cell debris from the hexane phase after which it was combined with the upper hexane phase from the first round of extraction. The crude quinone fraction was then air-dried overnight after which the quinone residue was dissolved in 100 µl *tert*-butylmethyl ether and transferred into a 0.5 ml of microcentrifuge tube. Then, 10 µl of the quinone fraction was spotted ten times (in order to concentrate the sample) per spot on silica gel 60 (Merck Millipore) TLC plates and left to dry before inserting into the TLC mobile phase (hexane:*tert*-butylmethyl ether, 80:20, v/v) to separate the different classes of quinones.

Once the run was complete, the plates were dried, viewed under UV light (Gel Doc™ EZ Imager (Bio-Rad) with the dark quinone spots circled with a pencil. The circled spots were

then scraped off and transferred into a labelled microcentrifuge tube. In order to elute the respiratory quinones from the scraped matrix, 0.5 ml of hexane:methanol (1:2, v/v) was added, vortexed and centrifuged for 1 min. This was done twice with both eluted fractions combined and chilled on ice. Then, 200 µl of a 0.3% NaCl solution was added and allowed to stand for a few minutes to allow the separation of the upper hexane phase. A 0.5 ml amount of hexane was added and the upper hexane phase was transferred into a glass vial and allowed to evaporate. After evaporation, the residue was suspended in 150 µl of methanol and transferred into a labelled glass HPLC vial. The quinones were then separated by HPLC on a C-18 column, using methanol:heptane (9:1, v/v) as mobile phase at a flow rate of 0.2 $ml.min^{-1}$ with the absorbance of the eluate monitored at 269 nm.

3.2.3.4.4. Liquid Chromatography Mass Spectrometry (LCMS) analysis

Samples were analysed on an ABSCIEX 4000QTRAP hybrid triple quadrupole mass spectrometer with Shimadzu front end. Ten microliters of each sample were injected into a Discovery HS C-18 reverse phase column (250 x 4.6 mm, Supelco) and separated using a 35% to 90% linear water (Solvent A) and acetonitrile (Solvent B) gradient over 25 min at 0.3 $ml.min^{-1}$ for a total runtime of 60 min including column re-equilibration. Eluting analytes were analysed in negative APCI ionization mode using an information dependent acquisition (IDA) method where ions between 500 and 700 Da with intensities above 300 000 counts per second (cps) originating from an enhanced MS (EMS) survey scan, were selected and fragmented in the collision cell and the fragments between 50 and 700 Da recorded following an enhanced product ion (EPI) scan.

3.2.3.4.5. Pigment extraction

The strains were streaked on NA plates and incubated to obtain sufficient growth for 48 h at 25 °C. The cells were then scraped and transferred to 2 ml Eppendorf tubes with 1 ml of acetone added. The tube was placed on an orbital shaker for 30 min at 180 rpm. Centrifugation was then carried out for 5 min at 3000 x *g* and the acetone extract was decanted into 2 ml HPLC vials and analysed on an Agilent 1200 HPLC with an Agilent

Zorbax Eclipse XDB C-18 column (4.6 mm x 250 mm) at 40 °C and a diode array detector. Separation was achieved using 50 mM NaH_2PO_4 (pH 4.5) as solvent A; methanol containing 0.1% (v/v) glacial acetic acid as solvent B; a flow rate of 1 $ml.min^{-1}$; and the following gradient: 0 – 5 min at 0%; increase to 75% solvent B at 10 min; increase to 100% solvent B at 30 min; remain at 100% solvent B for 45 min.

3.3. Results and discussion

3.3.1. PCR amplicons and 16S rRNA sequence analysis

The electrophoregrams of the PCR amplicon for the 16S rRNA gene of strains 1_F178^T and 5_R23647 gave bands of approximately 1500 bp (Fig. 3.1.). The 16S rRNA sequences obtained from Sanger sequencing were blasted on the NCBI GenBank (http://www.ncbi.nlm.nih.gov) and EzBioCloud (http://www.ezbiocloud.net/) databases and the results confirmed strains 1_F178^T and 5_R23647 belonging to the genus *Chryseobacterium* based on their high similarity values (Table 3.5).

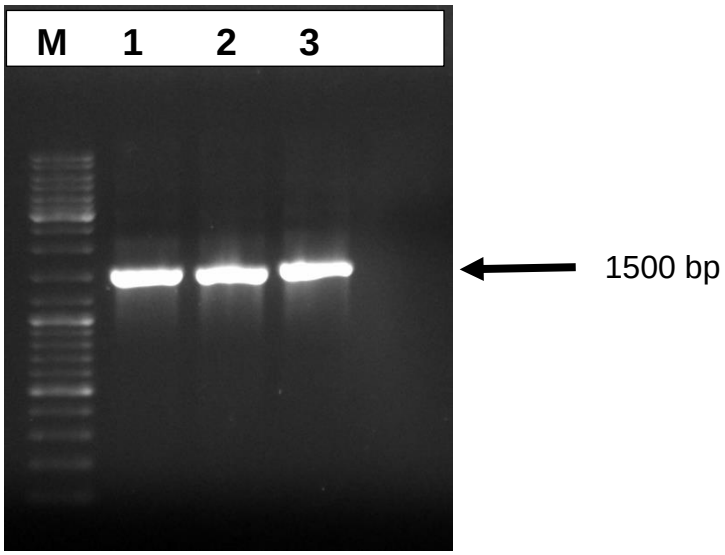


Fig. 3.1. Agarose (1%) gel photo showing ~1500 bp PCR products of the 16S rRNA regions of isolates 1, strain 1_F178^T; 2, strain 5_R23647; 3, strain 7_F195^T (not relevant to this study); M, DNA molecular marker.

Chryseobacterium jejuense and *C. nakagawai* were considered as the two closest relatives of strain 1_F178^T while *C piscium* and *C. balustinum* were considered as the closest relatives of strain 5_R23647 with high 16S rRNA gene sequence similarity values ranging between 98.68 to 99%. In 2006, Stackebrandt and Ebers proposed a threshold value of 98.7% to confirm strains belonging to separate species after establishing a correlation between 16S rRNA and DNA re-association. Considering this, it can be concluded that strains 1_F178^T and 5_R23647 do not represent new species since their closest relatives have similarity values equal to or greater than 98.7%. However, Stackebrandt and Goebel (1994) as well as Wayne *et al*. (1987) concluded that for strains with 16S rRNA sequence similarities greater than 97%, DNA-DNA hybridization will have to be performed to confirm separate species status.

3.3.2. Phylogenetic analysis

Phylogenetic trees constructed with both neighbour-joining (NJ; Fig. 3.2.) and maximum-likelihood (ML; Fig. 3.3.) methods confirmed *C. jejuense* and *C. nakagawai* as the nearest phylogenetic neighbours of strain 1_F178^T and *C. piscium* and *C. balustinum* as the nearest phylogenetic neighbours of strain 5_R23647 based on their 16S rRNA sequence similarity values. Strains 1_F178^T and 5_R23647 formed separate lineages from the other *Chryseobacterium* species on both trees although strain 1_F178^T had lower bootstrap values as compared to strain 5_R23647. For both treeing methods, strain 1_F178^T formed a sub-cluster with *C. rhizosphaerae* which is a soil isolate. This may explain its relation to strain 1_F178^T since it was isolated from chicken feather waste buried in the soil near a chicken processing plant. Strain 5_R23647 formed sub-clusters with *C. scophthalmum* and *C. indoltheticum* which are isolates of fish and marine mud respectively, quite different from chicken portions which is the origin of strain 5_R23647. These trees therefore revealed a clear affiliation of both 1_F178^T and 5_R23647 isolates to the genus *Chryseobacterium*.

Table 3.5 GenBank and EzBioCloud BLAST results for 16S rRNA gene sequence for strains 1_F178^T and 5_R23647.

Isolate	Description	Max % Identity	Accession
1_F178^T			
NCBI GenBank	*Chryseobacterium nakagawai* strain BIGb0215 Ga0304820_113, whole genome shotgun sequence	99.51 (2597/2597)	NZ_RKHU01000013.1
	Chryseobacterium jejuense strain NCTC 13492^T, whole genome shotgun sequence	99.09 (2564/2564)	NZ_UAWB01000001.1
	Chryseobacterium sp. StRB126 DNA, complete genome	98.67 (2531/15152)	NZ_AP014624.1
EzBioCloud	*Chryseobacterium jejuense* strain DSM 19299^T	99.10 (1426/1439)	Jgi.1085875
	Chryseobacterium nakagawai strain NCTC 13529^T	98.75 (1421/1439)	Jx100822
	Chryseobacterium lactis strain NCTC 11390^T	98.61 (1419/1439)	Jx100821
5_R23647			
NCBI GenBank	*Chryseobacterium piscium* strain CCUG 51923^T contig 138, whole genome shotgun sequence	98.79 (2495/2495)	NZ_QNVS01000138.1
	Chryseobacterium balustinum strain NCTC 11212^T whole genome shotgun sequence	98.72 (2490/2490)	NZ_UAVR01000023.1
	Chryseobacterium indoltheticum strain NCTC 13560^T, whole genome shotgun sequence	98.64 (2484/2484)	NZ_UFVS01000003.1
EzBioCloud	*Chryseobacterium piscium* strain LMG 23089^T	98.89 (1423/1439)	AM040439
	Chryseobacterium indoltheticum strain DSM 16778^T	98.75 (1421/1439)	Jgi.1096611
	Chryseobacterium balustinum strain DSM 16775^T	98.68 (1420/1439)	Jgi.1096607

A 16S rRNA sequence similarity value of 98.7% or below was used amongst other genotypic parameters to classify the strains as distinct species. It cannot be concluded that strains 1_F178^{T} and 5_R23647 are new species, basing classification only on 16S rRNA sequence similarity values, since their closest neighbours (*C. jejuense* (99.10%), *C. nakagawai* (98.75%) and *C. piscium* (98.87%), *C. balustinum* (98.68%) have 16S rRNA sequence similarity values above the threshold value. Due to the low resolution of the conservative nature of the 16S rRNA gene, the introduction of other genotypic indices like ANI and AAI should be considered in addition to 16S rRNA sequence similarity for classification to be effective (Richter & Rosselló-Móra, 2009).

Chryseobacterium jejuense, C. nakagawai, C. piscium and *C. balustinum* were chosen as the reference strains, based on the phylogenetic results, for the polyphasic studies of strains 1_F178^{T} and 5_R23647 in addition to *C. gleum*, as the type species for the genus *Chryseobacterium*.

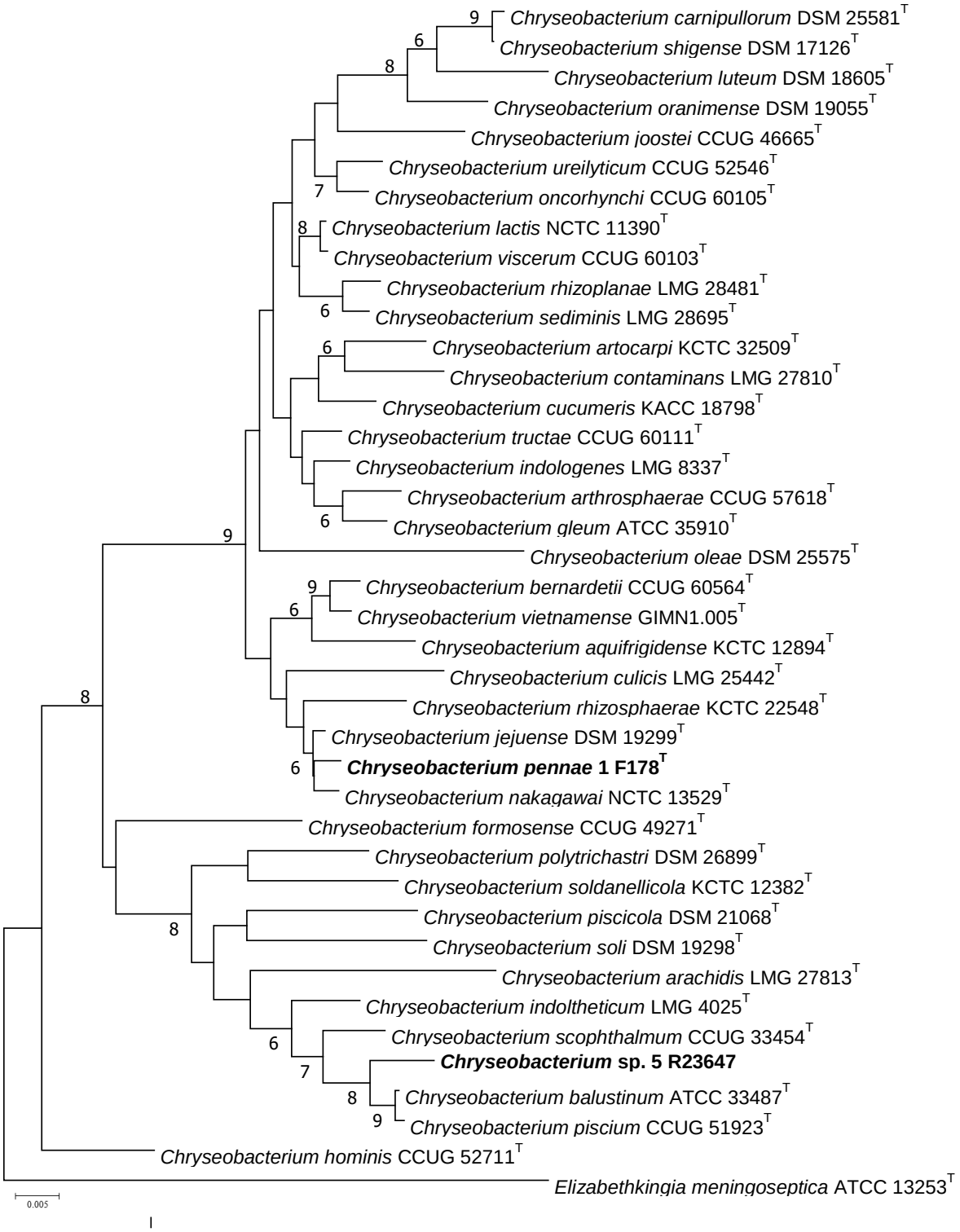


Fig. 3.2. Phylogenetic analysis of strains 1_F178^T and 5_R23647, nearest *Chryseobacterium* type species and outgroup (*Elizabethkingia meningoseptica*) based on 16S rRNA gene sequences available from the EzBioCloud database (accession numbers are given in parentheses). Multiple alignments were performed and evolutionary distances were computed using the Kimura 2-parameter method. Clustering was determined using the Neighbour-Joining method in the MEGA version 7 software package (Tamura *et al*., 2016). Bootstrap values >60%, based on 1000 replications, are given as percentages at the branching points. Bar, 0.0050 substitutions per nucleotide position.

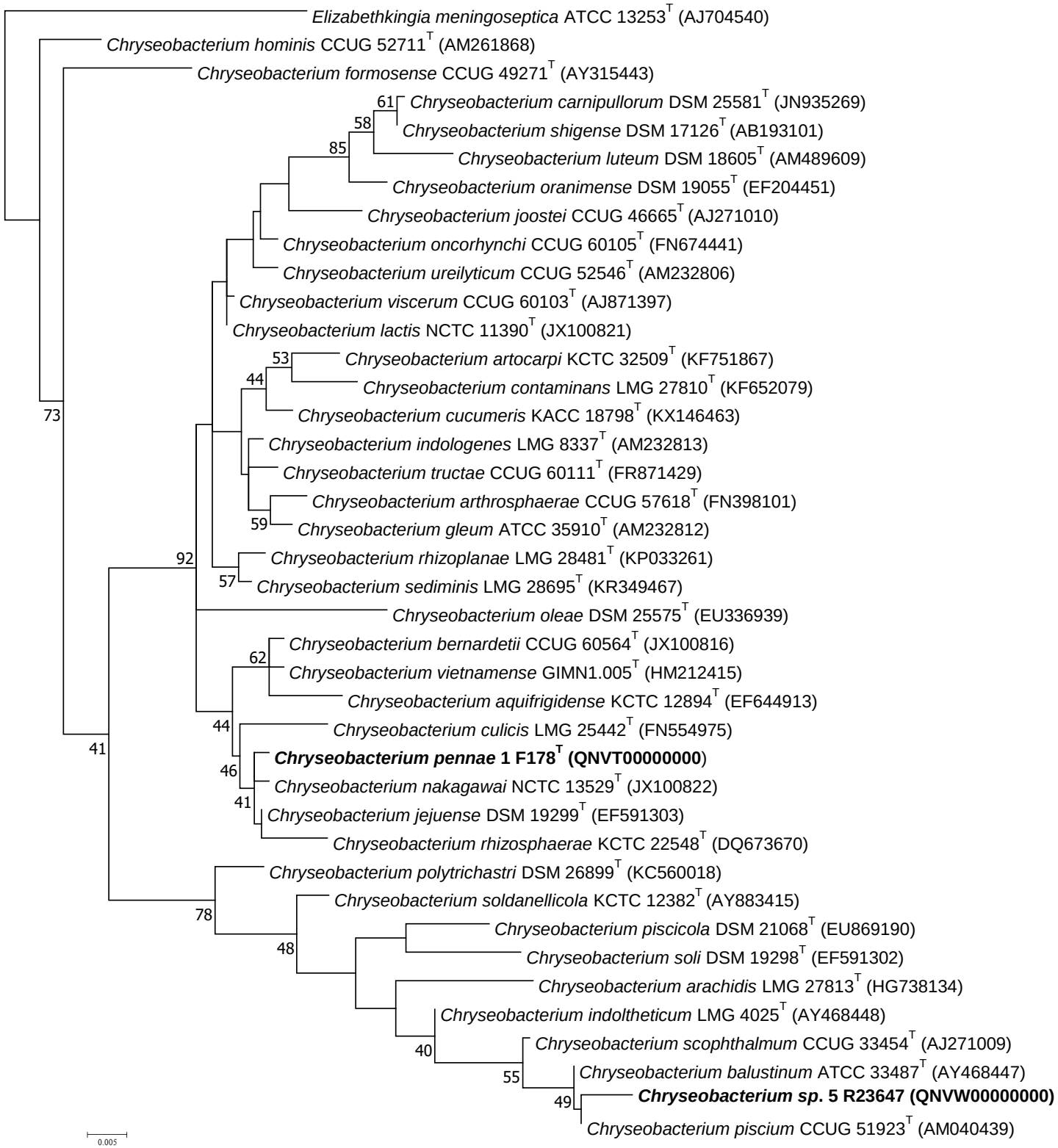


Fig. 3.3. Phylogenetic analysis of strains 1_F178^T and 5_R23647, nearest *Chryseobacterium* type species and outgroup (*Elizabethkingia meningoseptica*) based on 16S rRNA gene sequences available from the EzBioCloud database (accession numbers are given in parentheses). Multiple alignments were performed and evolutionary distances were computed using the Kimura 2-parameter method. Clustering was determined using the Maximum Likelihood method in the MEGA version 7 software package (Tamura *et al*., 2016). Bootstrap values >40%, based on 1000 replications, are given as percentages at the branching points. Bar, 0.0050 substitutions per nucleotide position.

3.3.3. Whole genome sequencing

Tables 3.6 show the general genome features of strains 1_F178^T and 5_R23647. Strain 1_F178^T has a much larger genome (6187872 bp) when compared to the average size of a *Chryseobacterium* genome (4.5 Mb), with a G+C content of 35.6 mol% (Table 3.6). The G+C contents of strains 1_F178^T and 5_R23647 fall within the range (29.7 – 42 mol%) of the G+C content of species belonging to the genus *Chryseobacterium* (Hugo *et al*., 2019), hence confirming their affiliation to this genus.

Table 3.6. Genome features of strains 1_F178^T and 5_R23647.

Genome features	**1_F178^T**	**5_R23647**
Genome size (bp)	6187872	4678357
Coding sequences (CDS)	5682	4424
Number of contigs	88	91
N50	162953	119221
Coverage	25.0x	30.9x
G+C (mol%)	35.6	33.5

Tables 3.7 and 3.8 show the DDH, ANI, AAI values and the 16S rRNA similarity values of the query strains strains 1_F178^T and 5_R23647 and their reference strains.

Stackebrandt & Ebers (2006) suggested a 16S rRNA gene sequence similarity value of 98.7–99% as the threshold value for the delineation of prokaryotic species. Strain 1_F178^T and its closest relatives, *C. nakagawai* and *C. jejuense*, have 16S rRNA similarity values (99.10% and 98.75%) above the threshold values (Tables 3.7 and 3.8) for novel species delineation. This implies that strain 1_F178^T might be the same as the reference species or it could be a different strain of the same species therefore disqualifying it as a novel species. However, 16S rRNA similarity values are not the lone genotypic parameters used for prokaryotic delineation because of their limited resolution due to high 16S rRNA sequence conservation. Based on this, for strains with 16S rRNA gene sequence similarity values greater than 97%, DDH is required.

A strain with a DNA-DNA similarity value of 70% or greater is considered to belong to the same species (Bernardet *et al*., 2002). The digital DDH values of *C. jejuense* and *C. nakagawai* (31.4% and 32.7%) as well as the seven other closest relatives had dDDH values well below 70% hence confirming the classification of strain 1_F178^T as a new species of *Chryseobacterium*. The results presented in Tables 3.7 are consistent with the findings of Stackebrandt & Ebers (2006) who concluded that two organisms with less than 98.5% 16S rRNA sequence similarity were unlikely to have DDH values greater than 70%. They also confirm that 16S rRNA gene sequence similarity have limited resolution (i.e. they have less discrimination/delineation power) since the region is quite highly conserved.

Table 3.7 16S rRNA similarity values and OGRIs of strain 1_F178^T in comparison with its nine closest *Chryseobacterium* relatives. dDDH, digital DNA-DNA hybridization; ANI (EzBioCloud), Average Nucleotide Identity; AAI, Average Amino acid Identity. Reference strains used for this study are indicated in bold; *, type species of the genus *Chryseobacterium*.

Chryseobacterium **strain**	**16S rRNA (EzBioCloud, %)**	**dDDH (%)**	**ANI (%)**	**AAI (%)**
C. jejuense	**99.10**	**31.4**	**86.4**	**89.3**
C. nakagawai	**98.75**	**32.7**	**86.6**	**89.6**
C. oncorhynchi	97.82	27.0	83.3	86.0
C. ureilyticum	97.92	27.4	83.5	85.7
C. indologenes	97.92	24.7	81.0	84.4
C. lactis	98.61	25.3	81.4	84.4
C. rhizosphaerae	98.16	25.1	81.4	84.3
C. viscerum	98.57	25.9	81.6	83.6
**C. gleum*	**97.36**	**25.3**	**81.2**	**83.5**

Table 3.8 16S rRNA similarity values and OGRIs of strain 5_R23647 in comparison with its nine closest *Chryseobacterium* relatives. dDDH, digital DNA-DNA hybridization; ANI (EzBioCloud), Average Nucleotide Identity; AAI, Average Amino acid Identity. Reference strains used for this study are indicated in bold; *, type species of the genus *Chryseobacterium*.

Chryseobacterium **strain**	**16S rRNA (EzBioCloud, %)**	**dDDH (%)**	**ANI (%)**	**AAI (%)**
C. piscium	**98.87**	**68.3**	**96.2**	**96.7**
C. balustinum	**98.68**	**51.7**	**93.0**	**92.9**
C. scophthalmum	97.78	41.6	90.2	92.2
C. indoltheticum	98.75	39.5	89.7	91.7
C. piscicola	97.36	24.1	79.4	80.4
C. soldanellicola	97.43	22.7	79.1	80.1
C. arachidis	96.94	22.8	78.9	79.2
C. soli	96.94	21.6	77.5	78.5
**C. gleum*	**99.10**	**21.4**	**76.7**	**77.2**

In order to avoid the taxonomic mislabelling of bacterial strains, Figueras and co-workers (2014) suggested the use of more than one ANI calculation method in the description of new species. In addition to EzBioCloud's Orthologous Average Nucleotide Identity Tool (OAT) ANI calculator, Kostas lab ANI calculator and JSpecies (ANIb) (Table 3.4) were therefore used to calculate the ANI of strains 1_F178^T and 5_R23647.

Table 3.9 clearly shows ANI values lesser than 95% for strain 1_F178^T and its closest relatives when all three methods are used, hence confirming the novelty of strain 1_F178^T as a new species of the genus *Chryseobacterium*. However, for strain 5_R23647, the ANI values for its closest relatives are less than the threshold value, except for *C. piscium* with ANI values above 95%.

Table 3.9 ANI value calculation of strains 1_F178^T, 5_R23647 and their reference strains using three different methods. OAT, Orthologous Average Nucleotide Identity Tool.

	ANI values (%)		
Chryseobacterium **strain**	**OAT** (EzBioCloud)	**Kostas lab**	**JSpecies (ANIb)**
1_F178^T			
C. jejuense	86.40	86.22	85.56
C. nakagawai	86.63	86.77	85.58
C. gleum	81.28	82.67	80.21
5_R23647			
C. piscium	96.27	96.37	95.84
C. balustinum	93.01	93.74	92.33
C. gleum	76.70	79.14	76.34

Richter & Rosello-Mora (2009) concluded that species with ANI values greater than 95% belong to the same species. The two closest relatives of strain 1_F178^T as well as the seven other closest relatives all have ANI values less than 95% hence confirming strain 1_F178^T as a new species of *Chryseobacterium*.

Species with AAI values less than 95% are considered to belong to the same species (Konstantinidis & Tiedje, 2005a). *Chryseobacterium jejuense* and *C. nakagawai* (89.3% and 89.6%) and the other seven closest relatives of strain 1_F178^T all have AAI values less than 95% hence confirming this strain as a novel species of *Chryseobacterium*.

For strain 5_R23647 the 16S rRNA sequence similarity value for *C. piscium* (98.87%) is greater than the threshold value for species delineation although that for *C. balustinum* (98.68%) falls within the range (Table 3.8). This therefore implies that strain 5_R23647 may not be a novel species of *Chryseobacterium*, although the dDDH values for its closest relatives are less than the threshold value for prokaryotic delineation. The more important genomic indices like ANI and AAI show a strong confirmation that strain 5_R23647 is not a novel species, with the ANI and AAI values of its closest relative, *C. piscium* (96.2%

and 96.7%, respectively) being greater than the threshold value (Table 3.9). Based on this finding, it can be concluded that strain 5_R23647 is not a novel species of *Chryseobacterium* but could rather be another strain of *Chryseobacterium piscium*.

The Venn diagram generated with the coding sequences with bidirectional best hits showed strain 1_F178^T to contain 1094 unique genes (Fig. 3.4). Strain 1_F178^T and its three closest relatives including the type species of the genus (*C. gleum*) shared a total of 2982 genes. *Chryseobacterium jejuense* shared the highest number of genes (165 genes) with strain 1_F178^T. This is interesting as *C. jejuense* is the most closest relative to strain 1_F178^T.

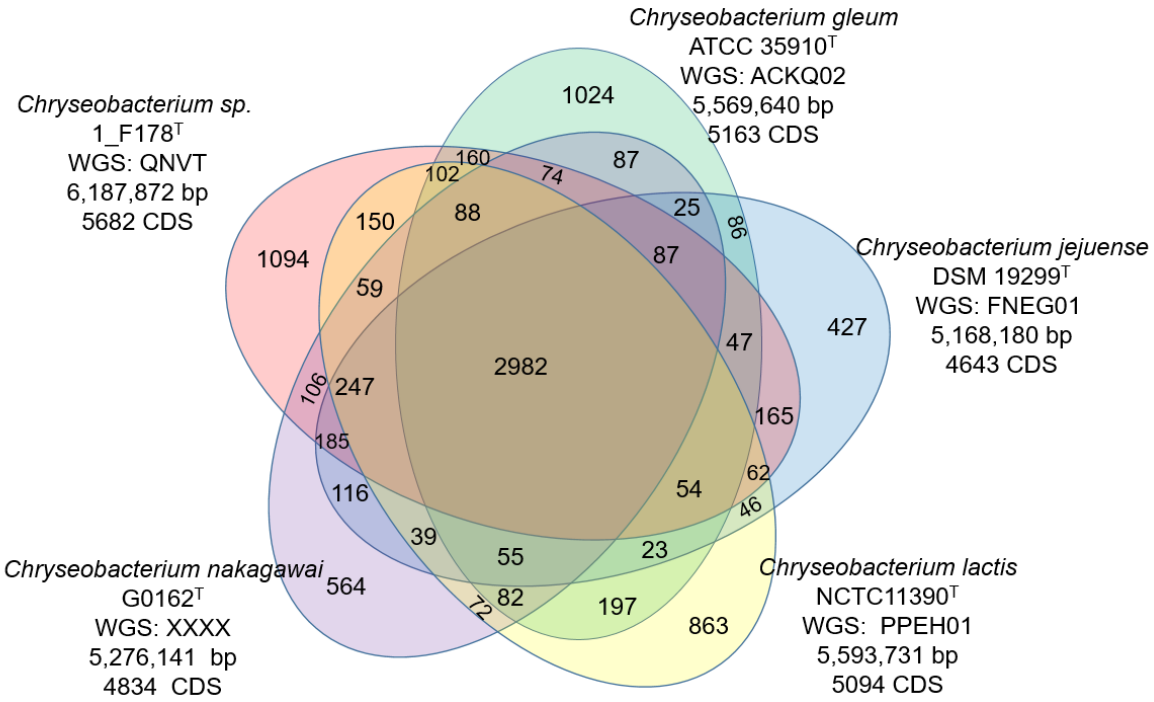


Fig. 3.4 Venn Diagram. Genomic comparison of strain 1_F178^T, its three closest relatives, and *C. gleum*, the type species for the genus *Chryseobacterium*. Each strain is represented by a colored oval. Number of predicted protein coding genes (CDSs) shared by all strains (i.e., the core genome) is in the centre. Overlapping regions show the number of CDSs conserved only within the specified genomes. Numbers in non-overlapping portions of each oval show the number of CDSs unique to each strain. The total number of protein coding genes within each genome is listed below the strain name.

The presence of ATP-dependent CIp protease and Zn-metalloprotease in the genome of *Chryseobacterium* strain 1_F178^T show the potential of this strain to degrade chicken feathers. Hence can be used as an eco-friendly alternative in chicken feather disposal in the poultry industry. Riffel and co-workers (2003) reported the complete degradation of chicken feathers by the Zn-metalloproteases of *Chryseobacterium* sp. strain kr6. Venter and co-workers (1999) reported spoilage in milk and milk products due to the presence of these proteases.

3.3.4 Conventional phenotypic tests

Table 3.9 shows the distinguishing phenotypic characteristics of the two isolates, their reference strains and *C. gleum* as the type species of the genus.

3.3.4.1 Isolate 1_F178^T and its reference strains

All the strains produced yellow, smooth, colonies with fruity-like odour; cells were negative for Gram-reaction and motility. They were all positive for growth at 32 °C and 35 °C; with 0 – 3% NaCl and at pH ranges of 4 – 6; for the production of oxidase, catalase, caseinase, phosphatase, DNase, gelatinase, tryptophanase and urease; Tween 80 and esculin hydrolysis; KCN tolerance; flexirubin production; growth on MacConkey, nutrient, tryptic soy, brilliant green, brain heart infusion and β-hydroxybutyrate agars; reduction of nitrate but not nitrite. All the strains were negative for the production of lecithinase, β-galactosidase (ONPG), arginine-, lysine- and ornithine decarboxylase, phenylalanine deaminase; acid production from citrate only; acid production from 10% glucose and lactose, L-arabinose, ethanol, fructose, lactose, malonate, sucrose, and xylose; ammonia production from arginine; 3-ketolactose production; methyl-red and Vogues-Proskauer reactions; growth on 0.4% selenite; gluconate oxidation; H_2S production; fluorescence on King's medium; anaerobic growth in a Gas-Pak, and aerobic growth at 42 °C.

Strain 1_F178^T could be distinguished from *C jejuense* and *C. nakagawai* by its ability to grow at 4 °C and 20 °C (weakly) on nutrient agar, produce acid from glycerol, mannitol

and Simmons' citrate, inability to grow at 37 °C nor produce acid from trehalose, but could grow on mannitol salt agar and not on cetrimide agar.

When compared to *C. gleum*, isolate1_F178^T grew at 4 °C and 20 °C. It did not grow at 37 °C nor produce acid from glucose and trehalose, but produced acid from mannitol, glycerol and maltose. It was capable of reducing nitrate but not nitrite, and did not grow on MSA and cetrimide agars.

3.3.4.2 Isolate 5_R23647 and its reference strains

All the strains appeared yellow, with smooth, fruity odour-like colonies, which were negative for Gram-reaction and motility. They were all positive for the growth at 32 °C and 35 °C; with 0–3% NaCl and at pH ranges of 4–6; production of oxidase, catalase, caseinase, phosphatase, DNase, gelatinase and urease; Tween 80, tyrosine and esculin hydrolysis; KCN tolerance; flexirubin production; growth on MacConkey, nutrient, tryptic soy, brilliant green, brain heart infusion and β-hydroxybutyrate agars; reduction of nitrate but not nitrite and brown pigment production. All strains were negative for the production of lecithinase, tryptophanase, β-galactosidase (ONPG), arginine-, lysine- and ornithine-decarboxylase, phenylalanine deaminase; acid production from 10% lactose and ethanol; acid production from 10% glucose, fructose, glucose, mannitol, glycerol, lactose, maltose, malonate, mannitol, trehalose and xylose; ammonia production from arginine; 3-ketolactose production; methyl-red and Vogues-Proskauer reactions; growth on 0.4% selenite; gluconate oxidation and H_2S production; fluorescence on King's medium; anaerobic growth in a Gas-Pak, and aerobic growth at 42 °C.

Strain 5_R23647 could be distinguished from *C. piscium* and *C. nakagawai* by its ability to grow at 4 °C and 20 °C, inability to grow at 35 °C and 37 °C, and ability to degrade Tween 20.

When compared with the type strain *C. gleum*, the following phenotypic differences were observed: grew at 4 °C and 20 °C, but not at 35 °C and 37 °C. Produced acid from glucose, mannitol, glycerol and maltose, but did not do so on Simmons' citrate. Unlike *C.*

gleum, 5_R23647 reduced nitrate but did not reduce nitrite. Strain 5_R23647 produced amylase but did not grow on cetrimide agar.

Table 3.10 Differential characteristics of strains 1_F178^T and 5_R23647 and their closest relatives. EMB, eosine methylene blue agar; MSA, mannitol salt agar; +, positive; -, negative; w, weakly positive.

Characteristic	**1_F178^T**	*C. jejuense*	*C. nakagawai*	**5_R23647**	*C. piscium*	*C. balustinum*	*C. gleum*
Temperature:							
4 °C	+	-	-	+	-	-	-
20 °C	w	-	-	+	-	-	-
35 °C	w	+	+	-	+	+	+
37 °C	-	w	+	-	+	+	+
Acid production from:							
Glucose	-	-	+	+	+	+	-
Mannitol	w	-	-	+	+	+	-
Glycerol	w	-	-	+	+	+	-
Maltose	w	-	w	+	+	+	-
Trehalose	-	+	+	+	+	w	+
Simmon's citrate	+	-	-	-	-	+	+
Christensen's citrate	+	+	+	w	-	+	+
Reduction of:							
Nitrate	+	+	+	+	+	+	-
Nitrite	-	-	-	-	-	+	+
Degradation of:							
Tween 20	-	+	-	-	+	+	-
Tyrosine	+	+	-	w	+	+	+
Production of:							
Amylase	-	+	-	+	+	+	-
Growth on:							
EMB	+	-	+	w	w	w	+
MSA	-	w	+	+	+	+	+
β-hydroxybutyrate	+	+	+	w	-	+	+
Cetrimide	-	+	+	-	-	-	+

3.3.4.3. Microscopy

Scanning electron microscopy (SEM)

Figure 3.5 depicts the images obtained for the scanning electron microscopy of strains 1_F178^T, 5_R23647 and *C. gleum*. A smooth surface was observed for strain 5_R23647 unlike strain 1_F178^T which showed string-like structures on its surface. The exact purpose and composition of these structures are unknown.

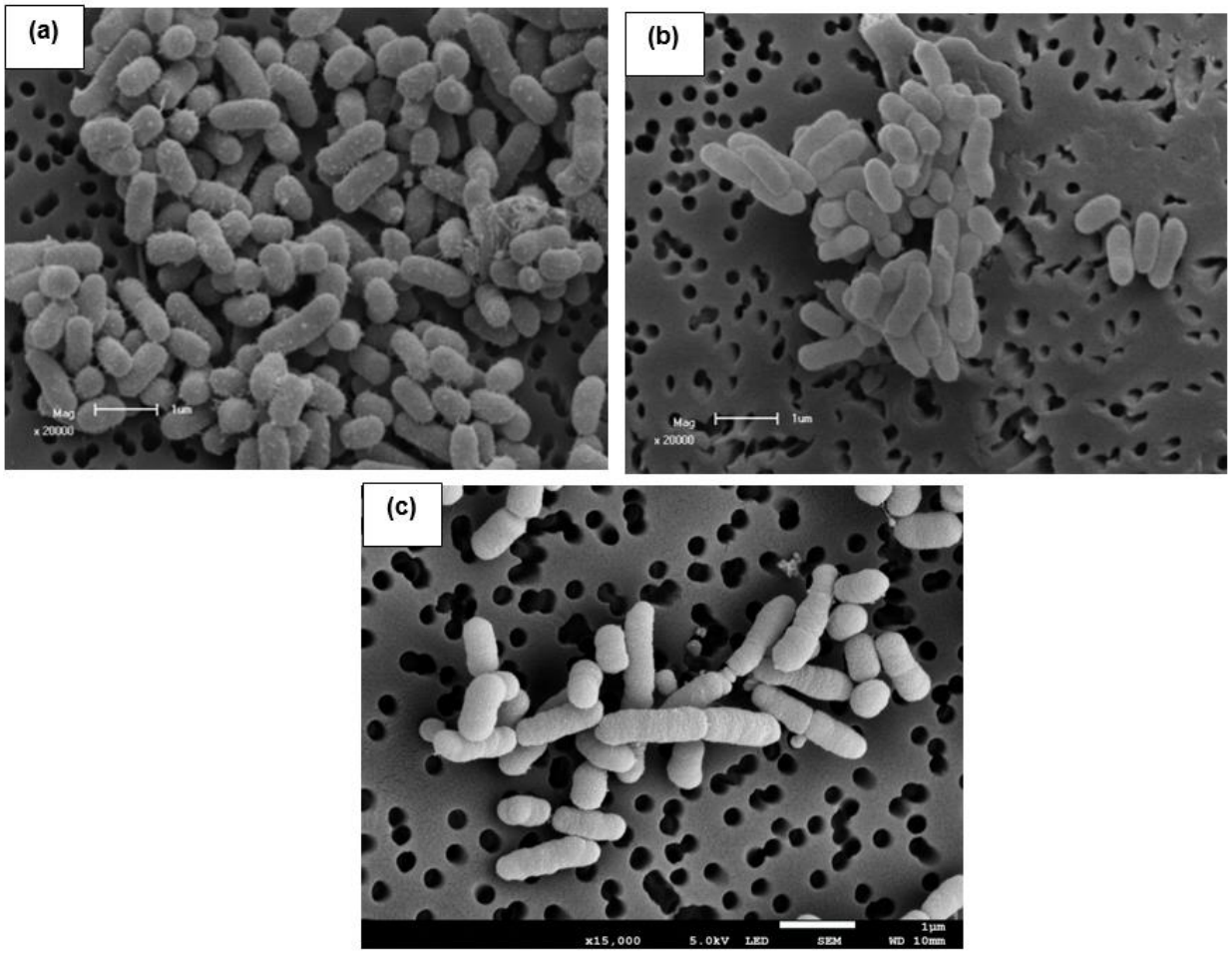


Fig. 3.5. Scanning electron micrographs of *Chryseobacterium* strains. Bar, 1 µm. (a) Strain 1_F178^T; (b) Strain 5_R23647; and (c) *Chryseobacterium gleum*.

The surfaces of strain 5_R23647 and the type strain *C. gleum* look similar when compared to that of strain 1_F178^T.

Transmission electron microscopy (TEM)

The images depicted in Fig. 3.6 show the transmission electron microscopy of strains 1_F178^T and 5_R23647. The string-like structures can still be seen on the surface of strain 1_F178^T as observed in the scanning electron microscopy image.

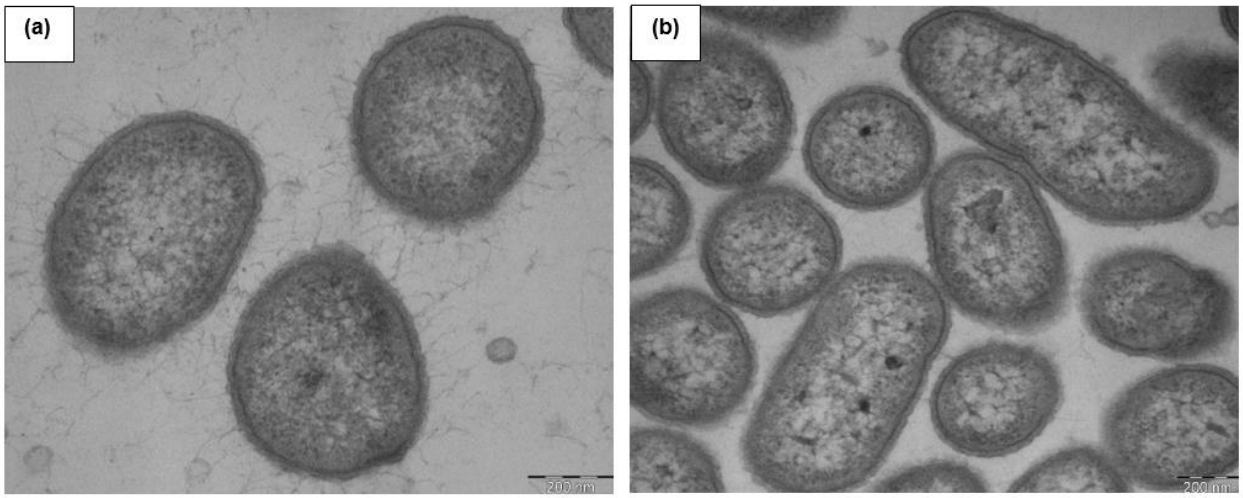


Fig. 3.6. Transmission electron micrographs of *Chryseobacterium* strains. Bar, 1 µm. (a) Strain 1_F178^T and (b) Strain 5_R23647.

3.3.5. Automated phenotypic tests

3.3.5.1. Biolog^{TM} OmniLog^{TM} Gen III phenotypic profiling

The Biolog^{TM} OmniLog^{TM} plates contain tests for metabolic response to sugars, amino acids, hexose acids, carboxylic acids, esters, fatty acids, and inhibitory substances. The results were obtained from the mean of triplicate experiments for strains 1_F178^T, 5_R23647 and their reference organisms.

Strain 1_F178^T and its reference strains were positive for the following substrates and conditions: dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, L-fucose, 1% NaCl, α-D-glucose, D-mannose, 1% sodium lactate, D-serine, glycerol, D-fructose-6-PO_4, troleandomycin, rifamycin SV, gelatin, glycyl-L-proline, L-aspartic acid, lincomycin,

guanidine HCl, D-galacturonic acid, D-glucuronic acid, tetrazolium violet, tetrazolium blue, Tween 40, acetoacetic acid, acetic acid, formic acid, sodium butyrate and aztreonam.

The following substrates were negative for strain 1_F178^T and its reference strains: sucrose, stachyose, D-raffinose, α-D-lactose, β-methyl-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, N-acetyl-D- galactosamine, N-acetyl neuraminic acid, 4 and 8% NaCl, fusidic acid, D-sorbitol, D-aspartic acid, niaproof 4, pectin, mucic acid, D-saccharic acid, *p*-hydroxy-phenylacetic acid, methyl pyruvate, D-lactic acid methyl ester, L-lactic acid, D-malic acid, L-malic acid, bromo-succinic acid, LiCl, γ-amino-butyric acid, α-hydroxy-butyric acid, β-hydroxy-D,L-butyric acid, α-keto-butyric acid, propionic acid and sodium bromate.

Strain 5_R23647 gave positive results for the following substrates: pH 6, 1% NaCl, 1% sodium lactate, gelatin, guanidine HCl, D-galacturonic acid, tetrazolium violet, tetrazolium blue, potassium tellurite, Tween 40, acetic acid and aztreonam.

The following substrates were negative for strain 5_R23647 and its reference strains: D-maltose, D-cellobiose, sucrose, D-turanose, stachyose, D-raffinose, α-D-lactose, D-melibiose, β-methyl-D-glucoside, D-salicin, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, N-acetyl-D- galactosamine, N-acetyl neuraminic acid, 4 and 8% NaCl, D-galactose, 3-methyl glucose, D-fucose, L-fucose, inosine, fusidic acid, D-serine, D-sorbitol, D-mannitol, D-arabitol, *myo*-inositol, glycerol, D-glucose-6-PO_4, D-fructose-6-PO_4, D-aspartic acid, minocycline, L-alanine, L-histidine, L-pyroglutamic acid, L-serine, niaproof 4, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, vancomycin, *p*-hydroxy-phenylacetic acid, D-lactic acid methyl ester, L-lactic acid, citric acid, α-keto-glutaric acid, D-,malic acid, L-malic acid, bromo-succinic acid, nalidixic acid, γ-amino-butyric acid, α-hydroxy-butyric acid, β-hydroxy-D,L-butyric acid, α-keto-butyric acid, propionic acid and formic acid.

Table 3.11. Biolog OmniLog Gen III system biochemical tests showing the differential characteristics of strain 1_F178^T and its closely related taxa. +, positive; -, negative; w, weakly positive.

Substrate	1_F178^T	*C. jejuense*	*C. nakagawai*	*C. gleum*
Sugar				
D-turanose	-	w	-	-
D-melibiose	w	-	-	-
D-fructose	+	+	-	+
D-galactose	+	w	-	w
3-methyl glucose	+	-	-	-
D-fuctose	+	w	-	-
L-rhamnose	+	w	-	-
D -mannitol	+	-	-	-
D -arabitol	+	-	-	-
myo-inositol	+	-	-	w
D-glucose-6-PO_4	+	-	w	+
Amino acids				
L-alanine	+	-	w	w
L-arginine	+	-	w	w
L-glutamic acid	+	-	+	+
L-histidine	+	-	-	-
L-serine	+	-	-	-
Hexose acids				
L -galacturonic acid lactone	+	-	w	w
Glucuronamide	+	w	-	-
Carboxylic acids, esters & fatty acids				
Citric acid	+	w	+	+
α-keto-glutaric acid	+	-	+	-
Inhibitory substances				
Minocycline	-	-	+	+
Potassium tellurite	-	-	+	+

Tables 3.11 and 3.12 show the differential characteristics of strains 1_F178^T and 5_R23647 and their reference strains divided into the aforementioned substrate categories.

Under the sugar category, strain 1_F178^T could be differentiated from its closest relatives by its ability to oxidise 3-methyl glucose, D-mannitol and D-arabitol.

In the amino acid category, strain 1_F178^T was the only *Chryseobacterium* strain which gave positive results for L-histidine, L-pyroglutamic acid and L-serine while *C. jejuense*, *C. nakagawai* and *C. gleum* were negative for these amino acids.

The hexose acids; D-gluconic acid and quinic acid showed weak positive results only for strain 1_F178^T while giving negative results for its closest relatives.

There were no differential characteristic for strain 1_F178^T under the carboxylic acids, esters and fatty acid category. *Chryseobacterium nakagawai* and 1_F178^T were both positive for α-keto-glutaric acid while *C. jejuense* and *C. gleum* were negative for α-keto-glutaric acid.

Under the inhibitory substance category, no clear differential characteristic was seen for strain 1_F178^T.

Strain 1_F178^T can be differentiated from the type strain *C. gleum* in the sugar category by showing positive results for 3-methyl glucose, D-fuctose, L-rhamnose, D-mannitol and D-arabitol. In the amino acid category, strain 1_F178^T can be differentiated from *C. gleum* by showing positive results for L-histidine and L-serine. In the hexose acid category, strain 1_F178^T can be differentiated from *C. gleum* by showing a positive result for glucuronamide. In the carboxylic acid category, strain 1_F178^T can only be differentiated from *C*. *gleum* by being positive for α-keto-glutaric acid production. Under the inhibitory substance category, strain 1_F178^T can be differentiated from *C. gleum* by showing negative results for the antibiotics, minocycline and potassium tellurite.

Table 3.12 Biolog OmniLog Gen III system biochemical tests showing the differential characteristics of strain 5_R23647 and its closely related taxa. +, positive; -, negative; w, weakly positive.

Substrate	5_R23647	*C. piscium*	*C. balustinum*	*C. gleum*
Sugar				
Dextrin	w	+	-	+
D-trehalose	-	-	+	+
Gentiobiose	-	+	+	+
α-D-glucose	-	+	+	+
D-mannose	-	+	+	+
D-fructose	-	-	+	+
L-rhamnose	-	-	+	-
Pectin	-	w	w	-
Amino acids				
Glycyl-L-proline	-	+	+	w
L-arginine	-	+	w	w
L-aspartic acid	-	-	w	+
L-glutamic acid	-	+	+	+
Hexose acids				
L-galacturonic acid lactone	-	+	-	w
Carboxylic acids, esters & fatty acids				
Methyl pyruvate	-	w	+	-
Acetoacetic acid	-	+	+	-
Inhibitory substances				
Troleandomycin	w	w	-	+
Rifamycin SV	-	+	-	+
Lincomycin	-	+	+	+
LiCl	-	+	-	w
Sodium butyrate	-	w	+	-
Sodium bromate	-	-	w	-

Strain 5_R23647 could be differentiated from its closest relatives in the carbohydrate substrate category by its inability to utilize gentiobiose, α-D-glucose, and D-mannose.

In the amino acid category, strain 5_R23647 was the only *Chryseobacterium* strain which gave negative results for glycyl-L-proline, L-arginine and L-glutamic acid, while its closest relatives, *C. piscium* and *C. balustinum* were positive for these amino acids.

The hexose acid profile did not show any differential characteristic between strain 5_R23647 and its closest relatives. However, strain 5_R23647 and *C. balustinum* gave negative results for L-galacturonic acid lactone while *C. piscium* and *C. gleum* gave positive results with *C. gleum* being weakly positive.

The carboxylic acid, esters and fatty acid profile did not show any differential characteristics as well between 5_R23647 and its closest relatives.

Strain 5_R23647 and *C. gleum* were negative for acetoacetic acid and methyl pyruvate production while *C. piscium* and *C. balustinum* were positive for the production of this carboxylic acid and ester.

Chryseobacterium piscium, *C. balustinum* and *C. gleum* showed sensitivity to the antibiotic, lincomycin while strain 5_R23647 was the only strain resistant to this antibiotic.

Strain 5_R23647 can be differentiated from the type strain *C. gleum* by its inability to oxidise the following sugars: D-trehalose, gentiobiose, α-D-glucose, D-mannose and D-fructose. In the amino acid category, strain 5_R23647 can be differentiated from *C. gleum* by its inability to oxidise glycyl-L-proline, L-arginine, L-aspartic acid and L-glutamic acid. The only difference between strain 5_R23647 and *C. gleum* in the hexose acid category is its inability to oxidise L-galacturonic lactone. No difference was observed between strain 5_R23647 and *C. gleum* under the carboxylic acid category. Strain 5_R23647 can be differentiated from *C. gleum* in the inhibitory substance category by its inability to grow in the presence of the antibiotics rifamycin SV and lincomycin and in the presence of the toxic compound lithium chloride.

3.3.5.2. API tests

Tables 3.13 and 3.14 show the differential characteristics of strains 1_F178^T and 5_R23647 and their closest relatives using API® NE and API® ZYM test strips.

The following API® NE tests produced negative results for strain 1_F178^T and its reference strains as follows: reduction of nitrates to nitrite; glucose fermentation; production of arginine dihydrolase and β-galactosidase; assimilation of capric acid, adipic acid, malate trisodium citrate and phenylacetic acid.

Table 3.13 Differential characteristics of strains 1_F178^T and 5_R23647 and their reference strains using API® NE test strips. +, positive; -, negative; w, weakly positive.

API® 20 NE							
Characteristic	1_F178^T	*C. jejuense*	*C. nakagawai*	5_R23647	*C. piscium*	*C. balustinum*	*C. gleum*
Production of:							
Urease	+	+	+	-	+	+	+
Assimilation of:							
Glucose	+	+	-	+	-	-	+
Arabinose	+	-	-	-	-	-	+
Mannose	+	-	-	+	-	-	+
Mannitol	+	-	-	+	-	-	+
N-acetyl-glucosamine	+	-	-	-	-	-	-
Maltose	+	+	+	+	-	-	+
Potassium gluconate	+	-	-	-	-	-	-
Adipic acid	-	-	-	+	-	-	-
Trisodium citrate	-	-	-	+	-	-	+

The following positive API® NE tests were obtained for strain 1_F178^T and its reference strains: indole and urease production; maltose assimilation; esculin and gelatin hydrolysis, and cytochrome oxidase production.

Strain 1_F178^T can be differentiated from its reference strains by its ability to assimilate arabinose, mannose, mannitol, N-acetyl-glucosamine and potassium gluconate.

Strain 1_F178^T can be differentiated from the type strain of *C. gleum* by its ability to utilize N-acetyl-glucosamine and potassium gluconate and its inability to utilise trisodium citrate

The following API® NE tests produced negative results for strain 5_R23647 and its reference strains: reduction of nitrates to nitrites; reduction of nitrates to nitrogen; glucose fermentation; production of arginine dihydrolase and β-galactosidase; assimilation of arabinose, N-acetyl-glucosamine, potassium gluconate, capric acid, malate and phenylacetic acid.

The following positive API® NE tests were produced for strain 5_R23647 and its reference strains: indole production; esculin and gelatin hydrolysis, and cytochrome oxidase production.

Strain 5_R23647 can be differentiated from its reference strains by its ability to assimilate glucose, mannose, mannitol, maltose, adipic acid, trisodium citrate and its inability to produce urease.

Strains 5_R23647 can be differentiated from the type strain of *C. gleum* by its inability to assimilate arabinose and produce urease, and its ability to assimilate adipic acid.

The following API® ZYM tests (Table 3.14) produced negative results for strain 1_F178^T and its reference strains: lipase; α-chemotrypsin; α-galactosidase; β-galactosidase; β-glucuronidase; β-glucosidase; α-mannosidase and α-fucosidase.

Positive API® ZYM tests were obtained for the following tests: alkaline phosphatase; esterase lipase; leucine arylamidase; valine arylamidase; acid phosphatase; naphtol-AS-BI-phosphohydrolase and α-glucosidase. Weak positive results were obtained for esterase and N-acetyl-β-glucosaminidase production.

Strain 1_F178^T can be differentiated from its reference strains by its inability to produce cystine arylamidase and trypsin.

No clear difference could be seen between strain 1_F178^T and the type strain of *C. gleum* using the API® ZYM tests.

Table 3.14 Differential characteristics of strains 1_F178^T and 5_R23647 and their reference strains using API® ZYM test strips. +, positive; -, negative; w, weakly positive.

	API® ZYM						
Characteristic	1_F178^T	*C. jejuense*	*C. nakagawai*	5_R23647	*C. piscium*	*C. balustinum*	*C. gleum*
Production of:							
Cystine arylamidase	-	w	w	w	w	w	-
Trypsin	-	+	+	+	+	+	-

The following API® ZYM tests produced negative results for strain 5_R23647 and its reference strains: lipase; α-chymotrypsin; α-galactosidase; β-galactosidase; β-glucuronidase; β-glucosidase; α-mannosidase and α-fucosidase.

Positive API® ZYM tests were obtained for the following tests: alkaline phosphatase; esterase lipase; leucine arylamidase; valine arylamidase; trypsin; acid phosphatase; naphtol-AS-BI-phosphohydrolase and α-glucosidase. Weak positive results were obtained for esterase and cystine arylamidase production.

Strain 5_R23647 did not show any differential characteristic from its reference strains on the API® ZYM test strips.

Strain 5_R23647 can be differentiated from the type strain of *C. gleum* by its ability to produce the enzymes cystine arylamidase and trypsinase.

3.3.6. Chemotaxonomic methods

3.3.6.1. Fatty acid methyl ester analysis

The presence of fatty acid methyl esters (FAME) is used as a chemotaxonomic marker in bacterial delineation both at the genus and species level (Bernardet *et al*., 2002). The presence of the fatty acids, iso-$C_{15:0}$, iso-$C_{17:1}\omega 9c$ and iso-$C_{17:0}$ 3-OH are usually associated with the genus *Chryseobacterium*. Table 3.15 shows the FAME profile of strains 1_F178^T and 5_R23647 and their reference strains. Cellular fatty acids most predominant in the novel strains were iso-$C_{15:0}$, iso-$C_{17:1}\omega 9c$ and iso-$C_{17:0}$ 3-OH thus confirming their affiliation to the genus *Chryseobacterium*.

A similar trend was observed in the increase or decrease of cellular fatty acid (CFA) composition for both strains 1_F178^T and 5_R23647. This is supported by Welch (1991) who mentioned that for some genera, CFA analysis allows the identification and differentiation of individual species or subspecies, while for others; different species have identical CFA profiles. He further illustrated that delineation using CFA was limited to the genus level as variations in cultivation conditions play a huge role in preventing differentiation at the species level.

When strain 1_F178^T is compared to its closest relatives, *C. nakagawai* had a greater amount of the major fatty acids except for summed feature 3, while *C. jejuense* had a slightly lower amount of the major fatty acids than strain 1_F178^T. Strain 1_F178^T could be differentiated from *C. gleum* in having slightly higher amounts of iso-$C_{15:0}$ and iso-$C_{17:1}$ $\omega 9c$.

In the case of strain 5_R23647, it had the highest amount of the major fatty acids when compared to its closest relatives except for summed feature 3, while *C. piscium* had the lowest amount of major fatty acids except for summed feature 3 Strain 5_R23647 could be differentiated from *C. gleum* in having slightly higher amounts of iso-$C_{15:0}$ and iso-$C_{17:1}$ $\omega 9c$. but lower amounts of iso-$C_{17:0}$ 3-OH.

Table 3.15 Fatty acid methyl ester profile (%) of strains 1_F178^T and 5_R23647 and the type strains of closely related species of the genus *Chryseobacterium*. Values are percentages of the total fatty acids. Fatty acids that are <1.0% in all strains are not shown. ECL, equivalent chain length; ND, not detected; Tr, Trace (<1.0%). The major fatty acids (>10%) are indicated in bold. Summed features are groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI system and include both peaks with discrete ECLs as well as those where the ECLs are not reported separately. Summed feature 3 is listed as iso-$C_{15:0}$ 2-OH/$C_{16:1}\omega7c$

Fatty acid (%)	1_F178^T	*C. jejuense*	*C. nakagawai*	5_R23647	*C. piscium*	*C. balustinum*	*C. gleum*
$C_{12:0}$	Tr	1.59	1.87	Tr	1.70	1.15	ND
iso-$C_{13:0}$	1.55	1.38	1.55	Tr	1.34	1.13	Tr
ECL 13.591	4.54	1.39	4.51	1.11	3.04	2.00	2.8
$C_{14:0}$	Tr	Tr	1.22	Tr	Tr	Tr	ND
iso-$C_{15:0}$	**37.99**	**34.16**	**38.94**	**36.01**	**31.53**	**34.10**	**35.7**
anteiso-$C_{15:0}$	Tr	6.59	1.68	5.10	4.67	2.75	Tr
iso-$C_{17:1}$ $\omega9c$	**24.64**	**15.62**	**30.35**	**28.23**	**24.73**	**27.12**	**23.6**
$C_{16:0}$	2.20	3.76	3.61	3.26	2.95	2.35	1.3
iso-$C_{15:0}$ 3-OH	2.22	3.02	2.91	2.41	2.35	2.04	2.8
Summed feature 3*	**9.31**	**10.79**	**10.41**	**8.23**	**11.54**	**7.34**	ND
ECL 16.587	1.29	Tr	1.90	1.07	Tr	Tr	1.7

Fatty acid (%)	1_F178^T	*C. jejuense*	*C. nakagawai*	5_R23647	*C. piscium*	*C. balustinum*	*C. gleum*
iso-$C_{16:0}$ 3-OH	Tr	1.26	1.63	1.03	1.62	Tr	ND
$C_{16:0}$ 3-OH	1.03	2.47	1.72	1.50	1.41	1.34	ND
iso-$C_{17:0}$ 3-OH	**15.14**	**12.36**	**19.93**	**10.00**	**9.32**	**8.41**	**15.1**
$C_{17:0}$ 2-OH	Tr	3.60	2.21	2.02	1.80	1.36	ND

3.3.6.2. Polar lipids

Polar lipids play an important role in the regulation and permeability of the plasma membrane, with the most common ones being phospholipids. Some microorganisms may also have glycolipids and amino acid amide lipids. The wide distribution of the phospholipids, phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol, have made them useful in bacterial taxonomy (Madl & Yip, 1999). Phosphatidylethanolamine is the most predominant polar lipid in *Chryseobacterium* species, and is usually present with or without aminophospholipids, aminolipids, glycolipids and other unidentified lipids (Kirk *et al*., 2013; Kämpfer *et al*., 2015c; Guo *et al*., 2016; Joeng *et al*., 2017; Lin *et al*., 2017). In strains 1_F178^T and 5_R23647 (Fig. 3.7), phosphatidylethanolamine was the most predominant polar lipid, in accordance with all other members of the families *Flavobacteriaceae* and *Weeksellaceae*, hence confirming the affiliation of these strains to the genus *Chryseobacterium*. Strain 1_F178^T also contained five unidentified lipids, three unidentified aminolipids and two unidentified glycolipids.

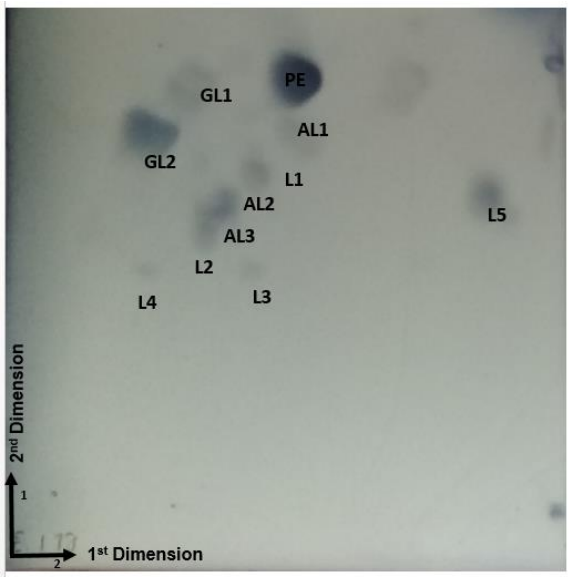


Fig. 3.7 Two-dimensional thin layer chromatogram sprayed with a 10% molybdophosphoric acid reagent showing the total polar lipids from strain 1_F178^{T}. Solvent systems: (1) chloroform:methanol:water (65:25:4, v/v/v); (2) chloroform:methanol:acetic acid:water (80:12:15:4, v/v/v/v). Arrows indicate direction of each chromatographic dimension. PE, phosphatidylethanolamine; AL1-AL3, uncharacterised aminolipids; L1-L5, uncharacterised lipids; GL1-GL2, uncharacterised glycolipids.

3.3.6.3 Respiratory quinones

Respiratory quinones have been used to characterize bacteria at different taxonomic levels based on variability of the side chains (differences in length, hydrogenation and saturation).

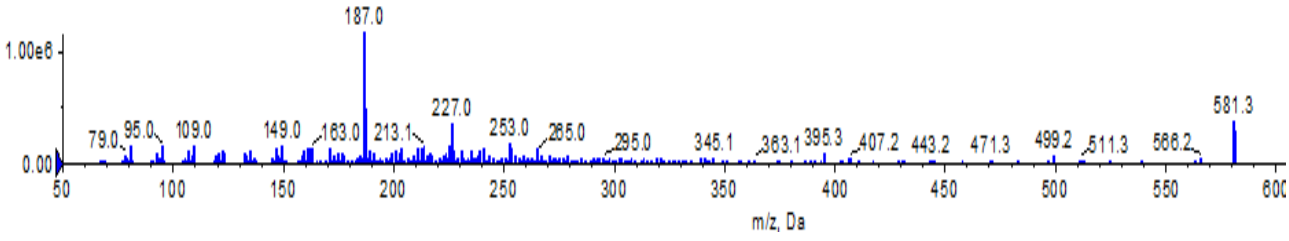


Fig 3.8 Fragmentary spectrum of MK-6 at 38.47 min for strain 1_F178$^{T.}$

In the genus *Chryseobacterium*, as in all other members of the *Flavobacteriaceae* and *Weeksellaceae*, menaquinone-6 (MK-6) is known as the only or major respiratory quinone (Divyasree *et al*., 2018; Lin *et al*., 2015; Wang *et al*., 2016b; Zhao *et al*., 2017b). It is apparent from Fig. 3.8 that strain 1_F178^T contains MK-6 as can be confirmed by an *m/z* ratio of 187.0. The presence of menaquinone-6 in strain 1_F178^T confirmed its affiliation to this genus.

3.3.6.4. Pigment analysis

There is limited information on the study of the flexirubin pigment from *Chryseobacterium* species. Most of the work done is based on bathochromic shift tests with 20% (w/v) KOH solution (Behrendt *et al*., 2008; Lin *et al*., 2015; Kim *et al*., 2016; Chaudhary & Kim, 2017; Divyasree *et al*., 2018). Flexirubins are unique bacterial pigments which are a class of polyene metabolites with a generalized 2,5-dialkylresorcinol (DAR) structure present as an aromatic ester (Nowak-Thompson *et al*., 2003).

Fig. 3.9 shows the pigment analysis of strain 1_F178^T by reverse-phase HPLC. The main pigments for strain 1_F178^T eluted between 37 and 43 min. The elution profile revealed a variety of compounds that absorbed light in the visible range. The peaks were detected optimally at a wavelength of 452 nm, which is typical of the elution wavelength for flexirubin and carotenoid pigments. A similar UV-visible absorbance spectrum was obtained by Bortniak and co-workers (2019) when they carried out pigment analysis on the acetone extracts of four chryseobacteria strains. In a study carried out by Venil and co-workers (2014) on the isolation and characterization of a flexirubin type pigment from *Chryseobacterium* sp. UTM-3^T, peaks for the orange pigment extracted in acetone eluted at a wavelength of 450 nm compared to the 452 nm obtained in this study and that of Stropko and co-workers (2014). The presence of flexirubin may be beneficial to strain 1_F178^T since it functions as a photo protective compound in bacteria (Venil *et al*., 2014b).

Flexirubin pigments have been shown to display a range of pharmacological effects such as antimicrobial and anticancer activities (Kim, 2013). They may also represent safe and

biodegradable natural colourants with potential as substitutes for synthetic colourants (Venil *et al*., 2014b). They possess terminal alkyl substitutions consisting of ω-phenyl octaenic acid chromophore esterified with resorcinol carrying two hydrocarbon chains and are used in the treatment for chronic skin disease, eczema, gastric ulcers, to name a few (Kim, 2013).

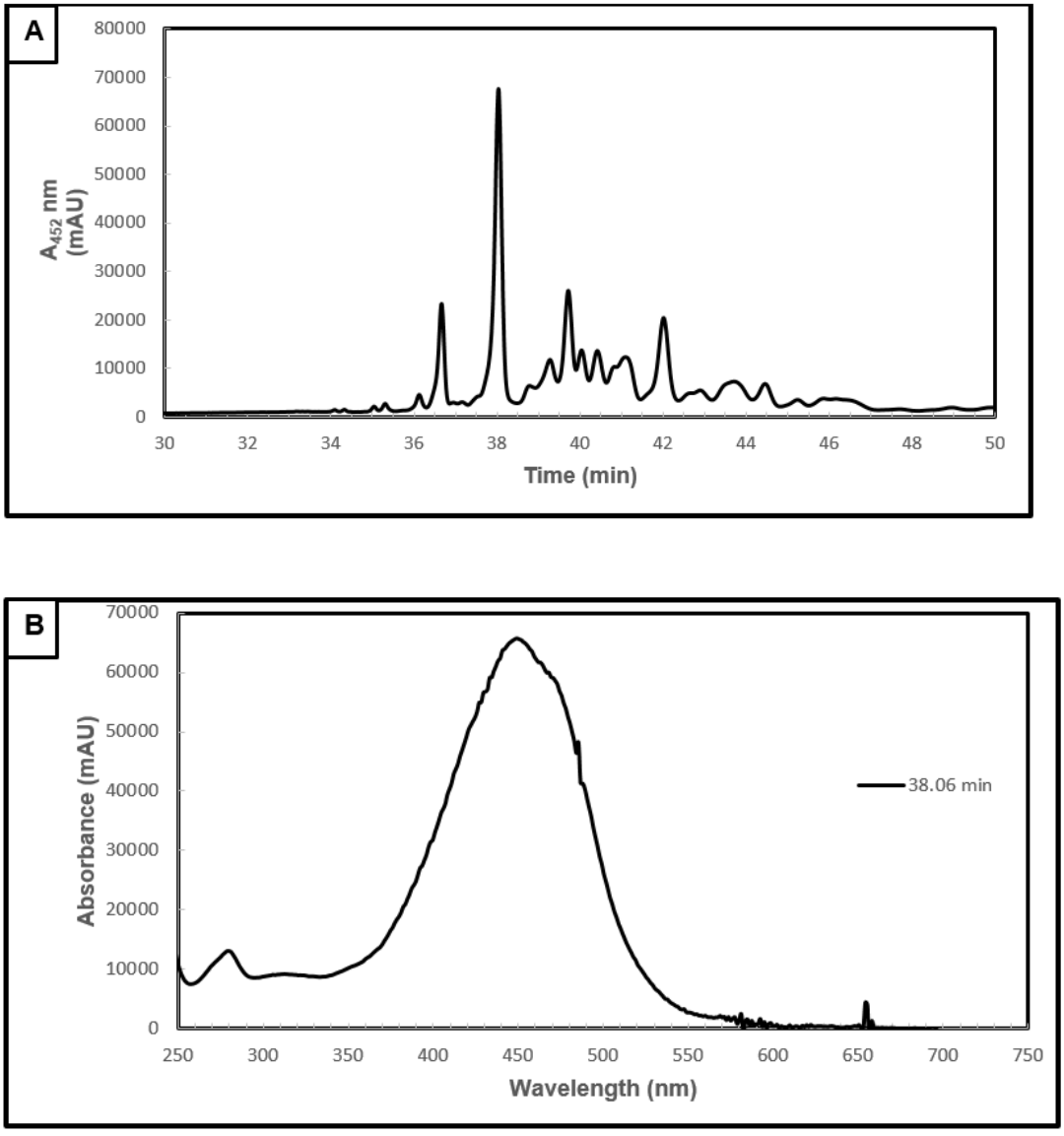


Fig. 3.9 Pigment analysis of (a) the acetone extract of strain 1_F178^T separated by reverse-phase HPLC. The elution profile at 452 nm is shown; (b) UV-visible absorbance spectra of the pigment peak of strain 1_F178^T at 38.06 min. mAU, milli absorption units.

All the above genotypic, chemotaxonomic and phenotypic data indicated that strain 7_F178^T was a novel species of *Chryseobacterium* and the name, *Chryseobacterium pennae* sp. nov., is proposed for this strain.

3.3.7. Description of *Chryseobacterium pennae* sp. nov. *Chryseobacterium pennae*

(pen'nae. L. gen. n. *pennae* of a feather).

Gram-negative-staining rods (approximately 1-3 µm in length devoid of flagella); non-gliding, non-motile and non-spore-forming cells with rounded ends. The colonies are yellowish and circular when cultivated at 25 °C for 48 h on nutrient agar (Oxoid CM003); produce a fruity odour, are translucent, non-fluorescent; have a flat and smooth entire surface and become mucoid on prolonged incubation. Cells produce flexirubin-type pigments; are strictly aerobic and grow at 4 °C but not at 37 °C and 42 °C. Optimal growth is at 25–30 °C. Grows on MacConkey, tryptic soy agar, Simmons citrate, Christensen's citrate, nutrient agar and β-hydroxybutyrate, but not on cetrimide and mannitol salt agars. Grows in 0–3% NaCl (optimal at 0% NaCl), but not in 4 – 6% NaCl; growth at pH 5 – 8 (optimal at pH 6) but not at pH less than 5; produce acid weakly from mannitol, glycerol and maltose, but not from glucose, fructose, arabinose, lactose, trehalose, sucrose and xylose.

Strain 1_F178^T produces caseinase, DNase, gelatinase, urease, but not amylase, lecithinase, tryptophanase, β-galactosidase (ONPG) and phenylalanine deaminase. The strain is negative for the production of 3-ketolactose and H_2S, and unable to utilize malonate.

Strain 1_F178^T hydrolyses the following substrates: esculin, gelatin, tyrosine (with the production of a brown pigment), casein, Tween 80, ethanol and 0.4% selenite. It is sensitive to KCN, does not reduce nitrite, but is positive for nitrate reduction. The strain is negative for methyl red, Vogues-Proskauer and gluconate oxidation.

Based on the BIOLOG Gen III system, strain 1_F178^T oxidises dextrin, D-maltose, D-trehalose, D-gentiobiose, α-D-glucose, D-mannose, D-fructose, D-galactose, 3-methyl glucose, D-fucose, L-fucose, L-rhamnose, inosine, 1% sodium lactate, D-mannitol, D-

arabitol, *myo*-inositol, glycerol, D-glucose-6-phosphate, D-fructose-6-phosphate, gelatin, D-galacturonic acid, L-galacturonic acid lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, citric acid, α-keto-glutaric acid, Tween 40, acetoacetic acid, acetic acid, formic acid, quinic acid and sodium butyrate.

Strain 1_F178^T is susceptible to troleandomycin, rifamycin SV, lincomycin, vancomycin (weakly), nalidixic acid (weakly) and aztreonam. The strain has a high reducing power to tetrazolium violet and tetrazolium blue.

Strain 1_F178^T oxidises the following amino acids (Biolog): D-serine, glycyl- L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, and L-serine.

On the API® 20 NE test strip, positive for: indole, urease and cytochrome oxidase production, esculin and gelatin hydrolysis, assimilation of glucose, arabinose, mannose, mannitol, N-acetyl-glucosamine, maltose and potassium gluconate.

On the API ZYM test strip, positive for: alkaline phosphatase, esterase lipase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and N-acetyl-β-glucosaminidase.

The predominant cellular fatty acids are iso-$C_{15:0}$, iso-$C_{17:1}\omega$9c and iso-$C_{17:0}$ 3-OH. Phosphatidylethanolamine is the most abundant polar lipid and menaquinone-6 is the only respiratory quinone.

The type strain is 1_F178^T, isolated in 2011 from chicken feather waste collected from the soil of an abattoir in Bloemfontein, Free State, South Africa.

The GenBank/EMBL/DDBJ accession for the 16S rRNA gene sequence of strain 1_F178^T is MH059518. General features of the genome assembly are as follows: strain 1_F178^T has a genome size of approximately 6187872 bp; 88 contigs, 5682 coding sequences (CDS), N50 value, 162953, 25.0x coverage and a G+C content of 35.6 mol%. The Whole Genome Shotgun project has been deposited at DDBJ/ENA/Genbank (https://www.ncbi.nlm.nih.gov) with accession number NZ_QNVT00000000. The version described in this paper is version QNVT01000000.

3.4. Conclusions

Based on the polyphasic studies, the following conclusions were drawn:

i. Cellular fatty acid analysis of strains 1_F178^T and 5_R23647 showed iso-C15:0, iso-$C_{17:1}$ *ω9c* and iso-$C_{17:0}$ 3-OH as the most predominant fatty acids, hence confirming their affiliation to the genus *Chryseobacterium*.

ii. Phosphatidylethanolamine was the most abundant polar lipid, with menaquinone-6 being the predominant respiratory quinone. These chemotaxonomic markers confirm strains 1_F178^T and 5_R23647 as members of the genus *Chryseobacterium*.

iii. Members of the genus *Chryseobacterium* have G+C contents ranging between 29 – 39 mol%. Strains 1_F178^T and 5_R23647 had DNA base compositions of 35.6 mol% and 33.6 mol% respectively confirming them as members of the genus *Chryseobacterium*.

iv. Based on prokaryotic classification, strains are considered novel when they have 16S rRNA sequence similarity values less than 98.7%. The closest relatives of strains 1_F178^T (*C. jejuense* and *C. nakagawai*) and 5_R23647 (*C. piscium* and *C. balustinum*) had 16S rRNA values ranging between 98.7 and 99.1% which did not confirm them as novel species.

v. The results for the dDDH of the closest relatives of strains 1_F178^T (*C. jejuense* 31.4% and *C. nakagawai* 32.7%) and 5_R23647 (*C. piscium* 68.3% and *C. balustinum* 51.7%) were less than 70% hence confirming them as novel species of *Chryseobacterium*.

vi. The results for the ANI of the closest relatives of strain 1_F178^T (*C. jejuense* 86.4% and *C. nakagawai* 86.6%) were less than 95% hence confirming strain 1_F178^T as a novel species of *Chryseobacterium* while the closest relative of strain 5_R23647 (*C. piscium* 96.2%) had an ANI value greater than the threshold value, hence disqualifying strain 5_R23647 as a novel species of *Chryseobacterium* but rather be classified as another strain of *C. piscium*.

vii. The closest relatives of strain 1_F178^T (*C. jejuense* 89.3% and *C. nakagawai* 89.6%) had AAI values below the threshold value (95%) hence confirming it as a

novel species of *Chryseobacterium* while the closest relatives of strain 5_R23647 (*C. piscium* 96.7% and *C. balustinum* 92.9%) had AAI values above the threshold value for species delineation, hence disqualifying strain 5_R23647 as a novel species of *Chryseobacterium*.

viii. Strain 1_F178^T had a larger genome with a genome size of 6,187,872 bp as compared to strain 5_R23647 with a genome size of 4,678,357 bp. Overall genome relatedness indices (OGRI's) confirmed strain 1_F178^T as a novel species of *Chryseobacterium* and disqualified strain 5_R23647 as a novel species of *Chryseobacterium*. Strain 5_R23647 was concluded to be another strain of *C. piscium* due to its OGRI.

ix. A novel species with the proposed name, *Chryseobacterium pennae* was described (strain 1_F178^T) as a new member of the genus *Chryseobacterium*.

CHAPTER 4

DETERMINATION OF THE GROWTH KINETICS OF A NOVEL *Chryseobacterium* SPECIES IN COMPARISON WITH *Chryseobacterium carnipullorum* AND *Pseudomonas fluorescens*

Abstract

Temperature-growth studies were conducted to investigate the growth kinetics of three chryseobacterial species, strain 1_F178^T (novel), 5_R23647 and *Chryseobacterium carnipullorum* in comparison with *Pseudomonas fluorescens* which is a major spoilage bacterium in the food industry. The different bacterial strains were grown in nutrient broth at different temperatures ranging from 12–47 °C and also at 4 °C on a temperature gradient incubator to monitor growth over the temperature range. Growth was monitored and measured periodically at an optical density of 600 nm. Linear regression analysis was used to determine the maximum specific growth rate (μ_{max}) at each temperature while an Arrhenius model was used to describe the linear relationship between specific growth rate and temperature. The Huang and Ratkowsky models were used to predict the minimum and maximum temperatures of growth for the four strains. The minimum temperature values obtained from the Huang model were closer to the biological minimum temperature of the microorganisms as determined by the temperature-growth studies as compared to the values obtained from the Ratkowsky model, hence suggesting the Huang model as a better predictive model. The results showed strain 1_F178^T having the highest μ_{max} of 0.64 h^{-1} at an optimum temperature of 33.2 °C followed by *P. fluorescens* with a μ_{max} of 0.60 h^{-1} at an optimum temperature of 30.5 °C. Growth was observed at 4 °C for all strains indicative of growth at refrigeration temperature with the possibility of food spoilage. The high growth rates of *P. fluorescens* confirm why they outgrow chryseobacteria in spoilt poultry samples.

4.1. Introduction

Food spoilage is defined as any sensory change (tactile, visual, olfactory or flavour) that the consumer considers unacceptable. This includes physical damage, chemical changes (oxidation, colour changes) or the appearance of off-flavours and off-odours resulting from microbial growth and metabolism in the product (Gram *et al*., 2002; Rawat, 2015). Major causes of food spoilage include physical (e.g. temperature, pH, light and mechanical damage), chemical (e.g. enzymatic reactions, non-enzymatic reactions, rancidity, chemical interactions), microbial (e.g. bacteria, yeasts, moulds) and other activities (e.g. insects, animals, rodents, birds). Bacteria, yeasts and moulds are the common cause of microbial food spoilage with bacterial contamination being not visible, though the food product is severely contaminated (Gram *et al*., 2002).

A number of factors affect the growth of microbes in food products. These factors include intrinsic (e.g. nutrients, water activity, pH, redox potential, inhibitors), extrinsic (e.g. temperature, humidity, atmosphere) and implicit (e.g. microbial interactions). Temperature is one of the most critical factors. Most of the spoilage manifest in the form of visible growth as well as the production of gas, slime, enzyme and off-flavours (Huis in't Veld, 1996).

The genus *Chryseobacterium* has been reported to cause spoilage in food products like butter, creamed rice and canned vegetables (Wolochow *et al*., 1942; Everton *et al*., 1968; Bean & Everton, 1969; Jooste *et al*., 1986a). Till date, there has been little research published on the effects of temperature on the growth of *Chryseobacterium* species. Bekker and co-workers (2015) did a study on the growth kinetics and proteolytic activities of *C. bovis*, *C. joostei* and *P. fluorescens*. They concluded that temperature had an effect on the growth of *Chryseobacterium joostei* which exhibited a great spoilage potential in milk. This therefore demonstrates that the role of temperature in food spoilage is vital and must be treated with caution.

With the use of predictive microbiology, the behavior of microbial populations can now be determined. The effect of different environmental factors like temperature can be predicted by the use of mathematical models derived from quantitative studies on

microbial populations (Fakruddin *et al*., 2011). Models like the Arrhenius, Huang and Ratkowsky can be used to describe the relationships between kinetic parameters like the maximum specific growth rate (μ_{max}) and environmental factors like temperature. Predictive microbiology models are currently being applied to improve food quality and safety (Ratkowsky *et al*., 1982; Fakruddin *et al*., 2011; Huang *et al*., 2011).

The aim of this study was to conduct temperature-growth studies in an attempt to determine the growth kinetics of a novel *Chryseobacterium* species in comparison with *Chryseobacterium carnipullorum* and the renowned food spoilage microbe, *Pseudomonas fluorescens*. The Arrhenius, Huang and Ratkowsky models were used to determine the relationships between the kinetic parameters and the temperature as an environmental factor, in order to predict the minimum and maximum temperatures of growth of the respective strains since the TGI only covered a limited temperature range.

4.2. Materials and methods

4.2.1. Cultures used and their maintenance

The details of the isolates and reference strains used in this study are given in Table 4.1. All reference strains were obtained in a freeze dried state in ampoules. For shorter-term maintenance, the isolates were freeze-dried on 5 mm diameter filter paper discs in sealed Petri dishes and stored at -20 °C. Before use, the strains were reactivated in 10 ml nutrient broth (NB; Oxoid CM67). Purity was checked by streaking on nutrient agar (Oxoid CM003) and Gram-staining. Incubation was at 25 °C for 48 h. The pure cultures on nutrient agar slants were stored at 4 °C for short-term maintenance and re-streaked every 4–6 weeks.

Table 4.1. Bacterial strains used for temperature-growth studies. DSM, Deutsche Sammlung von Mikroorganismen; UFSBC, University of the Free State Bacterial Collection, South Africa.

Genus and species	Culture collection	Source of isolation	Reference
Chryseobacterium strain 1_F178^T	UFSBC 707	Chicken feathers	Charimba, 2012
Chryseobacterium strain 5_R23647	UFSBC 702	Raw chicken	Charimba, 2012
Chryseobacterium carnipullorum	DSM 25581^T	Raw chicken	Charimba *et al*., 2013
Pseudomonas fluorescens	DSM 4358^T	Raw milk for cheese production	Migula, 1895

4.2.2. Preliminary growth studies

Preliminary growth studies were conducted to determine the late exponential phase of the bacterial strains. During the exponential phase, the growth rate is constant, hence the rate of increase of cells in the culture is proportional to the number of cells present at any particular time (Monod, 1949).

Freeze-dried filter paper discs containing the bacteria (Table 4.1) were revived in NB for 48 h at 25 °C, after which 5 ml was transferred into 500 ml Erlenmeyer side-arm flasks containing 95 ml of fresh NB. The flasks were placed on an orbital shaker at a shaking speed of 180 rpm at 25 °C for 24 h. Cell concentrations were monitored by measuring culture turbidity (optical density) against a blank medium with a Photolab S6 spectrophotometer (WTW, Wilheim, Germany) at 620 nm, every three hours.

When the exponential phase was reached for the individual strains, dry cell weight (biomass) was determined using triplicate 10 ml culture samples. These were centrifuged at 4000 *x g* for 15 min at 4 °C using an Eppendorf 5430 R centrifuge (Eppendorf AG,

Hamburg, Germany), washed twice with distilled water and dried to constant weight at 105 °C.

Biomass differences between the four different bacteria were determined by using a one way analysis of variance (ANOVA) procedure (Microsoft® Office Excel® 2013).

4.2.3. Determination of the effect of temperature on growth

4.2.3.1. Temperature-growth studies

Temperature-growth studies were conducted by reviving the cells in NB as described in 4.2.2. A volume of 5 ml was transferred into 500 ml Erlenmeyer flasks containing 95 ml of fresh NB. The flasks were placed on an orbital shaker at a shaking speed of 180 rpm at 25 °C for the duration of the predetermined exponential phase from the preliminary studies. Once the exponential phase was reached, 20 ml of culture was transferred into 500 ml Erlenmeyer flasks containing 400 ml of fresh NB. This was properly mixed and used as the inoculum to inoculate 30 sterile temperature gradient incubator (TGI) tubes. A volume of 10 ml was distributed into each of the L-shaped TGI tubes, made of optically selected glass and capped with loose-fitting metal caps. The tubes were placed on the TGI (Scientific Industries Inc., New York, USA) consisting of an aluminium bar that was cooled at one end and heated at the other to obtain a stable temperature gradient, as described by du Preez & Toerien (1978). The bar contained thirty equidistant sample wells on both sides, with one side containing the tubes with bacterial cultures and the other corresponding tubes containing water to be used for temperature measurements at different time intervals. The bar was rocked through a 30° arc at 60 oscillations.min^{-1}, providing mixing and aeration. The tubes were incubated at temperatures ranging between 12–47 °C with ± 2 °C increments between the 30 individual positions. Growth at 4 °C was carried out with the TGI placed in a 4 °C walk-in fridge, with measurements done every two to three hours for 24 h.

4.2.3.2. Growth measurements

Growth was monitored by measuring the optical density (OD) of the culture at 600 nm with a Biowave C0800 cell density meter (Walden Precision Apparatus Ltd., Cambridgeshire, UK). Measurements only commenced 30 min after incubation to allow the tubes to reach their respective incubation temperatures. During measurements, tubes were taken off the TGI without stopping the TGI, hence growth was only disrupted for a short period. The OD of the culture tubes were measured at 30–120 min time intervals with the subsequent measurement of the temperature using a handheld temperature meter (Crison TM 65 thermometer, Lasec, SA). Measurements were carried out until an OD value of 2 was obtained or when growth stopped. The experiment was performed in triplicate per bacterial strain.

4.2.3.3. Data analysis

The maximum specific growth rate at each temperature was determined by linear regression analysis of the exponential phase of the growth curve. The slope of the growth curve was calculated with the following equation with the help of a Microsoft Excel sheet (Microsoft Corporation, Washington, USA): $\mu_{max} = (\ln x_t - \ln x_0)/ t$; where x is optical density of the cell concentration, and *t* is time in hours.

An Arrhenius model ($\mu_{max} = Ae^{-(E/RT)}$) was used to describe the linear relationship between the specific growth rate and temperature by determining the activation energy of the microorganisms at a specific temperature range, with μ as the specific growth rate; A is the entropy constant; E the activation energy (temperature coefficient); R the universal gas constant (8.314 J mol^{-1} K^{-1}); and T the absolute temperature measured in Kelvin.

Ratkowsky and Huang models were used as additional models to predict the minimum and maximum temperature of growth of the microorganisms using the following equations;

Ratkowsky model: $\boldsymbol{\mu_{max} = [b\,(T - T_{min})(1 - e^{c(T-Tmax)})]^2}$

Huang model: $\boldsymbol{(\mu_{max} = \alpha\,(T - T_{min})^{1.5}(1 - e^{\beta(T - Tmax)})}$

Where μ_{max} is the maximum specific growth rate (h^{-1}),

b, α and β are coefficients,

T_{min}, T_{max} and T are the minimum, maximum and real time temperatures (°C)

4.3. Results and discussion

4.3.1. Preliminary studies

Figure 4.1 depicts the growth profiles for the bacterial strains. All the strains reached late exponential phase at around 10 h. The bacteria was then grown for 10 h before inoculation of the TGI tubes. *Chryseobacterium* strain 5_R23647 produced the highest biomass (16.4 g l^{-1}) followed by *P. fluorescens* (13.6 g l^{-1}), *Chryseobacterium* strain 1_F178^T (6.6 g l^{-1}) and *C. carnipullorum* (0.5 g l^{-1}).

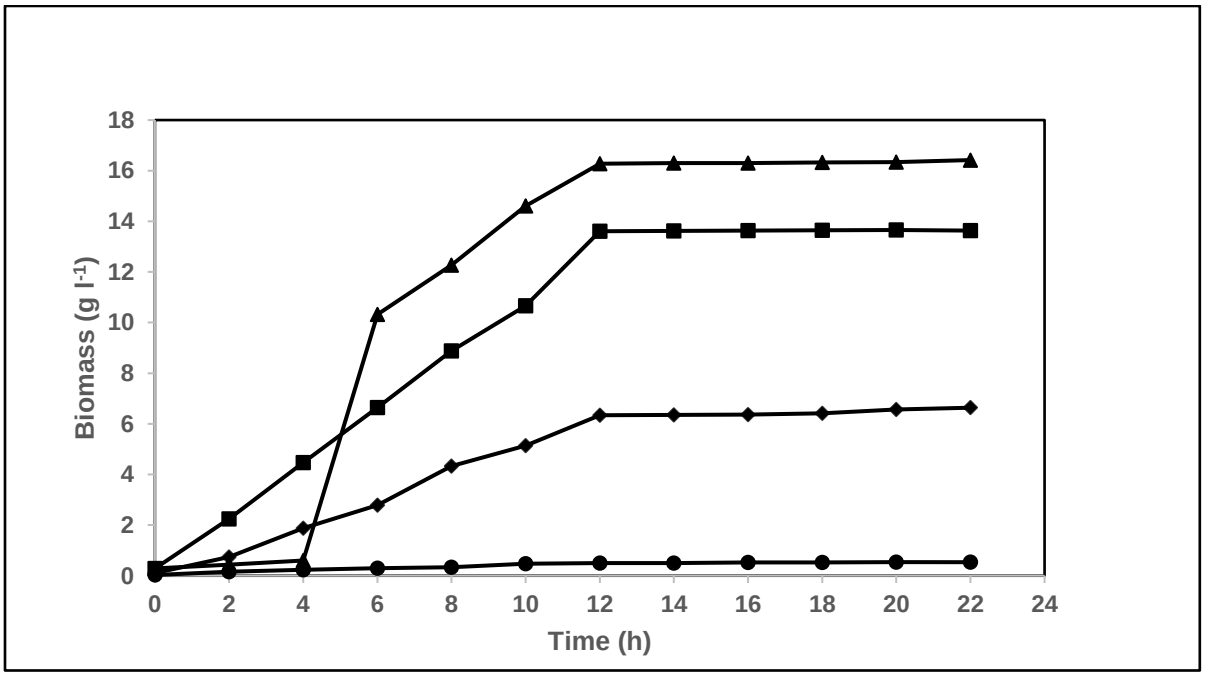


Fig. 4.1. Growth profiles of *Chryseobacterium* strain 1_F178^T, with biomass production (gl^{-1}) as a function of time (h). (♦); *Chryseobacterium* strain 5_R23647 (▲); *C. carnipullorum* (●) and *P. fluorescens* (■) grown in nutrient broth at 25 °C for 22 h. $P < 0.005$.

4.3.2. Growth Kinetics

Temperature is the main factor controlling the growth rate of microorganisms since other factors (water availability and nutrient status) are non-limiting, with microbial interactions only occurring after maximum cell densities have been reached. Based on their temperature of growth, microorganisms are divided into four main categories; psychrophiles, mesophiles, thermophiles and hyperthermophiles (Madigan *et al*., 2015). There was a statistically significant difference in biomass production between the four different bacteria. *Chryseobacterium carnipullorum* had the highest maximum growth temperature (38.7 °C) followed by *Chryseobacterium* strain 1_F178^T (37.0 °C), *P. fluorescens* (36.3 °C) and *Chryseobacterium* strain 5_R23647 (30.9 °C) (Table 4.2, Figure 4.2). *Chryseobacterium carnipullorum* had the lowest minimum growth temperature (14.9 °C) with *Chryseobacterium* strain 1_F178^T having the highest minimum growth temperature (15.3 °C).

Charimba and co-workers (2013) reported temperatures between 25–30 °C as optimal for the growth of *C. carnipullorum* on NA. No growth was noticed between 37 °C and 42 °C on NA plates. The ability of *C. carnipullorum* to grow at temperatures above 36°C on a TGI, but not grow above this temperature when grown on NA plates can be associated with the need for adequate aeration provided by TGI, and increase in nutrient availability which is not the case for the NA plates. Growth was also observed at 4 °C when all the strains were grown on the TGI. Interestingly, growth at 4 °C on NA was only observed for *Chryseobacterium* strains 1_F178^T and 5_R23647. This may mean adequate aeration at low temperatures is required for the growth of *C. carnipullorum* and *P. fluorescens*. Growth is generally not possible at and below the minimum temperature for growth, with the maximum specific growth rate being very low. In a study by Bekker and co-workers (2015), they also reported the growth of *C. joostei*, *C. bovis* and *P. fluorescens* at 4 °C, although the microorganisms were placed on a roller drum in a temperature controlled refrigerator.

Chryseobacterium strain 1_F178^T had the highest optimal temperature (33.2 °C) which means it is more likely to survive higher temperatures as compared to the other strains (Table 4.2, Fig 4.2A). *Chryseobacterium* strain 5_R23647 had the lowest optimal

temperature (26.5 °C), implying that it is more likely to cause spoilage at lower temperatures in comparison with the other strains (Table 4.2, Fig 4.2B). All these microorganisms are mesophilic since they grow in the temperature ranges between 20–40 °C. Most *Chryseobacterium* species are mesophiles with all growing at 30 °C, and most at 37 °C (Vandamme *et al*., 1994). Bekker and co-workers (2015) reported *C. joostei* having the highest optimum temperature (31.50 °C), and *P. fluorescens* having the highest maximum temperature (36.99 °C) in temperature-growth studies on a TGI.

As temperature increases, microorganisms grow faster as there is an increase in the rate of the key intracellular processes. A further increase in temperature results in a decline in growth as cellular metabolism is adversely affected. This happens until the maximum temperature is reached, after which growth cannot be sustained (Van Derlinden & Van Impe, 2012). The maximum specific growth rate as a function of temperature is asymmetrically oriented around the optimum growth temperature. There is a slower increase in the maximum specific growth rate (μ_{max}) when moving from the minimum temperature to the optimum temperature as compared to when moving from the maximum temperature to the optimum temperature. Hence the negative effect of high temperatures is much more visible than the effect of low temperatures (Van Derlinden & Van Impe, 2012).

The growth of the chryseobacterial strains and *P. fluorescens* over a wide temperature range indicate their ability to cause food spoilage over wide temperature ranges. Spoilage defects due to flavobacteria/chryseobacteria have been reported in various products including butter (Wolochow *et al*., 1942; Jooste *et al*., 1986a), creamed rice (Everton *et al*., 1968) and canned vegetables (Bean & Everton, 1969). Other food sources that have been reported to contain *Chryseobacterium* spp. include fish (*C. piscium*), meat and meat products, raw chicken (*C. vrystaatense* and *C. carnipullorum*) and dairy products (de Beer *et al*., 2005; Bernardet *et al*., 2006; de Beer *et al*., 2006; Charimba *et al*., 2013).

Pseudomonas fluorescens had the second highest maximum specific growth rate (μ_{max}), (0.60 h^{-1}) at 30.5 °C after strain 1_F178^T (0.64 h^{-1}) (Table 4.2, Fig 4.2D). *Pseudomonas fluorescens* is a spoilage specific organism (SSO), which has been isolated from a number of high-protein food like fish, meat and raw milk where they have been seen to

cause spoilage through the formation of biofilm, protease, lipase and siderophore production (Arslan *et al*., 2011; Lo *et al*., 2015; Li *et al*., 2018). *Pseudomonas fluorescens* has different maximum specific growth rates when grown in different media. They had a μ_{max} of 0.93 h^{-1} at 31.5 °C when grown in nutrient broth (Bekker *et al*., 2015), a μ_{max} of 0.48 h^{-1} at 25 °C when grown in skim milk (Jooste & Fischer, 1992), and a μ_{max} of 0.56 h^{-1} at 25 °C when grown in citrate mineral salts (Gügi *et al*., 1991). Bekker and co-workers (2015) associated these μ_{max} changes with strain variability and/or nutritional composition of the media. The high μ_{max} of *P. fluorescens* indicates that it will outgrow *Chryseobacterium* strains in spoiled food products. The higher μ_{max} of *Chryseobacterium* strain 1_F178^T at its optimum temperature in comparison with that of the other strains mean *Chryseobacterium* strain 1_F178^T may have the ability to cause spoilage at a faster rate as compared to the other strains.

There is very little information about the specific growth rate of chryseobacterial strains. Only Fischer (1987) and Bekker and co-workers (2015) have reported on this. The specific growth rates of *C. bovis, C. joostei* and *P. fluorescens* was reported by Bekker and co-workers (2015).

Table 4.2. The cardinal temperatures of the chryseobacterial strains and *P. fluorescens*.

Strain	**Cardinal temperature, °C**				**Maximum specific growth rate (μ_{max}, h^{-1})**
	[a]**4 °C**	**Minimum**	**Optimum**	**Maximum**	
Chryseobacterium strain 1_F178^T	4.1	15.3	33.2	37.0	0.64
Chryseobacterium strain 5_R23647	4.0	15.1	26.5	30.9	0.45
C. carnipullorum	3.7	14.7	29.8	38.7	0.55
P. fluorescens	3.4	14.9	30.5	36.3	0.60

[a:] Independent growth studies carried out at 4 °C on the TGI.

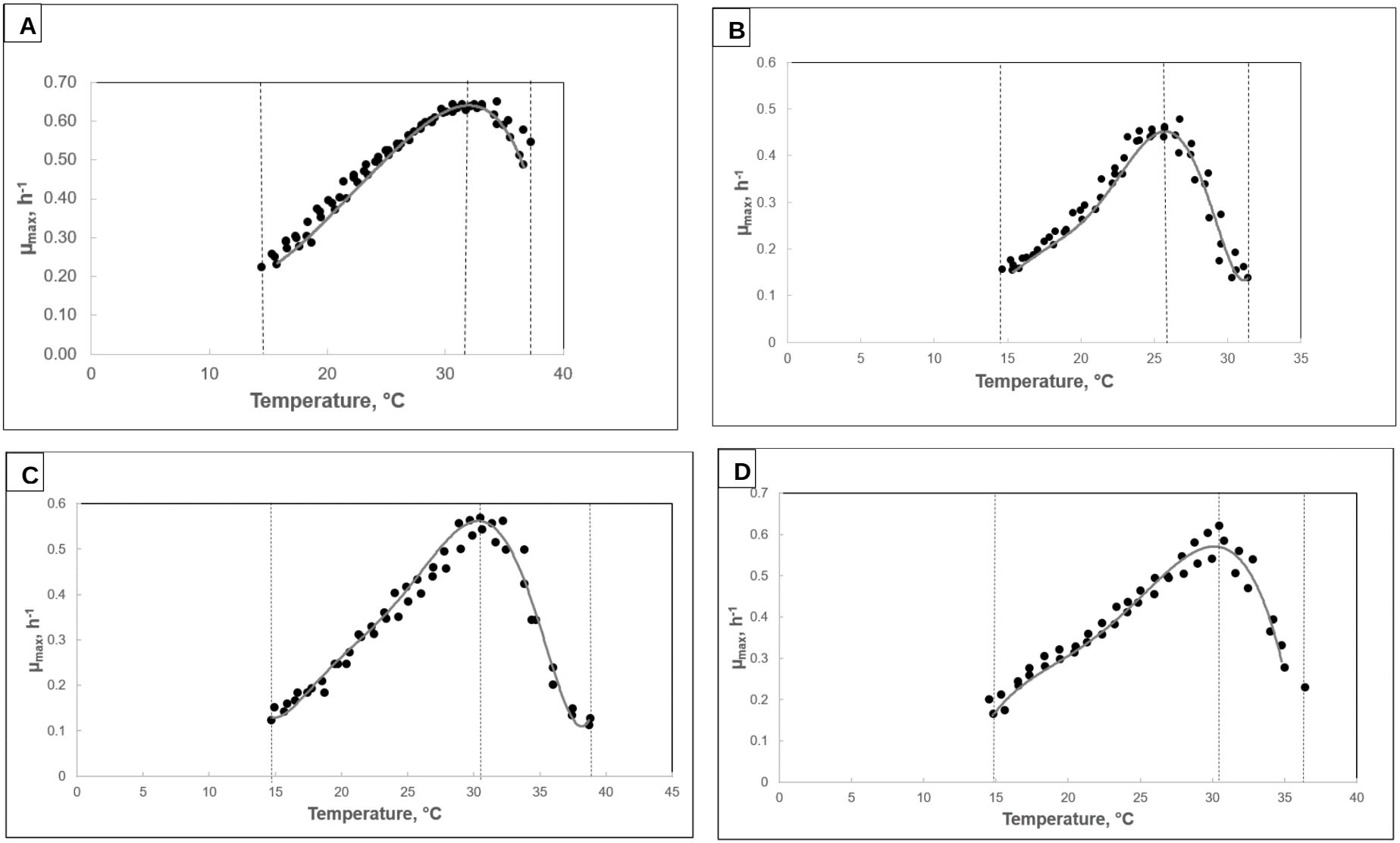


Fig. 4.2. Temperature profile of A: *S*train 1_F178^T, B: Strain 5_R23647, C: *C. carnipullorum*, D: *Pseudomonas fluorescens,* with maximum specific growth rate (μ_{max}) as a function of cultivation temperature. The broken vertical lines indicate the cardinal temperatures in Table 4.2.

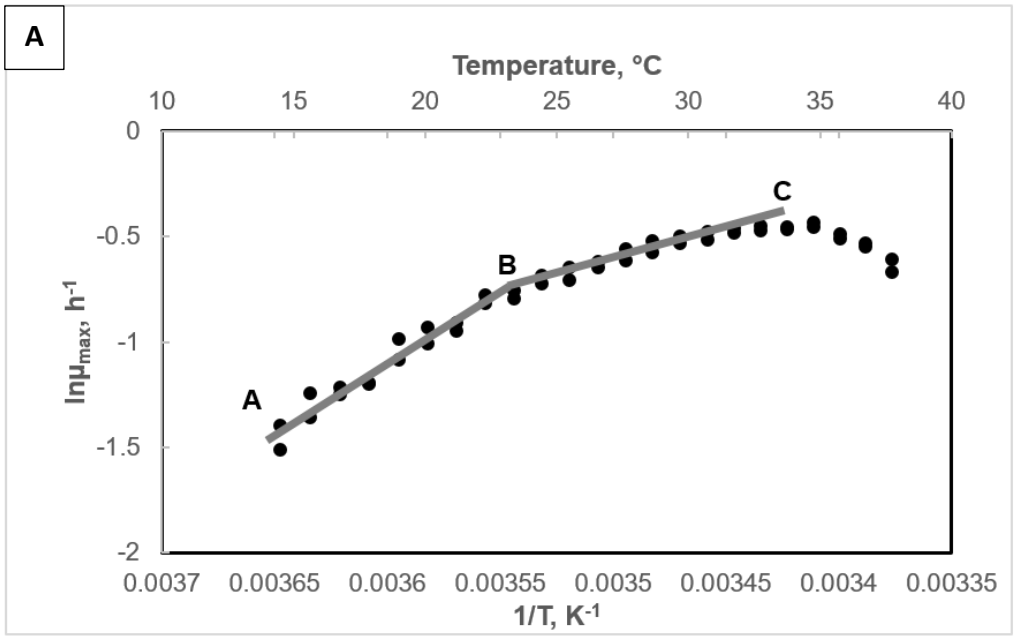
Arrhenius plots

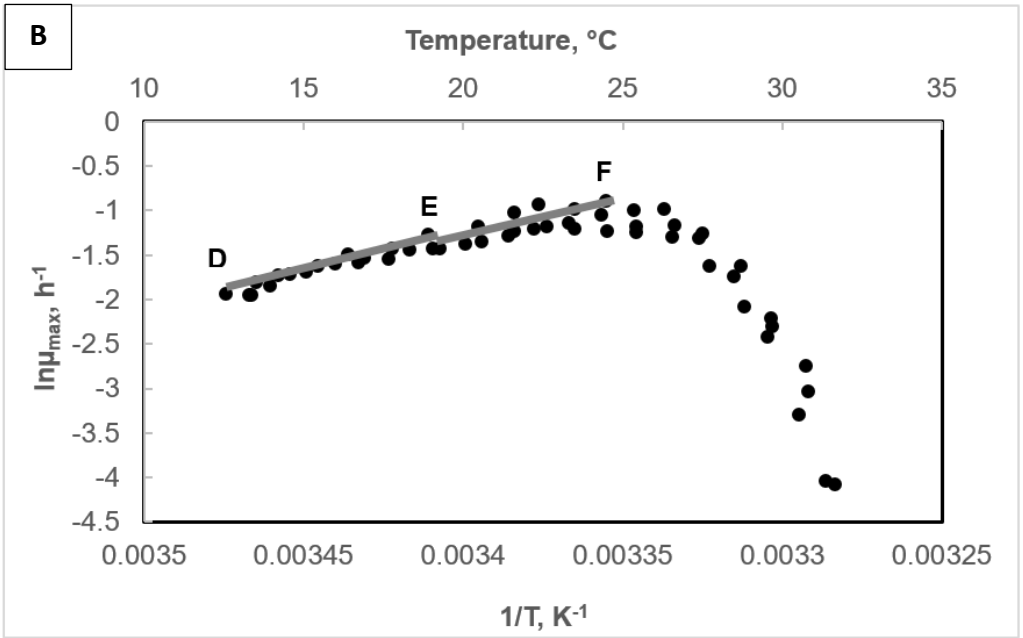
The Arrhenius plots of the chryseobacterial strains and *P. fluorescens* used in this study are indicated in Figure 4.3. The Arrhenius law has been used in chemical kinetics to describe the temperature dependence of simple chemical reactions. The Arrhenius model relates the constant *k* of a reaction to absolute temperature *T* (Van Boekel, 2008).

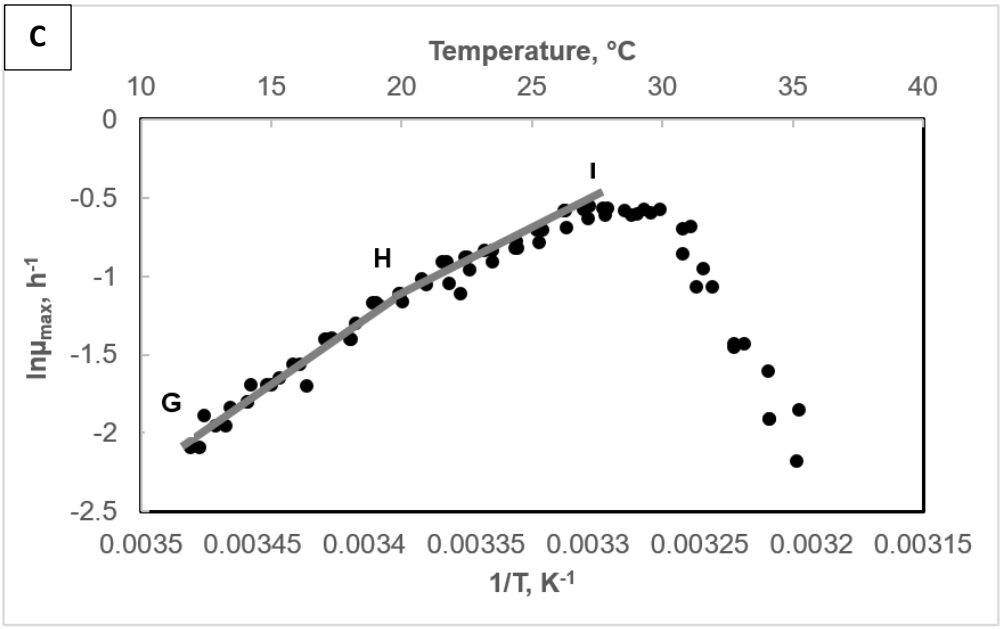
The activation energy (E_a) is defined by Van Boekel (2008) as the energy barrier that molecules need to cross in order to be able to react. The proportion of molecules that are able to react do so with an increase in temperature, which qualitatively explains the effect of temperature on the rate of reactions. The activation energy is used to predict the effect of temperature on the growth rate over a normal temperature range. Changes in the E_a indicate that differences in the rate-controlling reactions or metabolic regulations can occur (Pirt, 1975).

The activation energy was calculated based on the following formula: The slopes($\frac{\Delta ln\mu \max}{\Delta 1/T}$) of each zone on the ln$\mu_{max}$ vs 1/T graphs (Figures 4.3) were determined and multiplied with $\frac{R}{1000}$ (R is the universal gas constant, 8.314 J·mol^{-1}·K^{-1}) to calculate the activation energy in kJ·mol^{-1}.

Chryseobacterium carnipullorum had the highest activation energy (81.62 kJ·mol^{-1}) at temperatures between 12.3–19.8 °C (G–H) followed by *Chryseobacterium* strain 5_R23647 (71.49 kJ·mol^{-1}) at temperatures between 12.7–18.6 °C (D–E) and *Chryseobacterium* strain 1_F178^T (53.27 kJ·mol^{-1}) at temperatures between 13.6–22.2 °C (A–B) (Table 4.3, Fig 4.3). *Pseudomonas fluorescens* had the lowest activation energy (52.52 kJ·mol^{-1}) at temperatures between 14.3–23.4 °C (J–K). This implies that *P. fluorescens* is least sensitive to changes in temperatures in this region, and has the ability to grow over a wide temperature range. On the other hand, *C. carnipullorum* which has a higher activation energy, is most sensitive to changes at temperatures in the region between G–H and can only grow over a limited temperature range. Microorganisms with low activation energies like *P. fluorescens* will therefore have a higher spoilage potential in food since they can grow over wider temperature ranges.







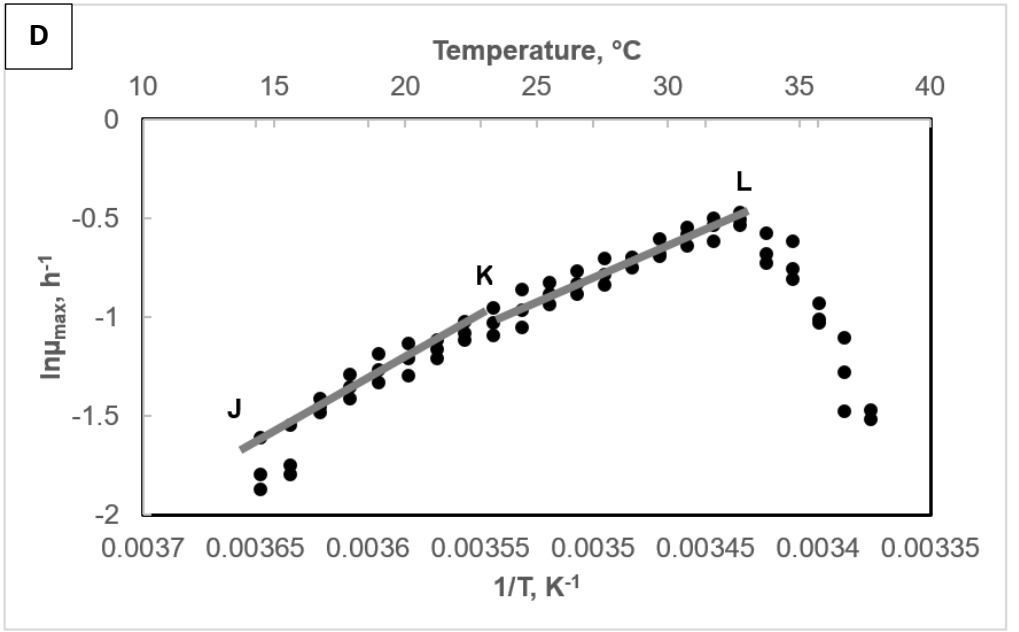


Fig. 4.3. Arrhenius plot for A: Strain 1_F178^{T}, B: Strain 5_R23647, C: *C. carnipullorum*, D: *Pseudomonas fluorescens* in nutrient broth with the maximum specific growth rate (μ_{max}) plotted against temperature expressed in Kelvin.

Bekker and co-workers (2015) reported higher E_a values (113.10 kJ·mol^{-1}) between 9.9–18.2 °C for *P. fluorescens* ATCC 13525^T as compared to the E_a values obtained for this study (52.52 kJ·mol^{-1}). This may be attributed to the different strains of *P. fluorescens* used for the respective temperature-growth studies or the difference in the temperature ranges where the E_a was calculated in these studies. All of the strains had E_a values above 20 kJ·mol-1. When the E_a is greater than 20 kJ·mol^{-1}, it suggests the occurrence of processes (like those that take place in foods during storage and cooking) involved in the breaking of primary chemical bonds. The E_a provides information about a reactions' mechanism (Laidler, 1972).

Table 4.3. Activation energies of *Chryseobacterium* strain 1_F178^T, *Chryseobacterium* strain 5_R23647, *C. carnipullorum* and *P. fluorescens* obtained from the Arrhenius plots.

Bacterium	Temp range (°C)	Zone	Slope	Activation energy (kJ·mol^{-1})
Chryseobacterium strain 1_F178^T	13.6–22.2	A–B	-6080.55635	53.27
	22.2–32.8	B–C	-2833.813637	25.27
Chryseobacterium strain 5_R23647	12.7–18.6	D–E	-8809.041949	71.49
	18.6–24.5	E–F	-6079.312493	59.23
C. carnipullorum	12.3–19.8	G–H	-8088.37403	81.62
	19.8–27.9	H–I	-5955.788265	59.16
P. fluorescens	14.3–23.4	J–K	-5974.370535	52.52
	23.4–33.7	K–L	-6300.735402	37.18

The results obtained indicate that the growth rate is less subject to temperature influences above 22.2 °C, but not exceeding 36.8 °C for *Chryseobacterium* strain 1_F178^T, 18.6 °C but not exceeding 30.7 °C for *Chryseobacterium* strain 5_R23647, 19.8 °C but not exceeding 37.4 °C for *C. carnipullorum* and 23.4 °C but not exceeding 36.3 °C for *P. fluorescens*.

A considerable amount of literature has been published on the use of the Arrhenius equations to describe the temperature effect on different biological processes (Frauenfelder *et al*., 1991; Lloyd & Taylor, 1994; Gillooly *et al*., 2001). Despite the benefits of this equation, like allowing good representations of growth rates at low temperatures, it also has some drawbacks. The Arrhenius equation is only valid over a small temperature range, excluding high temperatures where growth is inhibited. This means this model cannot represent the decreasing part of the thermal growth curve in the regions where an increase in temperature results in cell death (Slator, 1916; Grimaud *et al*., 2017). Other models can be used to predict the temperature in such regions. Secondary models like the Ratkowsky and Huang models have been used, and considered to be advantageous over the Arrhenius equation (Ratkowsky *et al*., 1982; Huang *et al*., 2011).

Ratkowsky and Huang models

Table 4.4 shows the estimated minimum and maximum temperatures obtained from the Ratkowsky and Huang models. The minimum temperature (T_{min}) values estimated using the Ratkowsky model were lower for *Chryseobacterium* strain 1_F178^T (-15.4 °C), *C. carnipullorum* (-1.0 °C) and *P. fluorescens* (-3.1 °C) when compared to their biological (or experimental) minimum temperatures obtained from the temperature-growth studies (4.1 °C, 3.7 °C and 3.4 °C, respectively), with the exception of *Chryseobacterium* strain 5_R23647.

The T_{min} values estimated using the Huang model were higher for *Chryseobacterium* strain 1_F178^T (4.8 °C), *Chryseobacterium* strain 5_R23647 (9.6 °C) and *C. carnipullorum* (7.9 °C). *P. fluorescens* was the only exception with its estimated minimum temperature less (2.8 °C) than its biological minimum temperature (3.4 °C) although not as low as the value obtained with the Ratkowsky model. These results are somewhat in agreement with that of Huang and co-workers (2011) who observed that the T_{min} values obtained from the Ratkowsky model systematically underestimated the minimum growth temperatures as opposed to the T_{min} values obtained from the Huang model. Huang and coworkers (2011) attributed the underestimation of T_{min} to the inability of the Ratkowsky model to provide a mathematical mechanism to reflect the accelerated decrease in

biological activities as the temperature approaches the biological minimum. They further suggested the use of the Huang model as it provides a mathematical mechanism to describe the rapidly reduced rate of growth as the temperature approaches the minimum bacterial growth rate. The Ratkowsky model has also been criticized by Ross (1993) due to its lack of fit at low temperature conditions hence resulting in the underestimation of T_{min}.

On the other hand, when the Ratkowsky and Huang models were used, the estimated T_{max} for *Chryseobacterium* strains 1_F178^T (38.4 °C and 38.0 °C) and 5_R23647 (32.6 °C and 31.8 °C) were higher than their biological maximum temperatures (37.0 °C and 30.9 °C). For *C. carnipullorum* and *P. fluorescens*, the estimated T_{max} values obtained from the Huang model were slightly lower (38.0 °C and 35.6 °C, respectively) than their biological maximum temperatures (38.7 °C and 36.3 °C, respectively). The Ratkowsky model yielded a lower estimated T_{max} (36.9 °C) for *C. carnipullorum*, and a slightly higher estimated T_{max} (36.6 °C) for *P. fluorescens*. This may suggest that both the Ratkowsky and Huang models have a good fit at high temperatures.

Table 4.4. Predicted minimum and maximum temperatures for *Chryseobacterium* strain 1_F178^T, *Chryseobacterium* strain 5_R23647, *C. carnipullorum* and *P. fluorescens* obtained from Ratkowsky and Huang models.

Parameter	*Chryseobacterium* strain 1_F178^T		*Chryseobacterium* strain 5_R23647		*C. carnipullorum*		*P. fluorescens*	
	γ = 2.0	**γ = 1.5**	**γ = 2.0**	**γ = 1.5**	**γ = 2.0**	**γ = 1.5**	**γ = 2.0**	**γ = 1.5**
T_{min} (°C)	-15.4	4.8	5.9	9.6	-1.0	7.9	-3.1	2.8
T_{max} (°C)	38.4	38.0	32.6	31.8	36.9	38.0	36.6	35.6

γ = 2.0; Ratkowsky model

γ = 1.5; Huang model

T_{min}; Minimum temperature

T_{max}; Maximum temperature

4.4. Conclusions

The temperature-growth studies to determine the effect of temperature on the growth of *Chryseobacterium* strains 1_F178^{T}, 5_R23647, *C. carnipullorum* and *P. fluorescens* showed *Chryseobacterium* strain 1_F178^{T} having the highest specific growth rate (0.64 h^{-1}) at an optimum temperature of 33.2 °C. This was followed by *P. fluorescens* with a specific growth rate of 0.60 h^{-1} at 30.5 °C. The high growth rate of *P. fluorescens* is not strange as it is a specific spoilage organism whose occurrence has been widely reported in spoiled food. It has been isolated from spoiled poultry samples together with some chryseobacterial species where it was reported to outgrow the chryseobacterial species. If confirmed as a spoilage microorganism, *Chryseobacterium* strain 1_F178^{T} will have a high spoilage potential due to its high specific growth rate.

Chryseobacterium carnipullorum had the highest maximum temperature (38.7 °C), with *Chryseobacterium* strain 1_F178^{T} having the highest optimum temperature (34.7 °C) and highest minimum temperature (15.3 °C). The independent growth studies of all the four strains on the temperature gradient incubator showed growth at 4 °C, indicative of growth at refrigeration temperature with possible food spoilage. The growth of *Chryseobacterium* over wide temperature ranges indicate their ability to cause food spoilage over wide temperature ranges. *Chryseobacterium carnipullorum* had the highest activation energy (81.62 kJ·mol^{-1}) between 12.3–19.8 °C, implying a high sensitivity to change in temperature in this region. The lowest activation energy was obtained for *P. fluorescens* (52.52 kJ·mol^{-1}) between 14.3–23.4 °C indicative of this microorganism being less sensitive to changes in temperature in this region, meaning it has the ability to grow over wider temperature ranges, and will therefore have a higher spoilage potential than the chryseobacterial strains in this study and as confirmed in literature.

Predictions to determine the minimum and maximum temperatures for growth using the Huang and Ratkowsky models for the four strains showed the Huang model to be a more effective model as the minimum temperature values of growth were closer to the biological minimum values as determined by the temperature growth studies. This research confirmed the findings of Huang and co-workers (2011) and once more confirmed the wide temperature range of growth for the four strains.

With the little information present on the effect of temperature on the growth of the chryseobacterial strains, this study adds to the body of knowledge in that regard. Knowledge of the effect of temperature on the rate of growth of spoilage bacteria may be used to monitor the time-temperature history of expired shelf life of the product.

Chapter 5

Phenotype microarray characterisation of *Chryseobacterium* strain 1_F178^T

Abstract

Phenotype microarray (PM) is used as a high-throughput technology for the characterisation and monitoring of microbial cellular phenotypes. *Chryseobacterium* strain 1_F178^T, and its closest relatives, *Chryseobacterium jejuense* DSM 19299^T and *Chryseobacterium nakagawai* NCTC 13529^T were subjected to PM analysis. Strain 1_F178^T differed from *C. jejuense* by 5.1% and from *C. nakagawai* by 8.9% based on the phenotypes gained and lost. Substrate utilisation of some carbohydrates, amino acids, carboxylic acids and polymers highlighted the spoilage potential of *Chryseobacterium* strain 1_F178^T. Based on parametric analysis, strain 1_F178^T showed resistance to only two antibiotics when compared to *C. jejuense*, and showed resistance mostly against cephalosporins and β-lactam antibiotics when compared to *C. nakagawai*. The resistance of strain 1_F178^T to certain pesticides and fungicides showed the possible application of this strain in the agricultural sector.

5.1. Introduction

The advent of proteomics and nucleic acid analyses only allowed for the cellular analysis of macromolecules that convey information flow from DNA to RNA to proteins (O'Farrell *et al*., 1975; Fodor *et al*., 1993). The genetic makeup of an organism determines the phenotype or cellular traits it displays, hence Bochner (1989) proposed the use of phenotype microarray (PM) which provides a two-dimensional array technology for the analysis of live cells (phenomics) (Bochner *et al*., 2001). Phenotype microarray measures

hundreds to thousands of cellular phenotypes using microplates for high-throughput assays.

Phenotypes are observable characteristics of cells. Growth phenotypes allow microbiologists to describe and differentiate cells. They define if and how fast a bacterium will grow, and are directly involved in fundamental aspects of cellular genome and organism evolution, hence remain the cornerstone of microbial taxonomy (Bochner, 2008).

Phenotype Microarray measure cellular phenotypes colorimetrically by making use of tetrazolium redox dye to measure cell respiration. Cell respiration is measured instead of growth because it is a more sensitive way to measure phenotypes. More so, cells may respond metabolically by respiring but not growing. In addition, it allows the measurement of more cellular pathways and can also be used to measure phenotypes of cells that cannot be cultured axenically (Bochner, 2008).

The PM system includes 96-well culture plates coated with a variety of chemicals. A total of 1920 different assays are available in 20 x 96-well PM plates. PM 01 and PM 02 are carbon source panels, consisting of about 200 assays of C-source metabolism. PM 03, 06, 07 and 08 are nitrogen source panels, consisting of 400 assays of N-source metabolism. PM 04 contains phosphate and sulphur sources, with about 100 assays of P-source and S-source metabolism. PM 05 contains various biosynthesis pathway end products and nutrient supplements with about 100 assays of biosynthetic pathways. PM 11 to PM 20 are chemical sensitivity assays including toxic compounds and ions with about 100 assays of ion effects and osmolality, 100 assays of pH effects and pH control with deaminases and decarboxylases, and 1000 assays of chemical sensitivity, each at four concentrations (Chojniak *et al*., 2015).

The PM technology is based on the chemistry of the tetrazolium dye. If the microorganism possesses a transport system and catabolic pathway of a substrate or chemical, then the substrate is catabolized with the production of NADP. The electron transport pathway in the cell then takes electrons from the NADH produced. Some of the electrons are transferred to the tetrazolium dye, eventually causing it to be reduced due to an increase in cell respiration, hence resulting in the formation of an irreversible purple colour in the

96-well culture plates (Fig. 5.1). The intensity of the purple colour is dependent on the rate of metabolism. A substrate that is strongly metabolized will rapidly form a dark purple colour while that which is weakly metabolized will slowly form a light purple colour. The OmniLog machine records the rates and extent of colour formation in each of the wells, producing colour-coded kinetic graphs with quantitative and kinetic information on the response of the cells to the PMs (Bochner, 1989; Bochner, 2008).

This colorimetric method of analysis is beneficial because firstly, the colour change is easy to monitor and quantitate, secondly, the colour change is very sensitive and highly reproducible, and lastly, because cell respiration can occur independent of cell growth and, in some cases, can measure phenotypes that do not lead to growth (Bochner *et al*., 2001).

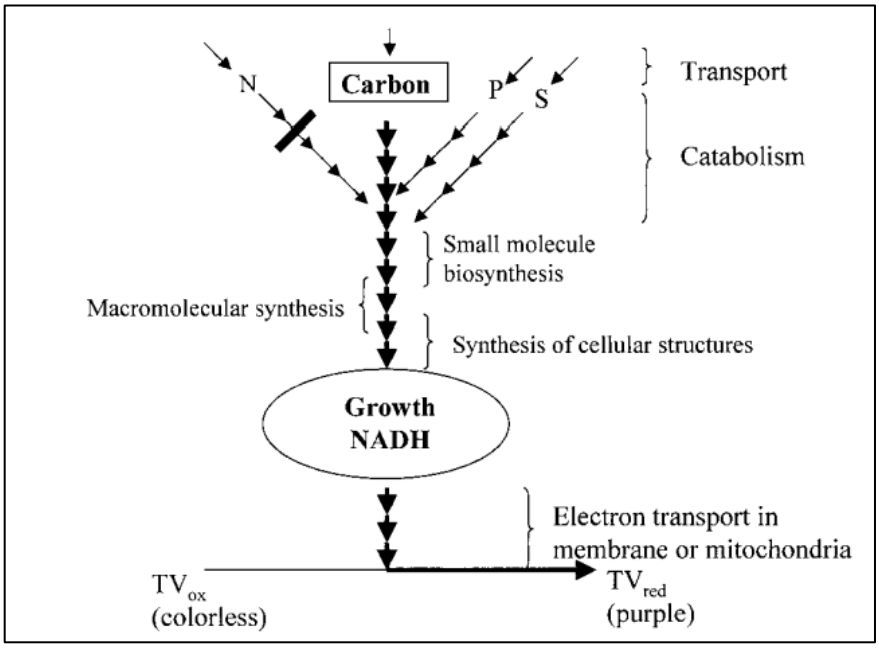


Fig 5.1 Respiration pathways coupled to cell physiology (Bochner *et al*., 2001).

Phenotype technology has been used to assay gene function. This is done by knocking out genes, then evaluating the effect the loss of genes has on the phenotypes of the cell. The use of PM to assay gene function has been so far its largest area of application. Funchain and co-workers (2000) used PM technology to detect phenotype changes

affecting carbon metabolism in 70% of mutated *E. coli* strains after about 1000 generations of growth. Zhou and co-workers (2003) assayed for phenotypic changes with PM to evaluate the efficiency of deleted mutants. Different biological changes were observed in two of the mutant clones, indicating the introduction of other genetic changes.

Phenotypic testing is universally applicable to culturable microorganisms and taxonomically predictive, and also provides useful information about the biological properties of cells. Taxonomists use PM 01, PM 02 and PM 09 plates consisting of over 300 different tests for carbon sources and osmolytes to compare most or all fast-growing bacterial species (Bochner *et al*., 2008).

In this study, a novel bacterial strain, *Chryseobacterium* strain 1_F178^T, was subjected to the BIOLOG OmniLog Combo System PM analysis and compared to its closest relatives, *C. jejuense* DSM 19299^T and *C. nakagawai* NCTC 13529^T.The aims were to determine the phenotypic differentiation between the novel strain and its closest relatives, in terms of phenotypes gained and phenotypes lost; to determine the potential food spoilage potential of the three strains in terms of PM substrates oxidised; to determine the resistance or sensitivity of the three strains to antimicrobials at four different concentrations, and to determine the potential applications of especially *Chryseobacterium* strain 1_F178^T.

5.2. Materials and methods

The test organism used was *Chryseobacterium* strain 1_F178^T and the reference strains used were *C. jejuense* DSM 19299^T and *C. nakagawai* NCTC 13529^T. The reference strains were obtained from international culture collections as indicated in Chapter 4. Strains were grown at 25 °C for 24 h in nutrient broth (Oxoid CM67), then streaked on Biolog Universal Growth agar (BUG^{TM}) for single colonies and incubated at 25 °C for 24 h. Cells were picked up with a sterile cotton swab and transferred into 20 ml sterile inoculation fluid (IF-0, Biolog, Inc.) to a cell density of 81% transmittance in the Biolog turbidimeter. The 20 PM plates were inoculated with 100 µl/well of the cell suspension. The methods followed were “PM procedures for *E. coli* and other GN bacteria” according

to the manufacturer's (Biolog Inc.) instructions. The 20 x 96-well plates were incubated for 24 h at 25 °C in the OmniLog incubator/reader (Biolog Inc., Hayward, USA).

To determine the phenotypic differences, the OmniLog® phenotype microarray software x 1.2 version was used. The intensity of the purple colour formation in each well was recorded as Omnilog™ units (OU). Kinetic and parametric data analysis were done using the OmniLog - OL_PM_FM/Kin 1.20.02: File Management/ Kinetic plot version (2005) and the OmniLog – OL_PM_Par 1.20.02: Parametric version (2005) respectively. A 20-panel comparison was done using the area under the curve parameter.

5.3. Results and discussion

5.3.1. Phenotypic differentiation

Figures 5.2, 5.3, 5.4 and 5.5 show the prominent phenotypes gained and lost by *Chryseobacterium* strain 1_F178^T when compared to its closest relatives, *C. jejuense* and *C. nakagawai* respectively. Tables 5.1, 5.2, 5.3 and 5.4 indicate other additional phenotypes gained and lost, in some cases not as prominent as depicted in Figures 5.2 – 5.4.

Most of the differentiating characteristics were observed with the chemical sensitivity tests from PM 10 to PM 20. PM 03 to PM 08 showed no clear differences in substrate oxidation, with PM 01 and PM 02 showing some differences in the oxidation of carbon sources (Fig. 5.2 - 5.4). *Chryseobacterium* strain 1_F178^T showed a total of 22 different phenotypes acquired, and 77 phenotypes lost when compared with *C. jejuense*, and showed a total of 164 different phenotypes gained and seven phenotypes lost when compared to *C. nakagawai*. The phenotypes that differed between strain 1_F178^T and *C. jejuense* was 5.1% (99/1920) and 8.9% (171/1920) in the case of *C. nakagawai*, hence confirms why *C. jejuense* is the closest relative to strain 1_F178^T when compared to *C*. *nakagawai*.

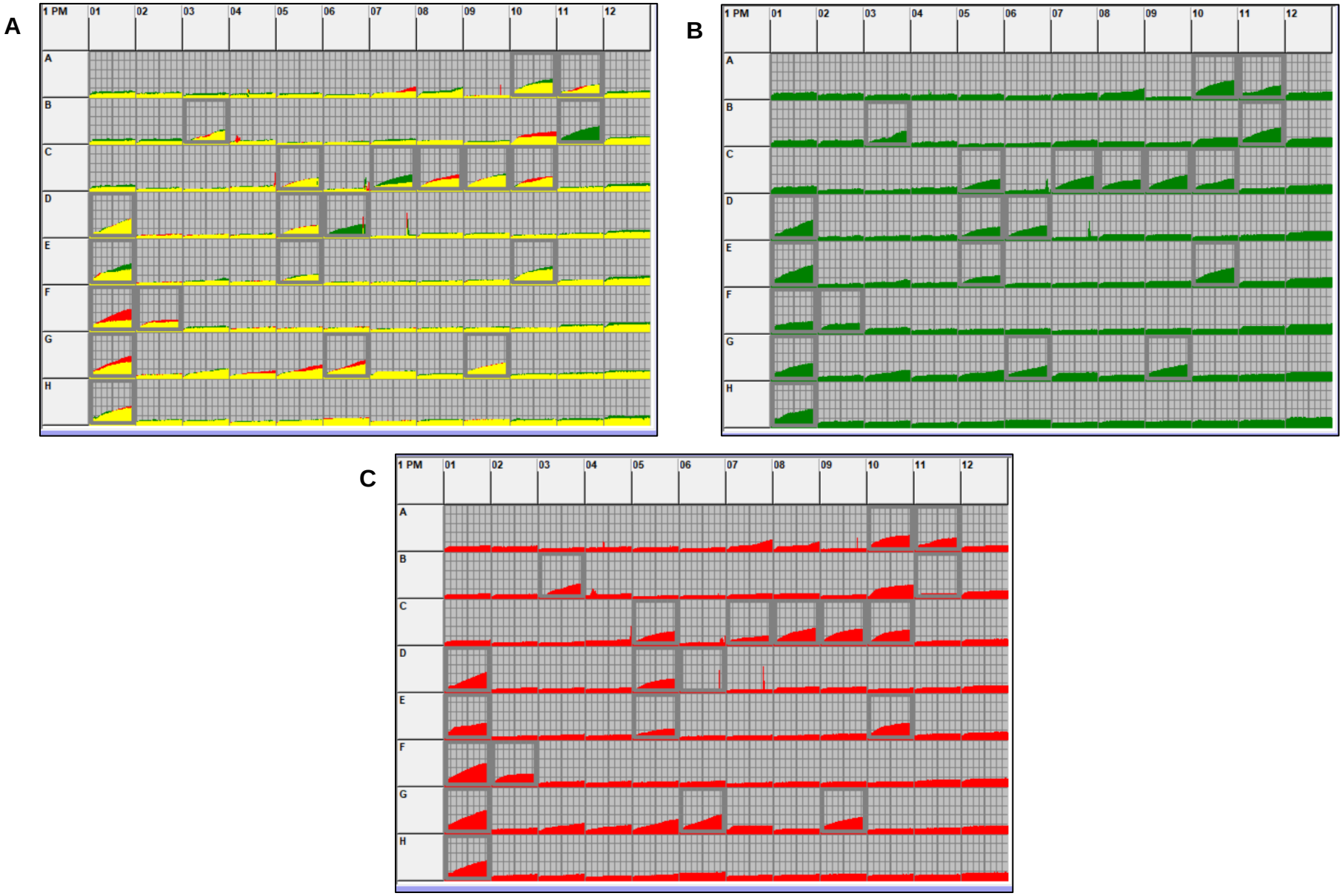


Figure 5.2. PM 01 showing the 96-well plates of A, strain 1_F178^{T} in comparison with *C. jejuense*; B, strain 1_F178^{T} only and C, *C. jejuense* only. Red indicates loss of phenotype; Green indicates gain of phenotype; Yellow indicates unchanged phenotype.

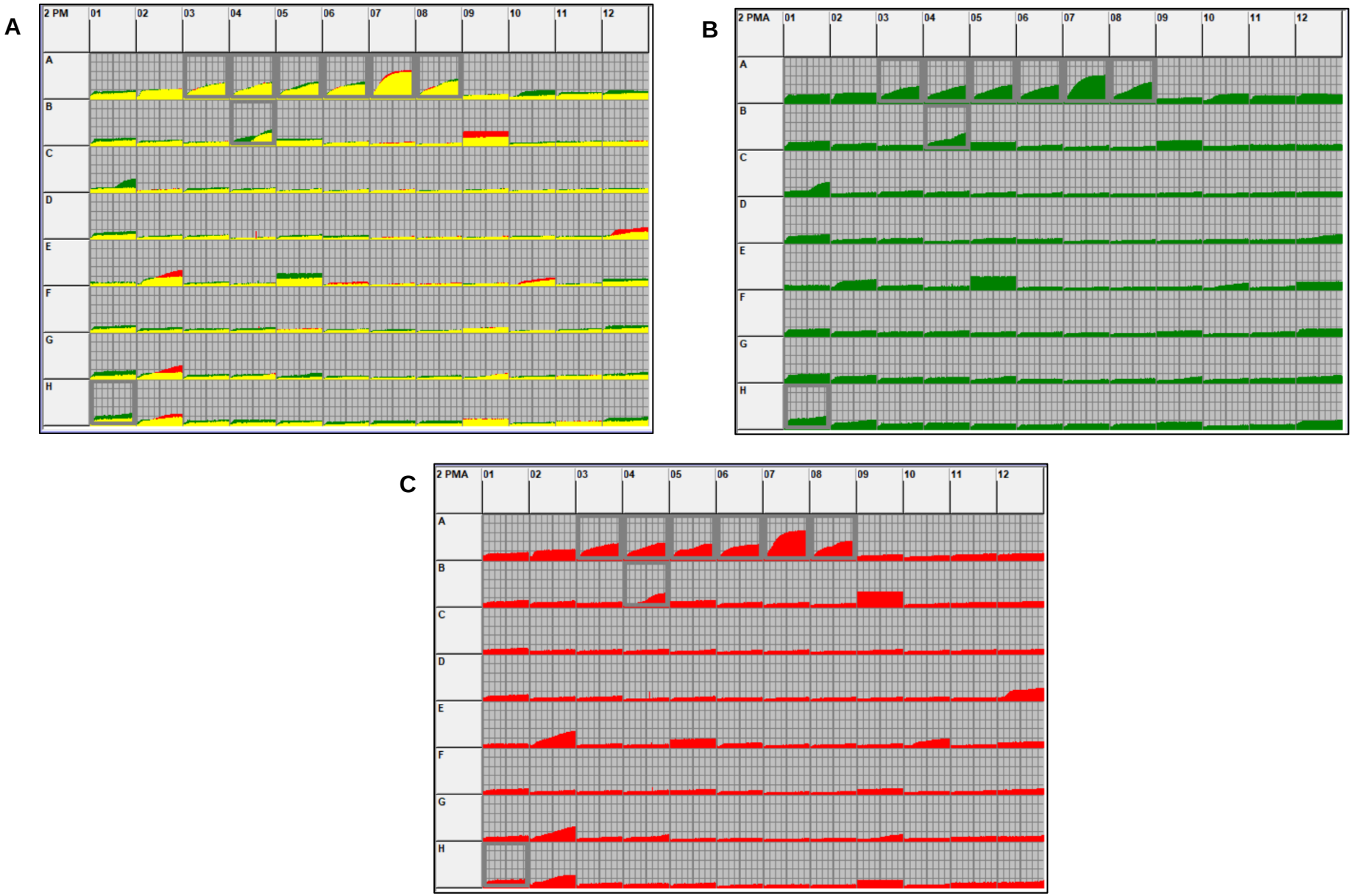


Figure 5.3. PM 02 showing the 96-well plates of A, strain 1_F178^{T} in comparison with *C. jejuense*; B, strain 1_F178^{T} only and C, *C. jejuense* only. Red indicates loss of phenotype; Green indicates gain of phenotype; Yellow indicates unchanged phenotype.

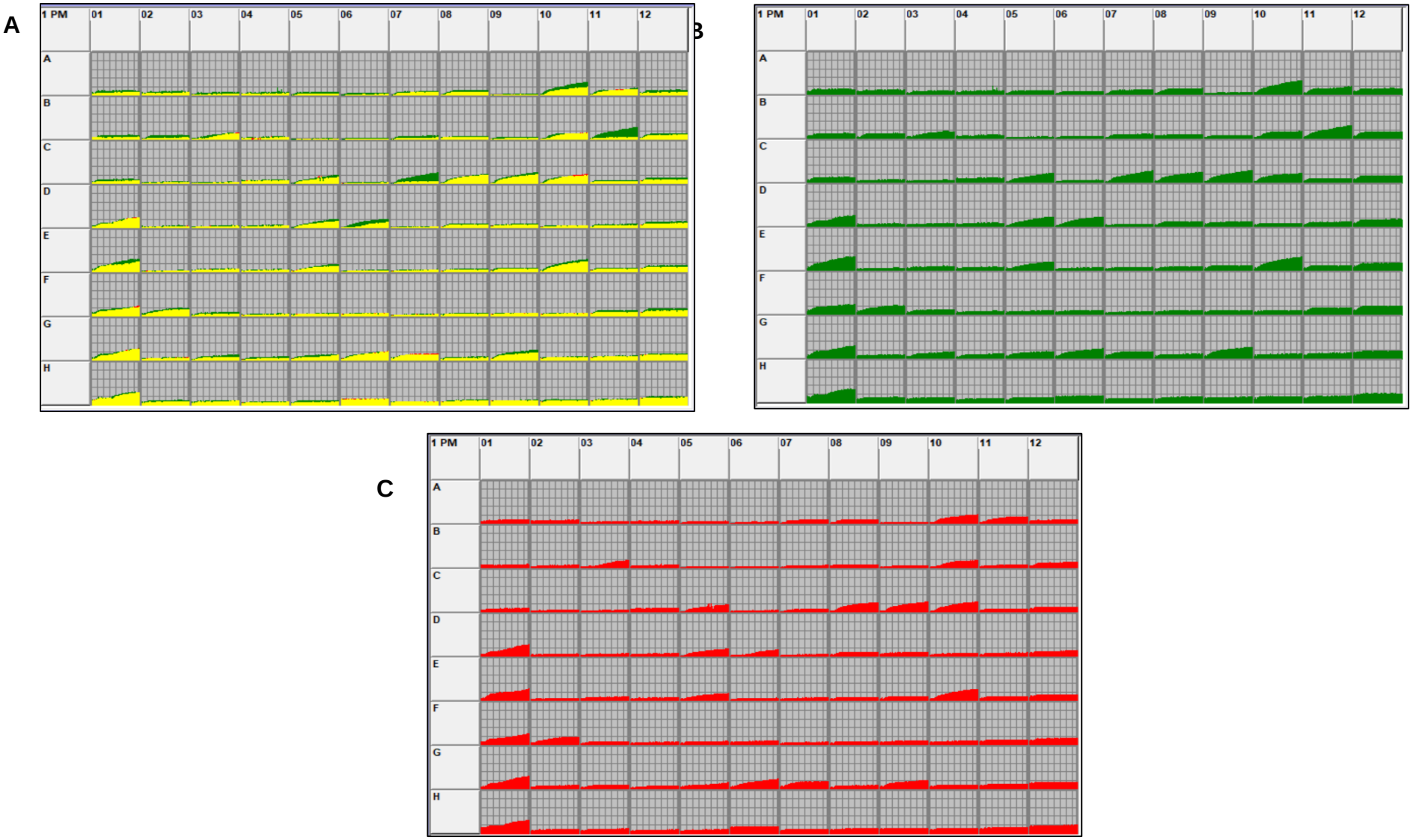


Figure 5.4. PM 01 showing the 96-well plates of A, strain 1_F178^{T} in comparison with *C. nakagawai*; B, strain 1_F178^{T} only and C, *C. nakagawai* only. Red indicates loss of phenotype; Green indicates gain of phenotype; Yellow indicates unchanged phenotype.

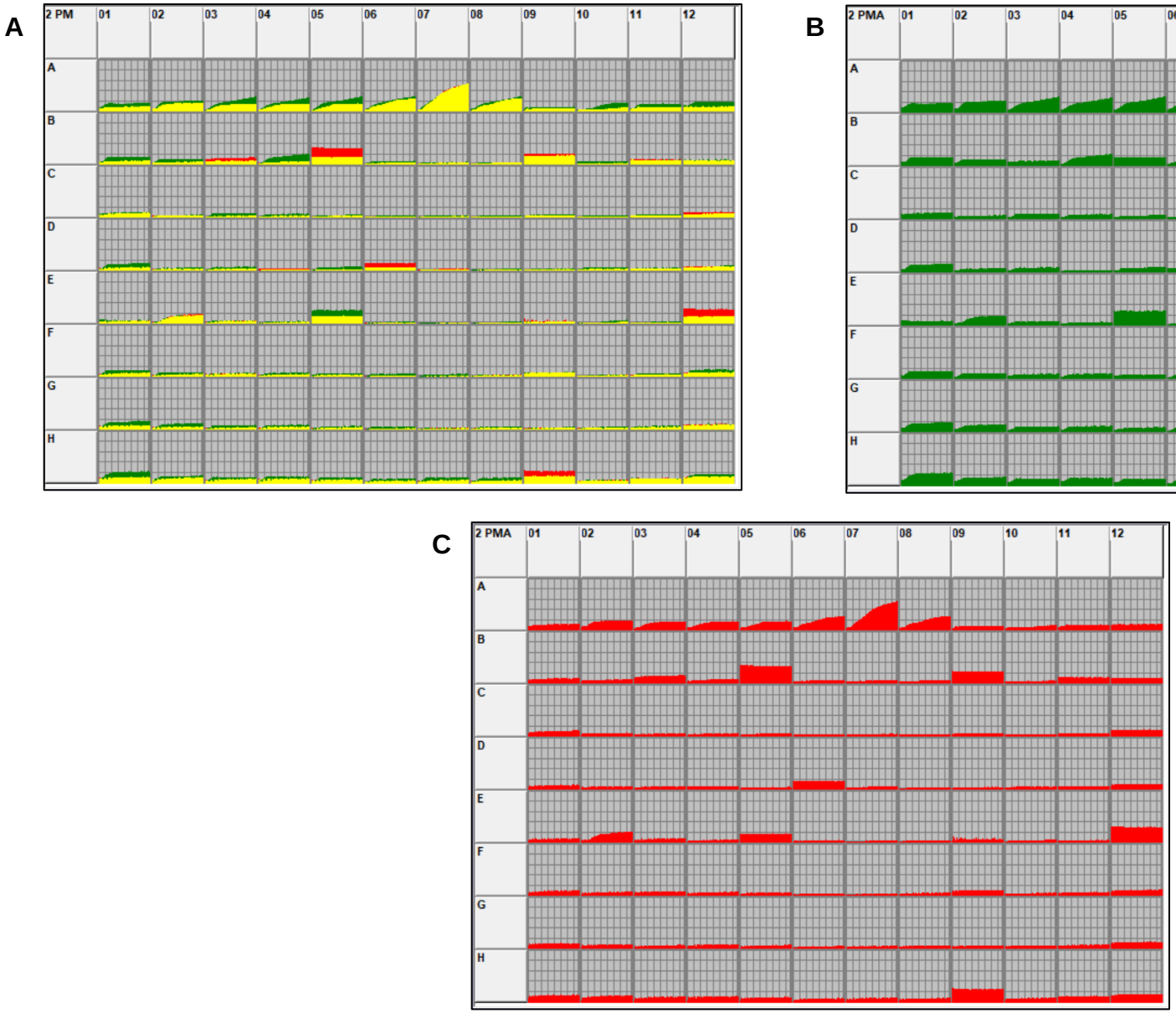


Figure 5.5. PM 01 showing the 96-well plates of A, strain 1_F178^{T} in comparison with *C. nakagawai*; B, strain 1_F178^{T} only and C, *C. nakagawai* only. Red indicates loss of phenotype; Green indicates gain of phenotype; Yellow indicates unchanged phenotype.

Table 5.1. Phenotypes gained (faster growth) by strain 1_F178^T compared to *C. jejuense*.

Plate type	Wells	Chemical	Importance
PM 01	C07	D-Fructose	C-source
PM 01	C08	Acetic acid	C-source
PM 01	C09	α-D-Glucose	C-source
PM 01	C10	Maltose	C-source
PM 01	D01	L-Asparagine	C-source
PM 01	D06	α-Keto-Glutaric acid	C-source
PM 01	E01	L-Glutamine	C-source
PM 01	A10	D-Trehalose	C-source
PM 01	A11	D-Mannose	C-source
PM 01	A08	L-Proline	C-source
PM 01	C05, D05, E05	Tween 20, 40, 80	C-source
PM 01	B03	Glycerol	C-source
PM 01	E10	Maltotriose	C-source
PM 01	E01	L-Glutamine	C-source
PM 01	F01	Gly-Asp	C-source
PM 01	F02	Citric acid	C-source
PM 01	G01	Gly-Glu	C-source
PM 01	G03	L-Serine	C-source
PM 01	G05	L-Alanine	C-source
PM 01	G06	L-Ala-Gly	C-source
PM 01	G09	Mono Methyl Succinate	C-source
PM 02	A03, A04, A05	α, β, ɣ-Cyclodextrin	C-source
PM 02	A06	Dextrin	C-source
PM 02	A07	Gelatin	C-source
PM 02	A08	Glycogen	C-source
PM 02	B04	Amygdalin	C-source
PM 02	D12	Caproic acid	C-source
PM 02	E10	α-Keto-Valeric acid	C-source
PM 02	G05	Glycine	C-source

Table 5.2. Phenotypes lost (slower growth) by strain 1_F178^T compared to *C. jejuense*.

Plate type	Wells	chemicals	Importance
PM 01	A07	L-Aspartic acid	C-source
PM 01	G04	L-Threonine	C-source
PM 02	G02	L-Alaninamide	C-source
PM 02	H02	L-Phenylalanine	C-source

Table 5.3. Phenotypes gained (faster growth) by strain 1_F178^T compared to *C. nakagawai*.

Plate type	Wells	chemicals	Importance
PM 01	A10	D-Trehalose	C-source
PM 01	A11	D-Mannose	C-source
PM 01	A08	L-Proline	C-source
PM 01	B10	Formic acid	C-source
PM 01	C05, D05, E05	Tween 20, 40, 80	C-source
PM 01	C09	α-D-Glucose	C-source
PM 01	C10	Maltose	C-source
PM 01	D01	L-Asparagine	C-source
PM 01	D06	α-Keto-glutaric acid	C-source
PM 01	E01	L-Glutamine	C-source
PM 01	E10	Maltotriose	C-source
PM 01	F01	Gly-Asp	C-source
PM 01	G01	Gly-Glu	C-source
PM 01	G03	L-Serine	C-source
PM 01	G05	L-Alanine	C-source
PM 01	G06	L-Ala-Gly	C-source
PM 01	G09	Mono methyl succinate	C-source
PM 01	H01	Gly-Pro	C-source
PM 02	A03, A04, A05	α-, β-, ɣ-Cyclodextrin	C-source
PM 02	A06	Dextrin	C-source
PM 02	A07	Gelatin	C-source
PM 02	A08	Glycogen	C-source
PM 02	D12	Caproic acid	C-source
PM 02	E10	α-Keto-Valeric acid	C-source

Table 5.4. Phenotypes lost (slower growth) by strain 1_F178^T compared to *C. nakagawai*.

Plate type	Wells	chemicals	Importance
PM 01	A07	L-Aspartic acid	C-source
PM 02	A10	Laminarin	C-source
PM 02	G02	L-Alaninamide	C-source
PM 02	H02	L-Phenylalanine	C-source

Table 5.5 indicates some of the chemicals *Chryseobacterium* strain 1_F178^T was resistant and sensitive to when compared to *C. jejuense*. Table 5.6 indicates only the

chemicals that strain 1_F178^T was resistant to when compared to *C. nakagawai*, since no prominent phenotypes were seen with regards to its sensitivity.

Chryseobacterium strain 1_F178^T was resistant to a number of antifungals like dodine, dichlofluanid, tolylfluanid, patulin and oxycarboxin which are mostly used against plant diseases (Tables 5.5 and 5.6).

Table 5.5. Resistance and sensitivity of strain 1_F178^T compared to *C. jejuense* to chemicals.

Plate type	Wells	Chemicals	Mode of action
		Resistance to chemicals	
PM14	B08	Fusaric acid	Chelator, lipophilic
PM16	C01	Dichlofluanid	Fungicide, phenylsulphamide
PM17	F11	Tannic acid	Antimicrobial, from plants
PM19	A05, A06	Gallic acid	Antimicrobial, from plants
PM 18	C09, C10, C11, C12	Pentachlorophenol	Fungicide
PM 14	D01, D02	Cadmium	Transport, toxic cation
		Sensitivity to chemicals	
PM15	B10	5,7-Dichloro-8-hydroxy-quinaldine	Chelator, lipophilic
PM16	A10, A11	5-Chloro-7-iodo-8-hydroxyquinoline	Chelator, lipophilic
PM20	A09, A11, A12	Benserazide	Fungicide
PM20	E07	Dodine	Fungicide, guanidine, membrane permeability
PM20	H05, H06, H07, H08	Tolylfluanid	Fungicide, phenylsulphamide

Table 5.6. Resistance of strain 1_F178^T compared to *C. nakagawai* to chemicals.

Plate type	Wells	Chemicals	Mode of action
		Resistance to chemicals	
PM20	H01, H02, H03, H04	Patulin	Antifungal, tubulin binding
PM20	A05, A06, A07, A08	Apramycin	Antimicrobial, aminocyclitol
PM17	F09, F10	Tannic acid	Antimicrobial, from plants
PM19	A07, A08	Gallic acid	Antimicrobial, from plants
PM14	H01, H02, H03	EGTA	Chelator, Ca++
PM15	C09	1,10-Phenanthroline	Chelator, Fe++, Zn++, divalent metal ions
PM15	B05, B06, B07	EDTA	Chelator, hydrophilic
PM14	B05, B06, B07, B08	Fusaric acid	Chelator, lipophilic
PM14	C05, C06, C07, C08	1-Hydroxy-pyridine-2-thione	Chelator, lipophilic
PM15	B09	5,7-Dichloro-8-hydroxy-quinaldine	Chelator, lipophilic
PM15	C01, C02, C03	5,7-Dichloro-8-hydroxyquinoline	Chelator, lipophilic
PM16	A09	5-Chloro-7-iodo-8-Hydroxyquinoline	Chelator, lipophilic
PM20	G09, G10, G11, G12	8-Hydroxyquinoline	Chelator, lipophilic
PM16	H05, H06, H07, H08	Chloroxylenol	Fungicide
PM19	D01, D02, D03, D04	Disulphiram	Fungicide
PM20	A10	Benserazide	Fungicide
PM17	D01, D02	Oxycarboxin	Fungicide, carboxamide, respiratory enzymes
PM20	E05, E06	Dodine	Fungicide, guanidine, membrane permeability

Plate type	Wells	Chemicals	Mode of action
		Resistance to chemicals	
PM16	C01	Dichlofluanid	Fungicide, phenylsulphamide

Chryseobacterium strain 1_F178^T showed resistance to 1, 10-phenanthroline and EDTA (Table 5.6) when compared to *C. nakagawai*. Venter and co-workers (1999) isolated a metalloprotease from *C. indologenes* Ix9a which was inhibited by the metal chelator, EDTA and the Zn^{2+}-specific chelator 1, 10-phenanthroline. A zinc metalloprotease was present in the genome of strain 1_F178^T (Chapter 3). The presence of the aforementioned inhibitors could also inhibit the production of this enzyme.

5.3.2. Antibiotic resistance/sensitivity

When compared to *C. jejuense, Chryseobacterium* strain 1_F178^T showed resistance to only two tetracycline antibiotics (doxycycline and rolitetracycline) (Fig. 5.6). It was mostly sensitive to β-lactams (penicillin, oxacillin, ampicillin and phenethicillin), macrolides (oleandomycin, josamycin and troleandomycin) and cefsulodin (Fig. 5.6).

Strain 1_F178^T was resistant to a number of cephalosporins (cefoxitin, cefotaxime, cefomandole, cetoperazone, cefsulodin, cefuroxime, moxalactam and cefmetazole), β-lactams (ampicillin, azlocillin, pipercillin, carbenicillin and phenethicillin), macrolides (oleandomycin, josamycin and troleandomycin), tetracyclines (rolitetracycline, doxycycline and oxytetracycline), aminoglycosides (puromycin and streptomycin), quinolones (norfloxacin and ciprofloxacin), glycopeptide (phleomycin) and sulfonamide (sulfamonomethoxine) when compared to *C. nakagawai* (Fig. 5.7). It only showed sensitivity to oxacillin, spiramycin and rifampicin.

Chryseobacterium indologenes and *C. gleum* were reported to produce carbapenem-hydrolysing β-lactamases, meaning they are resistant to extended-spectrum

cephalosporins and carbapenems. This may be the case of strain 1_F178^{T} as it showed a high resistance to a wide range of cephalosporins.

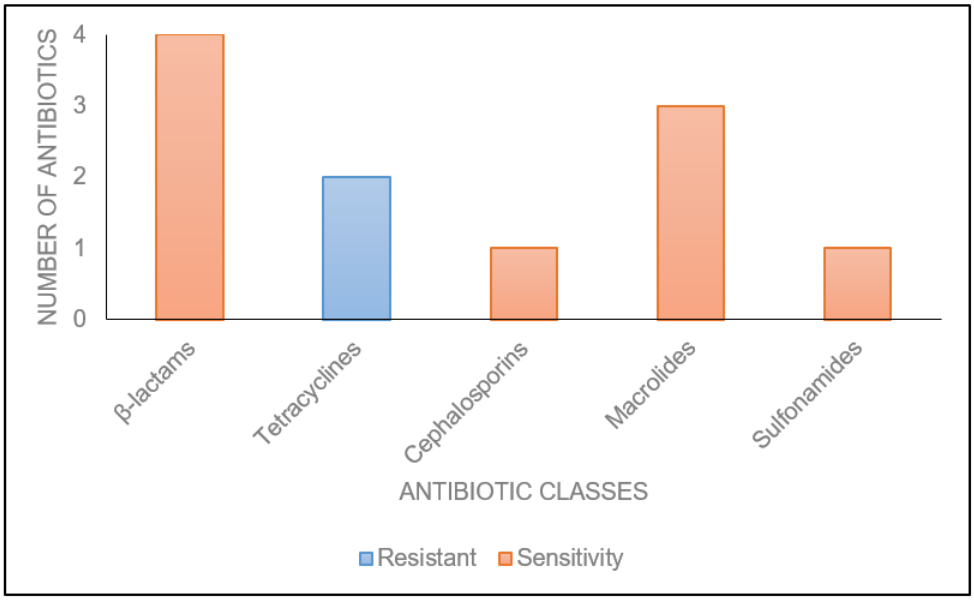


Fig 5.6 Antibiotics to which strain 1_F178^{T} was resistant and sensitive to when compared to *C. jejuense*.

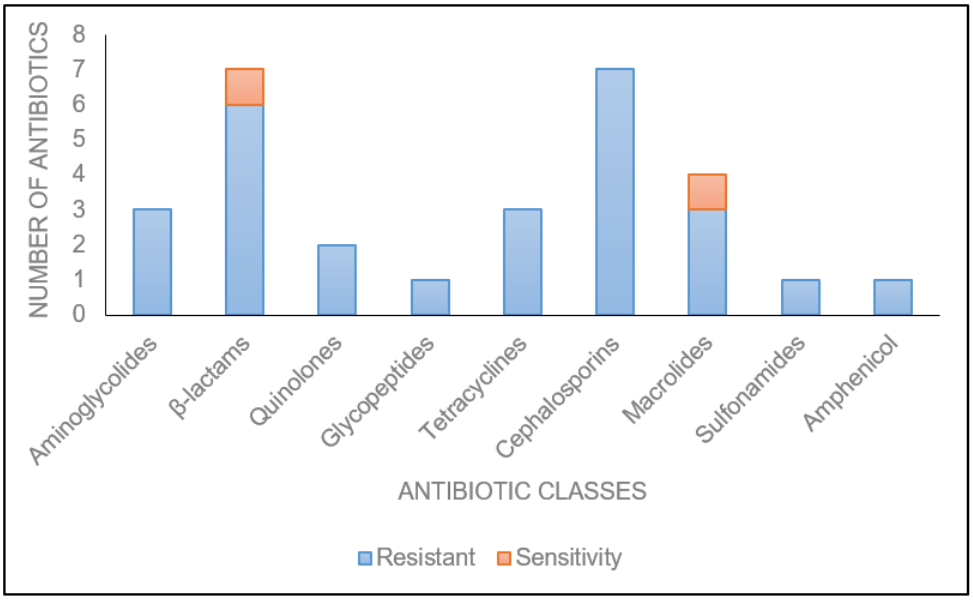


Fig 5.7 Antibiotics to which strain 1_F178^{T} was resistant and sensitive to when compared to *C. nakagawai*.

5.3.3. Potential food spoilage characteristics of substrate utilisation

A total of 70 carbohydrate substrates were tested with only 22.85%, 12.85% and 7.14% being oxidised by strain 1_F178^T, *C. jejuense* and *C. nakagawai,* respectively (Table 5.7). Strain 1_F178^T oxidised fatty acids as C-sources most (60.0%) followed by polymers (54.54%) and amino acids (40.0%).

Table 5.7. Classes of carbons oxidised by strain 1_F178^T and its closest relatives, *C. jejuense and C. nakagawai* (analysis of PM 01 and PM 02).

C-sources	Tested	F178^T		*C. jejuense*		*C. nakagawai*	
		Oxidised		Oxidised		Oxidised	
		Number	%	Number	%	Number	%
Carbohydrates	70	16	22.85	9	12.85	5	7.14
Amino acids	30	12	40.0	15	50.0	11	36.66
Carboxylic acids	60	7	11.66	6	10.0	5	8.33
Polymers	11	6	54.54	6	54.54	7	63.63
Alcohols	6	0	0	0	0	0	0
Amines	5	0	0	0	0	0	0
Amides	3	0	0	1	33.33	1	33.33
Fatty acids	5	3	60.0	3	60.0	3	60.0
Sum	190	44	23.15	40	21.05	32	16.84

No alcohols or amines were oxidised for all three organisms. The highest percentage of C-sources was oxidised by strain 1_F178^T (23.15%), hence indicating that this strain may have a higher spoilage potential when compared to its closest relatives.

Chryseobacterial species have been isolated from a variety of environmental sources (Jooste & Hugo, 1999). Spoilage defects due to flavobacteria/chryseobacteria have been reported in various products including butter (Wolochow *et al*., 1942; Jooste *et al*., 1986), creamed rice (Everton *et al*., 1968) and canned vegetables (Bean & Everton, 1969).

Chryseobacterium strain 1_F178^T oxidised simple carbohydrates like α-D-glucose, D-mannose, D-trehalose, gentiobiose, maltose, D-fructose and maltotriose (Fig. 5.8A). The oxidation of carbohydrates by bacteria results in the production of CO_2 and H_2O which do

not necessarily contribute to food spoilage. Food spoilage will only occur when there is a depletion in the availability of simple utilizable carbohydrates, hence resulting in off-odours in food (Dainty, 1996; Ellis & Goodacre, 2001). The physicochemical changes during spoilage usually occur in the aqueous phase of meat where low molecular weight compounds like glucose, lactic acid, nucleotides, certain amino acids, urea and water soluble proteins are found. These compounds are catabolized by the meat microflora. The random splitting of glycoside bonds result in textural defects due to softening and liquefaction (Chesson, 1980; Drosinos & Board, 1994; Nychas *et al*., 1998).

Chryseobacterium strain 1_F178^T showed the ability to oxidise polysaccharides like α-, β- and ɣ-cyclodextrins, dextrin and glycogen (Fig. 5.8C). During food spoilage, microorganisms usually hydrolyse complex di-, tri- or polysaccharides to simple sugars before utilisation. Polysaccharide oxidation produces metabolic products like CO_2, organic acids and alcohols which result in the production of off-odours, sourness, and bitter defects (Coultate, 1984; Banwart, 1989). The production of polysaccharides from various disaccharides present in food can form unpleasant slime in and on food.

Borch and coworkers (1991) observed that glucose limitation caused a switch from a saccharolytic to an amino acid-degrading metabolism in some species of bacteria. Nitrogenous compounds are usually the next best utilized substrates after carbohydrate depletion. Nitrogenous compounds lead to the formation of malodorous substances such as ammonia, dimethylsulphide and diacetyl. The production of these compounds, amongst others, lead to the characteristic changes associated with spoiled meat, such as malodours and alkalinization (Stanbridge & Davies, 1998).

Chryseobacterium strain 1_F178^T showed the ability to oxidise amino acids like glycyl-L-glutamic acid, L-alanine, L-alanyl-glycine, L-glutamine, L-isoleucine, L-serine, glycine and glycyl-L-proline (Fig. 5.7B). Amino acids are degraded to corresponding α-keto acids in bacteria. These α-keto acids are converted to various metabolites, such as aldehydes and sulphur compounds which may result in off-flavours (Gao *et al*., 1997). The incomplete metabolism of amino acids may result in putrescence, while the liberation of hydrogen sulphide from amino acids may result in a sulphide type of spoilage (Ayres *et al*., 1980). Decarboxylation of amino acids by *Chryseobacterium* strain 1_F178^T may

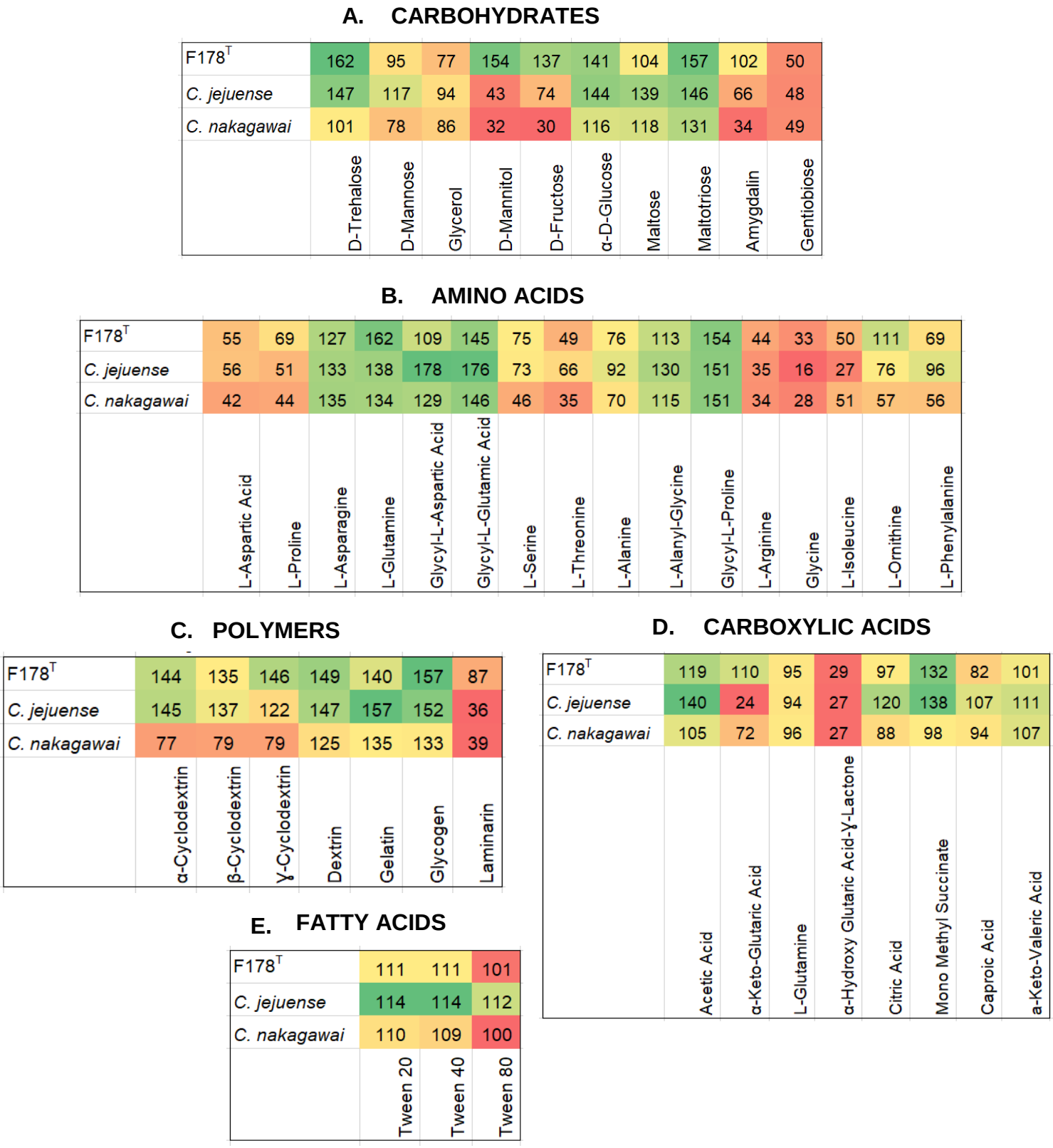


Fig. 5.8. Carbon sources (PM 01 and PM 02) oxidised by strain 1_F178^T and its closest relatives, *C. jejuense and C. nakagawai*. A, Carbohydrate oxidation; B, Amino acid oxidation; C, Oxidation of polymers; D, Carboxylic acid oxidation; E, Fatty acid oxidation. The response of each substrate is presented as a coloured scale (OmniLog units) ranging from a low response (red) to a high response (green). The values in the coloured blocks indicate the Omnilog Units.

result in the production of biogenic amines like cadaverine. Cadaverine is characterized by a foul smell, hence accounting for off-odours in spoiled food.

Chryseobacterium strain 1_F178^T oxidised carboxylic acids like α-keto valeric acid, α-keto-glutaric acid, acetic acid, caproic acid, formic acid and butyric acid (Fig. 5.8D). The metabolism of carboxylic acids by microorganisms may result in the coagulation of milk accompanied by a sour and bitter taste (Urbach, 1997). Butyric acid and ammonia impart obnoxious (rancid) odours in food (Banwart, 1989).

Chryseobacterium strain 1_F178^T showed positive results for Tween 20, 40 and 80 hydrolysis which serve as an indicator of lipase and other esterase activity (Harrigan & McCance, 1976) that can lead to spoilage of long-life dairy products during storage. Lipolysis by flavobacteria/chryseobacteria is known to break down fatty acids, resulting in rancid, butyric, bitter, soapy and astringent off-flavours and off-odours in some dairy products. Venter and co-workers (1999) reported the production of lipases and proteases by *Chryseobacterium indologenes.* The proteolytic activity of these enzymes led to the formation of short peptides which resulted in off-flavours and volume reduction in cheese, yogurt and other dairy products (Venter *et al*., 1999).

5.3.4. Potential applications

The pesticide, pentachlorophenol (PCP) is widely used in the agricultural sector. This comutagenic chemical has been found in the food chain and human exposure to this chemical poses serious health hazards. *Chryseobacterium* strain 1_F178^T showed resistance to this chemical (PM 18, well C09–C12; Table 5.5). Yu and Ward (1996) reported the use of *Chryseobacterium gleum* in bioremediation. A mixture of three strains (*Chryseobacterium gleum, Agrobacterium radiobacter* and *Pseudomonas* sp.) were used to degrade PCP, and *C. gleum* had the highest degradation rate when tested individually as compared to the other bacterial strains. The resistance of strain 1_F178^T to this chemical might mean it has the potential to be used in the biodegradation of PCP if it possesses the mechanism or enzymes for degradation. Strain 1_F178^T was also resistant to toxic chemicals like cadmium (PM 14, well D1 and D2; Table 5.5) which have been

found in heavily contaminated soils around the rhizospheres of plants colonizing mine tailings (Zhang *et al*., 2007).

Some strains of *Chryseobacterium* have been reported to offer protection to plants. They have been isolated from the rhizosphere of plants like cucumber, tomatoes and pepper where they inhibited the growth of pathogens and possessed plant growth-stimulating properties (Nishioka *et al*., 2016). *Chryseobacterium* strain 1_F178^T showed resistance to the fungicide, dodine (PM 20, well E05 and E06; Table 5.6). Dodine is used to control scabs on fruits like pears, apples and peaches, hence might not pose a threat to strain 1_F178^T, if it possesses plant growth-stimulating or plant-protective abilities.

5.4. Conclusions

Chryseobacterium strain 1_F178^T and its closest relatives, *C. jejuense* and *C nakagawai* were successfully subjected to PM analysis. So far, only Charimba (2012) and Chojniak and co-workers (2015) have reported the subjection of chryseobacterial strains to PM analysis. *Chryseobacterium* strain 1_F178^T differed more from *C. jejuense* (8.9%) when compared to *C. nakagawai* (5.1%) based on the phenotypes gained and lost, which explains why *C. jejuense* is more closely related to strain 1_F178^T than *C. nakagawai*.

Chryseobacterium strain 1_F178^T was able to oxidise a wide range of substrates ranging from fatty acids, carbohydrates, carboxylic acids, amino acids and polymers. Their oxidation and production of secondary metabolites could be linked to its spoilage potential, which includes the production of off-odours, off-flavours and bitterness in food products.

Most of the differentiating substrates fell in the antifungals, antibiotics and lipophilic chelator category. Strain 1_F178^T showed resistance to only two antibiotics when compared to *C. jejuense*, and 33 antibiotics when compared to *C. nakagawai*. Resistance to such a wide range of antibiotics may indicate that strain 1_F178^T possesses multidrug-resistance characteristics.

Chryseobacterial strains have been isolated from a number of clinical sources including hospital instruments. The resistance of strain 1_F178^T to a number of disinfectants and antibiotics means that controlling the growth of this bacterium might be problematic if found to be pathogenic.

Phenotype microarray technology therefore not only aids in bacterial delineation, but also may be used to identify possible applications in the food industry, agricultural sector as well as the medical environment.

CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

The genus *Chryseobacterium* has undergone several changes since its description. It is currently assigned to the family *Weeksellaceae* from its former family *Flavobacteriaceae* based on its inability to form a clade together with the type genus, *Flavobacterium* (García-López *et al*., 2019). The use of polyphasic taxonomy which incorporates phenotypic, genotypic and chemotaxonomic methods (Colwell, 1970), has been proven to be successful in bacteria delineation and several *Chryseobacterium* species have been described using this method.

The genus *Chryseobacterium* currently consists of 113 species (Parte, 2018). Members of this genus have been isolated from several sources ranging from water, soil, plants, animals, humans and food (Bernardet *et al*., 2002). Chryseobacteria are becoming significant in the food industry due to their ability to cause spoilage in poultry, meat, fish, milk and their products (Vandamme *et al*., 1994b; Bernardet *et al*., 2006).

In the first description of *Chryseobacterium* given by Vandamme *et al*. (1994) it was stated that members of the genus show strong proteolytic activity. This activity has been associated with their spoilage potential. Psychrotolerant flavobacteria produce lipolytic and proteolytic enzymes that are the main cause of spoilage in dairy products (Sørhaug & Stepaniak, 1997).

There is an increase in food spoilage in the food industry, and the use of predictive microbiology has been introduced to predict the behaviors of microorganisms under certain environmental conditions like temperature (Fakruddin *et al*., 2011). This can then be linked to the actual conditions of microbial growth, and used to address the issue of food spoilage.

Chryseobacterium species have a wide range of applications. They produce keratinolytic enzymes that degrade chicken feathers (Charimba, 2012). This provides an eco-friendly

and cost effective way to dispose chicken feathers produced by the poultry industry. They have been isolated from the rhizosphere of plants, where they were seen to offer protection against pathogens, with others possessing plant growth-stimulating properties. This makes them beneficial in both the agricultural and industrial sectors (Nishioka *et al*., 2016). Some species are able to degrade toxic compounds like flubendiamide, aniline and pentachlorophenol, hence they are applicable in the industrial sector (Yu & Ward, 1999; Radianingtyas *et al*., 2003; Jadhav & David, 2016).

The first aim of this study was to describe and classify two novel bacterial strains (1_F178^T and 5_R23647) isolated from poultry portions and feather waste respectively (Charimba, 2012) using a polyphasic approach. Phenotypic methods (both conventional and automated systems), chemotaxonomic (polar lipids, fatty acids and respiratory quinones) and genotypic methods (16S rRNA sequencing, whole genome sequencing, AAI, ANI and dDDH) were employed.

A comparison of the 16S rRNA gene sequences of the two organisms with the sequences of the type strains of the most closely related species of the genus *Chryseobacterium* showed the highest sequence similarities of strains 1_F178^T to *Chryseobacterium jejuense* (99.1%) and *Chryseobacterium nakagawai* (98.7%), and that of strain 5_R23647 to *Chryseobacterium piscium* (98.8%) and *Chryseobacterium balustinum* (98.6%). Strain 1_F178^T contained menaquinone MK-6 as the predominant respiratory quinone. The major fatty acids of the isolates were iso-$C_{15:0}$, iso-$C_{17:0}$ 3-OH and iso-$C_{17:1}$ *ω*9*c* which confirmed their affiliation to the genus *Chryseobacterium*. The polar lipid profile comprised of phosphatidylethanolamine and four aminolipids, two glycolipids and one lipid, which are presently uncharacterized.

The genome of both strains were sequenced and assembled with strain 1_F178^T and 5_R23647 having genome sizes of 6.2 Mbp (which is quite large for a typical *Chryseobacterium* species) and 4.7 Mbp and G+C contents of 35.6 and 33.5 mol%, respectively. Overall genome similarity metrics (ANI, AAI and dDDH) revealed greatest similarity of strain 1_F178^T to *Chryseobacterium jejuense* (86.4%, 89.3% 31.4%) and *Chryseobacterium nakagawai* (86.6%, 89.6%, 32.7%), and that of strain 5_R23647 to *Chryseobacterium piscium* (96.3%, 96.7%, 68.3%) and *Chryseobacterium balustinum*

(93.0%, 92.9%, 51.7%), hence disqualifying strain 5_R23647 as a new species since its ANI and AAI values were above the threshold value (95%). Based on evidence presented in this study, strain 1_F178^T is considered to represent a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium pennae* sp. nov. was proposed.

The second aim of this study was to conduct temperature-growth studies in an attempt to determine the growth kinetics of the novel *Chryseobacterium* species (1_F178^T) in comparison with *Chryseobacterium carnipullorum* and the renowned food spoilage microbe, *Pseudomonas fluorescens*. The Arrhenius, Huang and Ratkowsky models were used to determine the relationships between the kinetic parameters and the temperature as an environmental factor.

The results showed strain 1_F178^T having the highest μ_{max} of 0.64 h^{-1} at an optimum temperature of 34.7 °C followed by *P. fluorescens* with a μ_{max} of 0.60 h^{-1} at an optimum temperature of 30.5 °C. Growth was observed at 4 °C for all strains indicative of growth at refrigeration temperature with possibly food spoilage.

The growth of *Chryseobacterium* over wide temperature ranges indicated their ability to cause food spoilage over wide temperature ranges. *Chryseobacterium carnipullorum* had the highest activation energy (81.62 kJ·mol^{-1}) between 12.3 – 19.8 °C, implying a high sensitivity to change in temperature in this region. The lowest activation energy was obtained for *P. fluorescens* (52.52 kJ·mol^{-1}) between 14.3 – 23.4 °C indicative of this microorganism being less sensitive to changes in temperature in this region, meaning it has the ability to grow over wider temperature ranges, and will therefore have a higher spoilage potential than the chryseobacterial strains in this study and as confirmed in literature.

The high growth rates of strain 1_F178^T, and its ability to grow at temperatures as low as 4 °C is disadvantageous to the food industry, since this organism has a high spoilage potential and will cause spoilage in refrigerated food products.

Predictions to determine the minimum and maximum temperatures for growth using the Huang and Ratkowsky models for the four strains showed the Huang model to be a more effective model as the minimum temperature values of growth were closer to the

biological minimum values as determined by the temperature growth studies. Therefore, the Huang model will be a better model to be used in the insudtry for the prediction of possible spoilage temperatures of these organisms.

The third aim of this study was to subject *Chryseobacterium* strain 1_F178^T to the Biolog Omnilog Combo System PM analysis and compare it to its closest relatives, *C. jejuense* DSM 19299^T and *C. nakagawai* NCTC 13529^T to get an indication of potential food spoilage characteristics and potential applications of *Chryseobacterium* strain 1_F178^T.

Strain 1_F178^T differed from *C. jejuense* by 5.1% and from *C. nakagawai* by 8.9% based on the phenotypes gained and lost.

Chryseobacterium strain 1_F178^T was able to oxidise a wide range of substrates ranging from carbohydrates, carboxylic acids, amino acids and polymers. The utilisation of these substrates and production of secondary metabolites could be linked to its spoilage potential, which include the production of off-odours, off-flavours and bitterness in food products, hence being disadvantageous for the food industry.

Most of the differentiating substrates fell in the antifungals, antibiotics and lipophilic chelator category. Strain 1_F178^T showed resistance to only two antibiotics when compared to *C. jejuense*, and 33 antibiotics when compared to *C. nakagawai*. Resistance to such a wide range of antibiotics may indicate that strain 1_F178^T possesses multidrug-resistance characteristics. This is disadvantageous for the medicine or food industries with respect to the control of diseases associated with the presence of chryseobacterial species.

Chryseobacterium strain 1_F178^T was able to degrade the toxic chemical, pentachlorophenol, usually used as a pesticide, insecticide, herbicide and algicide in the agricultural sector. This is advantageous to this sector as it provides an eco-friendly alternative for toxic degradation.

Chryseobacterium strain 1_F178^T contained flexirubins and Zn-metalloproteases which have industrial applications. Flexirubin pigments display a range of pharmacological effects such as antimicrobial and anticancer activities. They also represent safe and

biodegradable natural colourants with potential as substitutes for synthetic colourants (Venil *et al*., 2014b).

The presence of Zn-metalloprotease in the genome of *Chryseobacterium* strain 1_F178^T shows the potential of this bacterium to degrade chicken feathers. Hence, it can be used as an eco-friendly alternative in chicken feather disposal in the poultry industry.

Future research

i. Based on genotypic studies, the presence of some metalloproteases were present in *Chryseobacterium* strain 1_F178^T. This strain can therefore be investigated for its ability to produce keratinolytic enzymes.
ii. The genome of *Chryseobacterium* strain 1_F178^T can be further investigated for the presence of food spoilage associated genes and the presence of unique genes and their functions.
iii. Other environmental parameters like water activity, pH, humidity, nutrient composition and oxygen can be investigated to determine the spoilage potential of *Chryseobacterium* strain 1_F178^T.

CHAPTER 7

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CHAPTER 8

SUMMARY

Chryseobacterial strains have been isolated from sources like soil, beer bottles, plants and hospital equipment. Other sources include poultry, meat, fish, milk, creamed rice and canned vegetables where they have been reported to cause food spoilage. They have a wide range of applications ranging from the production of keratinases for the degradation of chicken feather keratin, to the degradation of toxic environmental compounds like flubendiamide. They have also been reported to offer protection to plants where some protect plants against pathogens and possess plant growth-stimulating properties.

In the first section of this study, a polyphasic approach was used to describe two novel strains of *Chryseobacterium* (1_F178^T and 5_R23647). 16S rRNA sequencing was done and phylogenetic analysis confirmed *Chryseobacterium jejuense* and *Chryseobacterium nakagawai* as the closest relatives of strain 1_F178^T, and *Chryseobacterium piscium* and *Chryseobacterium balustinum* as the closest relatives of strain 5_R23647. Phenotypic, chemotaxonomic and genotypic methods confirmed the affiliation of both strains to the genus *Chryseobacterium*, and only confirmed strain 1_F178^T as a novel species of *Chryseobacterium*, to which the name *Chryseobacterium pennae* was proposed.

The growth kinetics of *Chryseobacterium* strain 1_F178^T, 5_R23647, *Chryseobacterium carnipullorum* and the spoilage specific microorganism, *Pseudomonas fluorescens* were investigated in the second section of this study, to determine their spoilage potential. *Chryseobacterium* strain 1_F178^T had the highest specific growth rate which indicated a higher spoilage potential. Its growth at temperatures as low as 4 °C was indicative of growth at refrigeration with possibly food spoilage. *Chryseobacterium carnipullorum* had the highest activation energy implying it has a high sensitivity to change in temperature. The lowest activation energy was obtained for *P. fluorescens* indicative of this microorganism being less sensitive to changes in temperature, hence can grow over wider temperature ranges, and will therefore have a higher spoilage potential than the

chryseobacterial strains in this study and as confirmed in literature. The Huang model was seen as a more effective model in predicting the minimum and maximum temperatures for growth.

Analysis using the Biolog^{TM} Phenotype MicroArray (PM) system showed the ability of *Chryseobacterium* strain 1_F178^{T} to oxidise a wide range of substrates ranging from fatty acids, carbohydrates, carboxylic acids, amino acids and polymers, which is linked to its spoilage potential. Strain 1_F178^{T} was able to oxidise a number of fungicides which showed potential of this organism for agricultural applications.

Keywords: *Chryseobacterium*, food spoilage, strain 1_F178^{T}, strain 5_R23647, polyphasic approach, taxonomy, whole genome sequencing, growth kinetics, phenotype microarray, application, predictive microbiology

Annexure

Validly named *Chryseobacterium* species isolated from different sources (Parte, 2018).

Species	G+C (mol%)	Source	References
C. aahli	34.1	Lake trout (*Salvelinus namaycash*)	Loch & Faisal, 2014
C. angstadtii	38.1	Newt tank	Kirk *et al*., 2013
[b] *C. antarcticum*	34.0	Antarctic soil	Yi *et al*., 2005; Kämpfer *et al*., 2009a
[b] *C. anthropi*		Human clinical specimens	Kämpfer *et al*., 2009c
C. aquaticum	38.3-38.5	Water reservoir	Kim *et al*., 2008
C. aquifrigidense	35.6	Water-cooling system	Park *et al*., 2008
[a] *C. arachidiradicis*	Not determined	Soil around peanut (*Arachishypogaea*)	Kämpfer *et al*., 2015a
C. arachidis	Not determined	Rhizosphere environment	Kämpfer *et al*., 2014a
C. arothri	36.5	Pufferfish *Arothron hispidus*	Campbell *et al*., 2008
C. arthrosphaerae	Not determined	Faeces of the pill millipede *Arthrosphaera magna* Attems	Kämpfer *et al*., 2010
C. artocarpi	34.8	Rhizosphere soil of *Artocarpus integer*	Venil*et al*., 2014
C. aurantiacum	36.3	Fresh water of a diseased Murray cod	Lou *et al*., 2018
C. balustinum	33.0	Heart blood of fresh water fish (dace, *Leuciscus*	Holmes *et al*., 1984a

Species	G+C (mol%)	Source	References
		leuciscus)	
C. bernardetii	37.0	Sputum	Holmes *et al*., 2013
[a] *C. bovis*	38.6	Raw cow's milk	Hantsis-*Zacharov et al.*, 2008b
[a] *C. caeni*	38.2	Bioreactor sludge	Quan*et al*., 2007
C. camelliae	41.7	Green tea leaves	Kook *et al*., 2014
C. carnipullorum	36.6, 36.7 & 36.9	Raw chicken portion	Charimba *et al*., 2013
[b] *C. carnis*	34.0	Beef	Holmes *et al*., 2013
[b] *C. chaponense*	Not determined	Atlantic salmon (*Salmo salar*)	Kämpfer *et al*., 2011
C. contaminans	Not determined	As a contaminant from an agar plate of a rhizosphere sample	Kämpfer *et al*., 2014b
C. cucumeris	36.1	Cucumber (*Cucumis sativus* L.) root	Jeong *et al*., 2017
C. culicis	Not determined	Midgut of the mosquito *Culex quinquefasciatus*	Kämpfer *et al*., 2010a
C. daecheongense	37.0	Lake Daecheong sediment	Kim *et al*., 2005b
C. daeguense	36.8	Wastewater of a textile dye works	Yoon *et al*., 2007
C. defluvii	Not determined	Activated sludge	Kämpfer *et al*., 2003
C. echinoideorum	36.4	Edible sea urchin	Lin *et al*., 2015
C. elymi	36.9	Rhizosphere of coastal sand dune plants	Cho *et al*., 2011

Species	G+C (mol%)	Source	References
C. endophyticum	37.2	Surface-sterilized maize leaf	Lin *et al*., 2017
C. flavum	37.2	Polluted soil	Zhou *et al*., 2007
C. formosense	Not determined	Rhizosphere of lettuce	Young *et al*., 2005
C. frigidisoli	33.7	Soil sample from a glacier forefield	Bajerski *et al*., 2013
C. frigidum	49.3	High-arctic tundra soil	Kim *et al*., 2016
C. gallinarum	Not determined	Chicken	Kämpfer *et al*., 2014
C. gambrini	37.8	Beer-bottling plant	Herzog *et al*., 2008
C. geocarposphaerae	Not determined	Environmental soil	Kämpfer *et al*., 2014
C. ginsengisoli	31.6	Ginseng roots	Nguyen *et al*., 2013
C. gingsengiterrae	37.9	Soil of a ginseng field	Hahnke *et al*., 2016
C. ginsenosidimutans	35.7	Soil of a *Rhus vernicifera*-cultivated field	Im*et al*., 2011
C. glaciei	34.0	Glacier surface	Pal *et al*., 2018
C. gleum	37.0	Human vaginal swab	Holmes *et al*., 1984b
C. greenlandense	39.6-41.6	Deep Greenland ice core	Loveland-Curtze *et al*., 2010
C. gregarium	38.4	Decaying plant material	Behrendt *et al*., 2008
C. gwangjuense	36.4	Soil	Park *et al*., 2013

Species	G+C (mol%)	Source	References
C. hagamense	Not determined	Rhizosphere of coastal sand dune plants	Cho *et al*., 2011
[b] *C. haifense*	37.8	Raw milk	Hantsis-Zacharov & Halpern, 2007b
C. halperniae	38.0	Raw cow milk	Shakéd *et al*., 2010
C. hispalense	37.2	Rain water pond	Montero-calasanz *et al*., 2013
[a] *C. hispanicum*	34.3	Drinking water distribution system	Gallego *et al*., 2006
[a] *C. hominis*	36.5	Clinical isolate	Vaneechoutte *et al*., 2007
C. humi	34.0	Industrially contaminated sediments	Pires *et al*., 2010
[a] *C. hungaricum*	37.5	Hydrocarbon-contaminated soil	Szoboszlay *et al*., 2008
C. indologenes	38.0	Human trachea at autopsy	Yabuuchi *et al*., 1983
C. indoltheticum	34.0	Marine mud	Bernardet *et al*., 2006
C. jejuense	41.4	Soil	Weon *et al*., 2008
[b] *C. jeonii*	36.0	Antarctic moss	Yi *et al*., 2005; Kämpfer *et al*., 2009a
C. joostei	37.0	Raw cow's milk	Hugo *et al*., 2003
C. koreense	Not determined	Human clinical specimens	Kämpfer *et al*., 2009b
C. kwangjuense	40.2	Root of a pepper plant	Sang *et al*., 2013

Species	G+C (mol%)	Source	References
C. lactis	34.5	Milk bottle	Holmes *et al*., 2013
C. lathyri	36.6	Rhizosphere of coastal sand dune plants	Cho *et al*., 2011
C. limigenitum	Not determined	Dehydrated sludge	Kämpfer *et al*., 2015d
C. lineare	29.7	Limpid stream	Zhao *et al*., 2017
C. luteum	Not determined	Phyllosphere of grasses	Behrendt *et al*., 2007
C. marinum	35.0	Antarctic seawater	Lee *et al*., 2007;Kämpfer *et al*., 2009a
C. meningosepticum	Not determined	Clinical isolates	Vandamme *et al.*, 1994a
C miricola	34.6	Condensation water of space station Mir	Li *et al*., 2003
[a] *C. molle*	39.2	Beer-bottling plant	Herzog *et al*., 2008
[b] *C. montanum*	37.7	Mountain soil	Li *et al*., 2016
C. nakagawai	35.0	Kidney abscess	Holmes *et al*., 2013
C. nepalense	38.6	Oil-contaminated soil	Chaudhary & Kim, 2017
C. oleae	38.2	Plant (ectorhizosphere of an organic olive tree	Montero-Calasanz *et al*., 2014
C. oncorhynchi	36.3	Gill & liver of rainbow trout	Zamora *et al*., 2012
C. oranimense	Not determined	Raw cow's milk	Hantsis-Zacharov *et al*., 2008a

Species	G+C (mol%)	Source	References
[a] *C. pallidum*	38.1	Beer-bottling plant	Herzog *et al*., 2008
[b] *C. palustre*	43.0	Industrially contaminated sediments	Pires *et al*., 2010
C. piperi	38.6	Freshwater creek	Strahan *et al*., 2011
C. pennipullorum	38.6	Chicken feather waste	Oosthuizen *et al*., 2019
C. populi	36.6	Rhizosphere and endosphere of cottonwood tree	Bortniak *et al*., 2019
C. piscicola	32.3-32.5	Diseased salmonid fish	Ilardi *et al*., 2009
C. piscium	33.6	Fish	de Beer *et al*., 2006
C. polytrichastri	37.3	*Polytrichastrum formosum* obtained from Gawalong glacier	Chen *et al*., 2015
C. profundimaris	40.7	Atlantic ocean sediment	Xu *et al*., 2015
C. psychrotolerans	34.4	Alpine permafrost	Ge *et al*., 2015
C. proteolyticum	37.0	Soil, rice field	Yamaguchi & Yokoe, 2000
C. reticulitermitis	39.9	Gut of a wood-feeding lower termite *Reticulitermes aculabialis*	Zhao *et al*., 2017
C. rhizoplanae	Not determined	Rhizoplane of a field-grown *Zea mays* plant	Kämpfer *et al*., 2015b
C. rhizosphaerae	35.9	Rhizosphere of coastal sand dune plants	Cho *et al*., 2011

Species	G+C (mol%)	Source	References
C. rigui	37.9	Estuarine wetland of the Han River	Park *et al*., 2013
C. salipaludis	34.0	Sediment sample obtained from a wild ass sanctuary	Divyasree *et al*., 2018
C. scophthalmum	34.0	Gills of diseased turbot (*Scophthalmus maximus*)	Mudarris *et al*., 1994
C. sediminis	Not determined	River sediment	Kämpfer *et al*., 2015c
C. shandongense	37.0	Soil	Yang *et al*., 2015
C. shigense	36.6	Lactic acid beverage	Shimomura *et al*., 2005
C. solani	35.3	Field-grown eggplant rhizosphere soil	Du *et al*., 2015
C. soldanellicola	28.8	Roots of sand-dune plants	Park *et al*., 2006
C. soli	39.9 and 41.4	Soil samples	Weon *et al*., 2008
[b] *C. solincola*	40.9	Soil	Benmalek *et al*., 2010
C. taeanense	32.1	Roots of sand-dune plants	Park *et al*., 2006b
C. taichungense	Not determined	Tar-contaminated soil	Shen *et al*., 2005
C. taihuense	36.8	Algal bloom	Wu *et al*., 2013
C. taiwanense	36.8	Soil	Tai *et al*., 2006

Species	G+C (mol%)	Source	References
C. takakiae	37.3	*Takakia lepidozioides* collected from Gawalong glacier	Zhao *et al*., 2015
C. taklimakanense	41.5	Desert soil	Kim *et al*., 2016
C. tenax	37.5	River epilithon	O'Sullivan *et al*., 2006)
[b] *C. treverense*	Not determined	Human blood	Yassin *et al*., 2010
C. tructae	33.6- 36.1	Diseased rainbow trout	Zamora *et al*., 2012
C. ureilyticum	36.4	Beer-bottling plant	Herzog *et al*., 2008
C. vietnamense	42.1	Forest soil sample	Li & Zhu, 2012
C. viscerum	38.6	Gills & livers of diseased rainbow trout	Zamora *et al*., 2012
C. vrystaatense	37.1	Chicken-processing plant	de Beer *et al*., 2005
C. wanjuense	37.8	Greenhouse soil	Weon *et al*., 2006
C. xinjiangense	33.5	Soil	Zhao *et al*., 2011
C. xixisoli	33.3	Bank-side soil of the Xixi wetland	Feng *et al*., 2014
[b] *C. yonginense*	31.3	Mesotrophic artificial lake	Joung & Joh, 2011
[a] *C. zeae*	ND	Rhizosphere environment	Kämpfer *et al*., 2014a

a Transferred to the genus *Epilithonimonas*

b Transferred to the genus *Kaistella*