

**TAXONOMY, GROWTH AND FOOD SPOILAGE CHARACTERISTICS
OF A NOVEL *Chryseobacterium* SPECIES**

By

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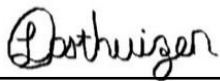
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DECLARATION

I declare that the dissertation hereby submitted by me for the M. Sc. Degree in the Faculty of Natural and Agricultural Science at the University of the Free State is my own independent work and has not previously been submitted by me at another university/faculty. I furthermore cede copyright of the dissertation in favour of the University of the Free State.



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November, 2018

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
A	Absorbance
A	Entropy constant
AAI	Amino Acid Identity
ANI	Average Nucleotide Identity
APC	Aerobic Plate Counts
API	Analytical Profile Index
ATCC	American Type Culture Collection, Rockville, Maryland
a_w	Water activity
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
<i>C.</i>	<i>Chryseobacterium</i>
CDS	Coding sequences
CFU	Colony Forming Units
DDBJ	DNA Data Bank of Japan
dDDH	digital DNA-DNA hybridization
DDH	DNA-DNA hybridization
DMC	Direct Microscopic Counts
DSM	Deutsche Sammlung von Mikro-organismen
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
E	Activation energy/ Temperature coefficient
<i>E.</i>	<i>Elizabethkingia</i>

e.g.	For example
ECL	Equivalent Chain Length
Eh	Oxidation-reduction potential
EMBL-EBI	European Bioinformatics Institute
<i>et al.</i>	<i>(et alii)</i> and others
etc.	<i>Et cetera</i>
g	Gram
G+C	Guanine and Cytosine
GC	Gas chromatography
GGDC	Genome-Genome Distance Calculator
GOLD	Genomes Online Database
h	Hour(s)
h⁻¹	Per hour
H₂S	Hydrogen sulphide
HACCP	Hazard Analysis Critical Control Point
HCl	Hydrochloric acid
HPLC	High-Performance Liquid Chromatography
IF	Inoculating fluid
KCTC	Korean Collection of Type Cultures
KOH	Potassium hydroxide
KP2	Kimura two
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
ml	Millilitre
mm	Millimetre
mol%	Mole percentage
MPN	Most Probable Numbers
mRNA	Messenger RNA
NA	Nutrient agar
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
NCTC	National Collection of Type Cultures
NGS	Next-Generation Sequencing
nm	Nanometer
NNI	Nearest neighbour interchange
O/R	Oxidation-reduction potential
OD	Optical Density
OGRI	Overall Genome Related Index
ONPG	O-nitrophenyl-beta-D-galactopyranoside
PacBio	Pacific Biosciences
PCR	Polymerase Chain Reaction
PE	Phosphatidylethanolamine
R	Universal gas constant

RAST	Rapid Annotation with Subsystems Technology
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
SEM	Scanning Electron Microscope
SPC	Standard Plate Counts
Spp.	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
UHT	Ultra Heat-Treated
UPGMA	Unweighted Pairgroup Method
ΔT_m	Melting temperature
μm	Micrometer
μ_{max}	Maximum growth rate

CHAPTER 1

INTRODUCTION

Some species of *Chryseobacterium*, a genus of the family *Flavobacteriaceae*, were originally classified as members of the genus *Flavobacterium*, but were later reclassified through a polyphasic study (Vandamme *et al.*, 1994a). *Chryseobacterium* has been isolated from environments ranging from terrestrial, aquatic, diseased animals, humans and food (Bernardet *et al.*, 2002) and some species may play a significant role in food spoilage (Bekker *et al.*, 2016). Characteristics of food spoilage caused by *Chryseobacterium* species have been studied less extensively than this genus's taxonomy and nomenclature.

The spoilage of food is defined as any change in a product that will make the product unacceptable for humans to consume (Hayes, 1985). Spoilage can be caused by chemical damage (oxidation and colour changes), insect damage, physical damage (bruising, pressure, freezing, drying and radiation) and growth/metabolism of microorganisms that cause off-odours and off-flavours (Gram *et al.*, 2002). Gram and co-workers (2002) termed the ability of a pure culture to produce the metabolites that are associated with spoilage as the potential of an organism to grow and spoil food.

Studies on flavobacteria and chryseobacteria in the Food Science Department of the University of the Free State have been ongoing since the 1980's. These organisms have been investigated in milk and butter (Jooste *et al.*, 1985, 1986a, 1986b; Welthagen & Jooste, 1992; Hugo *et al.*, 2003); fish (de Beer *et al.*, 2006); fresh beef (Hugo & Jooste, 2012) and chicken meat (de Beer, 2005; de Beer *et al.*, 2005). In the most recent study by Charimba (2012), *Chryseobacterium* strains from raw poultry portions and poultry feather waste have been isolated and some of these isolates belonged to a new species, *C. carnipullorum* (Charimba *et al.*, 2013). The other isolates from this study remain to be classified and described by taxonomic studies. The significance of these isolates in terms of food spoilage should also be investigated.

Taxonomic information helps scientists understand the relationship between living organisms and the biodiversity in different environments, it is therefore essential to investigate (Gevers *et al.*, 2005). Polyphasic taxonomy uses a combination of phenotypic, chemotypic and genotypic methods to describe a microorganism (Prakash *et al.*, 2007). Phenotypic methods in the past were the cornerstone of bacterial taxonomy, but today DNA and RNA relatedness are more reproducible, easy to use and have high discriminatory power and are, therefore, more commonly used. Chemotaxonomic methods are used to characterize bacteria. These methods will be further discussed in Chapter 2.

In microbiology, kinetics includes growth, survival, death, mutation, adaptation, formation, cell cycles, biological interactions and environmental effects (Panikov, 1991). Predictive modelling is used for the development of mathematical equations to describe the behaviour of microorganisms under different environmental conditions (Fakruddin *et al.*, 2011). The conditions can be intrinsic, e.g., pH, or extrinsic, e.g., salinity and temperature. The results are expressed as an equation that can be used to predict a combination of conditions that have not yet been tested (Hajmeer & Cliver, 2002). Combining the potential of a microorganism to spoil food and e.g., the food product's temperature history provides optimization of food quality through the development of management systems (Giannakourou *et al.*, 2001; Koutsoumanis *et al.*, 2002; Koutsoumanis *et al.*, 2003). This will increase the safety of food when consumed. Kinetic models can lead to a better understanding of food and food spoilage at molecular level and at microbial level (Van Boekel, 2008).

Chapter 2 aims to investigate the literature on the history, characteristics and significance of *Chryseobacterium* species in food spoilage. *Chryseobacterium* species are regarded as food spoilage organisms due to their proteolytic characteristics (Charimba, 2012; Bekker *et al.*, 2015). This chapter will focus on literature describing a novel *Chryseobacterium* species through polyphasic taxonomy that will include genotypic, phenotypic and chemotypic methods. Attention will also be placed on the growth phases of a microorganism, methods for measuring growth and factors influencing microbial growth. Different growth kinetic models will be investigated especially using the Arrhenius equation to determine the effect of temperature on microbial growth.

Chapter 3 will focus on describing a novel *Chryseobacterium* species isolated from poultry feather waste in a previous study (Charimba, 2012). This chapter will especially focus on the following: genotypic methods; phylogenetic treeing methods; microscopy; phenotypic tests using conventional and automated methods and chemotaxonomic methods.

Chapter 4 will focus on growth kinetics, determined through kinetic modelling, of the novel *Chryseobacterium* species isolated from poultry feather waste (Charimba, 2012) in comparison to *C. carnipullorum* that was isolated from a raw chicken portion (Charimba *et al.*, 2013) and *C. vrystaatense* (de Beer *et al.*, 2005) that was isolated from chicken portions from a poultry abattoir in South Africa.

Chapter 5 will conclude with general discussions and conclusions.

Purpose and objectives of this study

Purpose:

In a previous study (Charimba, 2012) in the Food Science Department at the University of the Free State, several bacterial isolates from chicken feather waste were classified as belonging to the *Chryseobacterium* genus, but these isolates could not be identified to species level. The question was also whether these isolates from the feathers could spoil the poultry meat.

In order to determine the spoilage potential of the unidentified and novel *Chryseobacterium* isolates, polyphasic taxonomic studies need to be performed in order to describe and name the novel species. The novel species will then be used to determine their spoilage potential by determining the kinetic growth profile of the novel species and compare it to the growth profiles of other *Chryseobacterium* species that were isolated from poultry.

This study will provide valuable information about the effect *Chryseobacterium* may have on the quality of food products. Understanding the growth patterns and knowing the spoilage potential of *Chryseobacterium* will help prevent and control food spoilage due to these organisms. Knowledge about the role of

Chryseobacterium species in the ecology of food spoilage will be improved in this study.

Objectives:

- I. Literature review
- II. Describing a novel *Chryseobacterium* species that was isolated from poultry feather waste in a previous study (Charimba, 2012) using polyphasic taxonomy. This will include: conventional 16S rRNA sequencing and whole-genome sequencing; phylogenetic treeing methods; DNA-DNA hybridization, ANI and AAI values; mol% G+C; conventional and automated phenotypic tests; fatty acid methyl ester, polar lipid and respiratory lipoquinone analysis. This will finally be combined into a species description of the novel *Chryseobacterium* species.
- III. Determination of the growth kinetics of the novel *Chryseobacterium* species in comparison with *C. carnipullorum* and *C. vrystaatense* will include focusing on specific growth rate, survival and determining the effect of temperature as an environmental parameter.

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Chryseobacterium is a genus that originated in the *Flavobacterium* genus and was reclassified as a new genus in 1994 (Vandamme *et al.*, 1994a). Psychrotolerant and proteolytic spoilage microorganisms like members of the genus *Chryseobacterium* have been found in food sources, e.g., poultry, red meat, milk and fish (Vandamme *et al.*, 1994b; Bernardet *et al.*, 2011). In the Food Science department of the University of the Free State, *Chryseobacterium* strains have been isolated from a wide variety of food sources (Jooste & Hugo, 1999; Hugo *et al.*, 2003; de Beer *et al.*, 2005, 2006; Charimba *et al.*, 2013).

In order to determine the role and significance of these isolates in food spoilage, these isolates first have to be identified by polyphasic taxonomy. Colwell proposed polyphasic taxonomy in the 1970s and it is based on the combination of phenotypic, chemotypic and genotypic methods to describe a microorganism (Prakash *et al.*, 2007). Polyphasic taxonomy can be seen as an integration of any significant information on characteristics of an organism; the more information, the better one can understand the organism's biological reality (Vandamme *et al.*, 1996a). It is an empirical and consensus type of classification.

Phenotypic methods were the cornerstone of bacterial taxonomy before molecular techniques were developed. Examples of phenotypic tests include evaluation of growth at different temperatures, pH ranges, salinity ranges and utilisation of different carbon sources. These characteristics of a novel species can be determined conventionally or by automated methods, e.g. API systems or the Biolog/Omnilog® systems (Bernardet *et al.*, 2002; BIOLOG, 2013). Phenotypic methods can characterize organisms up to strain-level (Prakash *et al.*, 2007).

Bacteria can be identified to the rank of genus using chemotaxonomic methods (Prakash *et al.*, 2007). These include methods for the determination of cellular fatty acids, polar lipids, respiratory quinones, polyamines and cell wall components. These methods are used to group and compare a large set of strains in a short period (Vandamme *et al.*, 1996a).

Genotypic methods using DNA and RNA relatedness are reproducible, easy to use and have high discriminatory power (Prakash *et al.*, 2007). They are more commonly used today where classification in the past relied more on morphological and physiological characteristics (Gevers *et al.*, 2006). Studies revealed that comparing the phylogeny of microorganisms based on a part of the genome that is conserved is more stable than using phenotypic traits (Prakash *et al.*, 2007). The 16S rRNA molecule is mostly used and other examples are 5S and 23S rRNA (Prakash *et al.*, 2007).

Although 16S rRNA gene sequencing and DNA-DNA hybridization (DDH) were regarded as the basis of prokaryotic taxonomy until recently (Stackebrandt *et al.*, 2002), whole-genome sequencing of bacterial genomes has become very important (Land *et al.*, 2015). Genome sequence similarities, e.g., digital DNA-DNA hybridization (dDDH) and Average Nucleotide Identity (ANI) will potentially replace DDH (Kim *et al.*, 2014). Advantages of whole-genome sequencing are that it focuses on a broader range of genes, which provides better taxonomic resolution and shows less sensitivity to horizontal gene transfer (Land *et al.*, 2015). Phylogeny inference and inferring functional pathways are improved and are more accurately determined through genome-scale modelling than using gene-based modelling. Although whole-genome sequencing is of great value, it has not yet been fully integrated into bacterial taxonomy (Land *et al.*, 2015).

There are no straightforward or easy guidelines for performing a polyphasic study (Vandamme *et al.*, 1996a) but once a bacterial isolate has been identified by a polyphasic taxonomic approach; it can then be evaluated for its role and significance in food spoilage. Food spoilage and the inappropriate handling of food can be a significant problem for consumers and the industry due to financial losses (Egan *et al.*, 2006; Cairo *et al.*, 2008). The specific microbiota in food is dependent on the environment in which the food is produced, the raw materials the food consists of,

the processing conditions and the storage conditions of the food (Stellato *et al.*, 2015). Microorganisms that colonise food are not only dependent on the compositional characteristics of the food itself but also on environmental conditions and the interaction it has with the food. Food quality and microbiological safety, therefore, rely on the principles of microbial ecology applied in food systems (Cairo *et al.*, 2008).

In order to evaluate the food spoilage characteristics of an organism, growth kinetic studies may be employed. The word kinetics is derived from Greek and means forcing to move (Panikov, 1991). It investigates the rates of growth of an organism and the mechanisms of any food processing system, e.g., physical, chemical and biological. The term Predictive Microbiology (Brul, 2007) was first proposed by Roberts and Jarvis (1983). The first predictive model used in the food industry, namely the log-linear microbial death model, was developed by Bigelow and co-workers (1920), Bigelow (1921) and Esty & Meyer (1922) who used the model to describe the thermal death of *Clostridium botulinum* type A spores. The log-linear model defines that the specific death rate of the bacteria is constant with time at a given temperature (Fakruddin *et al.*, 2011).

The classification of predictive models are as follows: Firstly, by the microbiological event that consists of kinetic and probability models (Roberts, 1989) and secondly by the modelling approach that includes empirical and mechanistic models (Roels & Kossen, 1978) and lastly by variables that are classified into primary, secondary and tertiary models (Whiting & Buchanan, 1993). Predictive modelling can be applied in Hazard Analysis Critical Control Point (HACCP) procedures, risk assessment, microbial shelf life studies, product research and development, temperature function integration and hygiene regulatory activity, education and design of experiments (Fakruddin *et al.*, 2011).

The aims of this literature review were to study the genus *Chryseobacterium* in terms of its history, characteristics and isolation from food sources. To investigate polyphasic taxonomic techniques used to describe and characterize a new species in this genus; study food spoilage in terms of microbial growth kinetics, measurement of growth and factors influencing growth by using predictive microbiology.

2.2. The genus *Chryseobacterium*

2.2.1. History

The *Chryseobacterium* genus belongs to the *Flavobacteriaceae* family, which was proposed by Jooste in 1985. The characteristics of the genus *Flavobacterium*, e.g., having pigments ranging from yellow to orange or no pigments, no gliding movement and being strictly aerobic, were used to describe the family *Flavobacteriaceae* in Bergey's Manual (Reichenbach, 1989). In 1996 a polyphasic study was performed to create an extensively revised description of the family *Flavobacteriaceae* and the genus *Flavobacterium* (Bernardet *et al.*, 1996). The following genera were included in the family: *Flavobacterium* (the type genus); *Bergeyella*; *Capnocytophaga*; *Chryseobacterium*; *Empedobacter*; *Ornithobacterium*; *Riemerella*; *Weeksella*; *Myroides* and *Tenacibaculum*. The List of Prokaryotic Names with Standing in Nomenclature (LPSN) reports that at the time of writing the number of genera belonging to the family *Flavobacteriaceae* are 158 (<http://www.bacterio.net/-classifphyla.html#flavobacteriaceae>, accessed 2018/10/15).

The genus *Chryseobacterium* was proposed in 1994 (Vandamme *et al.*, 1994a). Six bacterial species namely *C. balustinum*, *C. gleum*, *C. indologenes*, *C. indoltheticum*, *C. meningosepticum* and *C. scophthalmum* that were previously included in the genus *Flavobacterium*, were reclassified as members of the genus *Chryseobacterium*. *Chryseobacterium gleum* was identified as the type species of the genus *Chryseobacterium* after phenotypic and genotypic studies revealed that all twelve strains of *C. gleum* were homogeneous (Holmes *et al.*, 1984). *Chryseobacterium meningosepticum* and *C. miricola* (Li *et al.*, 2003) were later placed into the new genus, *Elizabethkingia* as *E. meningoseptica* while another species, *E. miricola* was also described (Kim *et al.*, 2005b). *Chryseobacterium* sp. CDC group IIb (Ursing & Bruun, 1991; Bernardet *et al.*, 2011) includes the group of strains that have not been assigned to named species and was first known as *Flavobacterium* CDC group IIb (King, 1959). The LPSN reports that at the time of writing the number validly published *Chryseobacterium* species are 112 (<http://www>.

bacterio.net/chryseobacterium.html, accessed 2018/10/15). The *Chryseobacterium* species and their source of isolation are given in Annexure 1.

2.2.2. Characteristics

Chryseobacterium cells are small Gram-negative straight rods with parallel sides and rounded ends about 0.5 µm wide and 1-3 µm long (Bernardet *et al.*, 2011). Ring-shaped cells are not formed, but under certain conditions, some species can produce cells that are filamentous and flexible (Bernardet *et al.*, 2002). Cells do not show swarming or gliding, are non-motile and do not have flagella (Bernardet *et al.*, 2011). The colony colour ranges from pale to bright yellow due to the presence of flexirubin-type pigments that do not fluoresce and do not diffuse. *Chryseobacterium* species grow on commercial media without growth factors. Nutrient agar (NA) is an example of a medium for culturing *Chryseobacterium* isolated from the environment, marine fish, freshwater fish and food-associated sources (Hugo & Jooste, 2012). Some *Chryseobacterium* species can grow on MacConkey agar, but ceftrimide agar shows no growth or weak growth. Growth temperatures of environmental members range from 5°C (most), 15-30°C (all) to 37°C (several) while clinical isolates grow at 5°C (none), 15-37°C (all) and 42°C (some). Most species can grow at a pH range of 5 to 7 or others from pH 8 to 10 (Hugo & Jooste, 2012).

Although most *Chryseobacterium* species prefer 0 to 1% (w/v) sodium chloride in the growth medium, some species can grow in media that contain 3-5% (w/v) NaCl concentration. This organism is positive for catalase and oxidase production. *Chryseobacterium* species can oxidize several carbohydrates. Esculin but not agar is hydrolysed and the organism shows a strong proteolytic activity. Most strains show resistance towards a wide range of antimicrobials e.g.: *C. indologenes* and *C. gleum* show resistance against a spectrum of cephalosporins and carbapenems; others show resistance to erythromycin, tetracyclines, and linezolid including intermediate resistance to vancomycin and clindamycin; fish pathogens have shown resistance to ampicillin, oxytetracycline, polymyxin B, and chloramphenicol (Bernardet *et al.*, 2011).

Previous research reported that Summed feature 4 (consisting of iso-C_{15:0} 2-OH and/or C_{16:1} ω 7c/t), iso-C_{15:0}, iso-C_{17:0} 3-OH and iso-C_{17:1} ω 9c are the predominant cellular fatty acids in members of the genus *Chryseobacterium* (Bernadet *et al.*, 2011). Recent studies, however, report that the presence of fatty acid iso-C_{15:0} 2-OH is no longer recorded in descriptions of novel species and iso-C_{17:1} ω 9c is reported as part of summed feature 9 rather than alone (Montero-Calasanz *et al.*, 2013). *Chryseobacterium* has G+C values ranging from 29-39 mol% (Bernadet *et al.*, 2011). Phosphatidylethanolamine is the most abundant polar lipid in *Chryseobacterium* species (Wu *et al.*, 2013) and menaquinone-6 is reported to be the major or only respiratory quinone (Bernadet *et al.*, 2002).

2.2.3. Significance of *Chryseobacterium* species in food

Several studies on *Chryseobacterium* species isolated from food sources have been done globally and in the Department of Food Science at the University of the Free State. The *Chryseobacterium* species isolated from food sources are listed in Table 2.1. *Chryseobacterium* species are regarded as food spoilage organisms due to their proteolytic characteristics (Charimba, 2012; Bekker *et al.*, 2015).

Contamination of poultry or other animal meat can originate from the food processing environment and may originate from soil or water, the animal's skin and mucous membranes, otherwise, the meat is sterile (Molin, 2000; Forsythe, 2000). Metabolites that cause spoilage are produced when the microorganism utilizes carbohydrates, amino acids and carboxylic acids (de Beer, 2005). Proteins in poultry meat are degraded into indole, dimethyl sulphide and ammonia compounds that aid in volatile and off-flavours (Banwart, 1989). Rancid off-flavours are caused by the chemical oxidation of unsaturated lipids (Forsythe, 2000). The main genera present on poultry meat are *Achromobacter*, *Acinetobacter*, *Aerobacter*, *Alcaligenes*, *Bacillus*, coryneforms, *Cryptococcus*, *Eberthella*, *Enterobacteriaceae*, *Escherichia*, *Flavobacterium* (possibly also *Chryseobacterium*), *Micrococcus* (Molin, 2000), *Moraxella*, *Oospora*, *Penicillium*, *Proteus*, *Pseudomonas*, *Psychrobacter*, *Rhodotorula*, *Salmonella*, *Sarcina*, *Staphylococcus*, *Streptococcus* and

Streptomyces (Mountney & Gould, 1998). *Chryseobacterium carnipullorum* was isolated from a raw chicken portion (Charimba *et al.*, 2013) and *C. vrystaatense* (de Beer *et al.*, 2005) was isolated from chicken portions from a poultry abattoir in South Africa.

Chryseobacterium gleum and *C. indologenes* are often present on raw red meat (Bernardet *et al.*, 2005). The function of *C. indologenes* and *C. gleum*, isolated from various raw meat sources is uncertain and vague (Dworkin *et al.*, 2006). *Chryseobacterium* spp. made up 18% of the total bacterial isolates in a study that evaluated the bacterial population on fresh beef (Hugo & Jooste, 2012).

Spoilage in fish is similar to spoilage in poultry and red meat (Leisner & Gram, 1999). However, raw fish mostly contain the metabolite trimethylamine that has an ammonia-like “fishy” odour (Gram & Dalgaard, 2002). Phenylalanine deaminase and urea produced by *C. piscium* are reported to spoil fish (de Beer *et al.*, 2006). An example of an organism isolated from fish on farms and in the wild is *C. balustinum* (Dworkin *et al.*, 2006). The origin of *C. gleum* and *C. indologenes* that caused spoilage in Cape marine fish is still uncertain (Dworkin *et al.*, 2006).

Spoilage of milk and milk products can be associated with the production of heat-stable metalloproteases (Venter *et al.*, 1999). Milk and butter are sources from which *Flavobacterium* was isolated during a research project in the Department of Food Science at the University of the Free State (Jooste, 1985; Hugo *et al.*, 2003) and some of these isolates were later classified to be *Chryseobacterium* species (Hugo *et al.*, 2003; Tsôeu *et al.*, 2016). Fresh South African cow’s milk (Jooste, 1985; Jooste *et al.*, 1985, 1986a; Welthagen & Jooste, 1992; Hugo *et al.*, 2003) and a lactic acid beverage from Japan (Shimomura *et al.*, 2005) have been sources of *Chryseobacterium* species. *Chryseobacterium indologenes*, CDC group IIb, *C. gleum* and *C. joostei* have been identified from milk by Hugo and Jooste (1997) and Hugo *et al.* (1999, 2003). Other examples of *Chryseobacterium* species isolated from milk are *C. haifense*, *C. oranimense* and *C. bovis* (Hantsis-Zacharov & Halpern, 2007a, 2007b; Hantsis-Zacharov *et al.*, 2008a, 2008b).

Table 2.1. *Chryseobacterium* species isolated from a food source.

Species	Sources	Reference(s)
<i>C. aahli</i>	Lake trout (<i>Salvelinus namaycush</i>) and brown trout (<i>Salmo trutta</i>)	Loch & Faisal, 2014
<i>C. angstadtii</i>	Environmental - freshwater	Kirk <i>et al.</i> , 2013
<i>C. aquaticum</i>	Water reservoir	Kim <i>et al.</i> , 2008
<i>C. aquifrigidense</i>	Water-cooling system	Park <i>et al.</i> , 2008
<i>C. arothri</i>	Pufferfish (<i>Arothron hispidus</i>)	Campbell <i>et al.</i> , 2008
<i>C. bovis</i>	Raw cow's milk	Hantsis-Zacharov <i>et al.</i> , 2008a
<i>C. carnipullorum</i>	Raw chicken	Charimba <i>et al.</i> , 2013
<i>C. chaponense</i>	Atlantic salmon	Kämpfer <i>et al.</i> , 2011
<i>C. echinoideorum</i>	Edible sea urchin (<i>Tripneustes gratilla</i>)	Lin <i>et al.</i> , 2015
<i>C. elymi</i>	Wild rye (<i>Elymus</i>)	Cho <i>et al.</i> , 2010
<i>C. gallinarum</i>	Animal (Chicken)	Kämpfer <i>et al.</i> , 2014b

Species	Sources	Reference(s)
<i>C. gambrini</i>	Beer-bottling plants	Herzog <i>et al.</i> , 2008
<i>C. haifense</i>	Raw milk	Hantsis-Zacharov & Halpern, 2007b
<i>C. halperniae</i>	Food	Hahnke <i>et al.</i> , 2016
<i>C. hispanicum</i>	Drinking water distribution system	Gallego <i>et al.</i> , 2006
<i>C. joostei</i>	Raw milk	Hugo <i>et al.</i> , 2003
<i>C. lactis</i>	Milk	Holmes <i>et al.</i> , 2013
<i>C. lineare</i>	Freshwater	Zhao, Z. <i>et al.</i> , 2017
<i>C. molle</i>	Beer-bottling plants	Herzog <i>et al.</i> , 2008
<i>C. oleae</i>	Olive tree (<i>Olea europaea</i> L.)	Montero-Calasanz <i>et al.</i> , 2014
<i>C. oncorhynchi</i>	Rainbow trout, <i>Oncorhynchus mykiss</i>	Zamora <i>et al.</i> , 2012a
<i>C. oranimense</i>	Raw cow's milk	Hantsis-Zacharov <i>et al.</i> , 2008b
<i>C. pallidum</i>	Beer-bottling plants	Herzog <i>et al.</i> , 2008
<i>C. piperi</i>	Freshwater Creek	Strahan <i>et al.</i> , 2011

Species	Sources	Reference(s)
<i>C. piscicola</i>	Diseased salmonid fish	Ilardi <i>et al.</i> , 2009
<i>C. piscium</i>	Fish	de Beer <i>et al.</i> , 2006
<i>C. scophthalmum</i>	Gills of diseased turbot (<i>Scophthalmus maximus</i>)	Vandamme <i>et al.</i> , 1994a
<i>C. sediminis</i>	Freshwater	Kämpfer <i>et al.</i> , 2015b
<i>C. shigense</i>	Lactic acid beverage	Shimomura <i>et al.</i> , 2005
<i>C. tructae</i>	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Zamora <i>et al.</i> , 2012b
<i>C. ureilyticum</i>	Beer-bottling plants	Herzog <i>et al.</i> , 2008
<i>C. viscerum</i>	Fish	Zamora <i>et al.</i> , 2012c
<i>C. vrystaatense</i>	Raw chicken	de Beer <i>et al.</i> , 2005

2.3. Description of novel *Chryseobacterium* species using a polyphasic approach

In order to describe novel species in the genus *Chryseobacterium*, the following genotypic, phenotypic and chemotaxonomic tests are employed as part of a polyphasic approach.

2.3.1. Genotypic methods

Genotypic identification is used in combination with other methods and to complement phenotypic techniques and usually include 16S rRNA sequencing, whole-genome sequencing, DNA-DNA hybridization and mol% G+C determination (Tang *et al.*, 1998).

16S rRNA sequencing

The 16S rRNA sequencing method is a versatile and highly accurate method for identifying bacteria up to species level, even species that are not easily identified through biochemical methods (Tang *et al.*, 1998). This method is still commonly used because there is at least one copy in every bacterial genome and it provides information on the family, genus and mostly species level of bacteria (Land *et al.*, 2015). The 16S rRNA sequence provides insight into the evolution and taxonomy of prokaryotes (Tindall *et al.*, 2010).

Organisms that have a 16S rRNA gene sequence similarity value of 97% or more are classified as members of the same species (Tindall *et al.*, 2010) but it is necessary to compare several strains of a species because sequence similarities can differ up to 5% between strains of the same species (Bernardet *et al.*, 2002). The phylogenetic hypothesis will be more reliable and provide an estimation of how much diversity exists in a new taxon. Higher 16S rRNA similarity values of 98.7–99.0% (Stackebrandt & Ebers, 2006) and 98.2–99.0% (Meier-Kolthoff *et al.*, 2013) have been recommended for species delineation. Analysing the 16S rRNA sequence was initially made possible by cataloguing (Fox *et al.*, 1977), secondly by reverse transcriptase-sequencing (Sanger *et al.*, 1977; Lane *et al.*, 1988) and finally, gene sequencing based on polymerase chain reaction (PCR) (Saiki *et al.*, 1988).

After 16S rRNA sequencing, phylogenetic trees should be constructed. At least two methods should be used when constructing a phylogenetic tree (Bernardet *et al.*, 2002). The following methods are available: maximum-likelihood, maximum-

parsimony, unweighted pair group method (UPGMA) and the neighbour-joining method. Maximum-parsimony uses sites that contain at least two or more nucleotides or amino acids that are different while the UPGMA method accepts that the rate of nucleotide and amino substitutions of all evolutionary lineages are the same (Nei & Kumar, 2000). A tree created through the maximum-likelihood method, is first built using e.g., the neighbour-joining method then the data likelihood is maximized by adjusting the branch lengths. The Nearest Neighbour Interchange (NNI) method is used to create variants. Maximum-likelihood branch lengths are computed and only variants that have the highest likelihood are retained (Nei & Kumar, 2000).

The reliability of branching of the phylogenetic tree is confirmed through bootstrap analysis (Bernardet *et al.*, 2002) and when the bootstrap value of the interior branch has a value of 95% or higher, only then can the topology of the branch be regarded as correct (Nei & Kumar, 2000). The significance of the phylogenetic treeing method used will be enhanced if all the related organisms are included in the phylogenetic tree (Bernardet *et al.*, 2002). The phylogenetic relationship will be more reliable and insight into the genomic diversity will be provided if sequences of strains in a species are compared. The type strain is used for comparison between known characteristics and the new species and should be included when a new species is described (Bernardet *et al.*, 2002).

The family *Flavobacteriaceae* contains an extensive database that is used to organise species on a phylogenetic tree (Bernardet *et al.*, 2002). Sequences of 16S rRNA that are new should be incorporated into a database that is well-known, e.g., GenBank, EMBL-EBI (European Bioinformatics Institute) and DDBJ (DNA Data Bank of Japan) (Tindall *et al.*, 2010). The organism's description and accession number should be included in the database. Confusion should be avoided by depositing the new 16S rRNA gene sequences under the laboratory code or culture collection number rather than using the binomial name of the organism (Bernardet *et al.*, 2002). Another database, EzBioCloud, is a new database that contains 16S rRNA gene and genome sequences of *Bacteria* and *Archaea* (Yoon *et al.*, 2017).

The 16S rRNA gene sequencing method, however, has some limitations when it comes to interpretation, incomplete databases in some cases and the inability to

assign a species for organisms that have diverged recently. More comprehensive approaches e.g., whole-genome sequencing are becoming more important (Land *et al.*, 2015).

Whole-genome sequencing

A complete set of an organism's genes in its DNA is defined as its genome (Lawrence, 2011). In 1995 (Fleischmann *et al.*, 1995; Fraser *et al.*, 1995) two complete bacterial genome sequences were published for the first time and from then on sequencing technology improved dramatically (Land *et al.*, 2015). Bacterial genome-sequencing is done more often nowadays because of its cost reduction and affordability to more laboratories (Shendure & Ji, 2008). Binnewies and co-workers (2006) reported that 300 bacterial genomes had been sequenced from 1995 to 2006 and only two metagenomic projects have been published. Land and co-workers (2015) reported that over 30 000 sequenced bacterial genomes and thousands of metagenomic projects are publicly available on the National Center for Biotechnology Information (NCBI, 2014) and the Genomes Online Database (GOLD, 2014), respectively.

At the time of writing NCBI reports that 76 known *Chryseobacterium* species' genomes have been sequenced (<https://www.ncbi.nlm.nih.gov/genome/?term=Chryseobacterium>, accessed 2018/10/15). Their genome size ranges from 2 to 5 Mb.

Primary methods, e.g., 16S rRNA sequencing, mostly used for taxonomic assignment and creating phylogenetic trees (Mizrahi-Man *et al.*, 2013) are gradually being replaced with methods that are more comprehensive and give a better understanding of genetic relationships (Land *et al.*, 2015). Proteomes, reference genomes, whole genomes and conserved protein groups are used rather than only focussing on one gene. Computers play a critical role in the interpretation and handling of sequence data (Land *et al.*, 2015). In the future, improving the density, capacity, stability, reliability and speed of storage of computers and databases will continue. Bioinformatic tools that are fast and robust will be needed when

sequencing replaces other diagnostic tests and mechanisms used for detection (Land *et al.*, 2015).

Three generations of sequencing have developed over the past years (Land *et al.*, 2015). Sanger sequencing, a first generation method, was mostly performed by robots and selected whole-genome shotgun libraries. These robots selected the templates, performed the sequencing reactions and electrophoresis on the samples. A high-quality draft genome was then created. This method is, however, labour intensive and expensive (Land *et al.*, 2015).

Next-Generation Sequencing (NGS), a second-generation method, involves four main steps namely: library preparation, cluster generation, sequencing and data analysis (Illumina, 2016). Deoxyribonucleotide triphosphates that are fluorescently labelled are incorporated into a DNA template strand during consecutive cycles of DNA synthesis. Fluorophore excitation is used to identify the nucleotides at the point of incorporation during each cycle. The advantage is that this process is repeated across millions of fragments in a parallel fashion instead of sequencing a single fragment. Millions of reads are generated, this refers to the data strand consisting of A, T, C, G bases that correspond to the sample DNA. Overlapping sequencing reads are aligned to create contigs which are continuous stretches of DNA sequences. The coverage level is the average amount each base in a genome was sequenced and the recommended value is $\geq 50X$ (Chun *et al.*, 2018). Sequencing systems available, range from the Miniseq, Miseq, Nextseq, Hiseq and Hiseq X. Although this method is more cost-effective, an increase in coverage for assembly and larger numbers of contigs are necessary to change a genome status from only being a draft to complete (Land *et al.*, 2015).

Single-molecule sequencing, a third generation method, produces longer reads and is, therefore, more cost-effective and can eliminate draft genomes in the future. Examples of third generation methods are PacBio (Brown *et al.*, 2014; Terabayashi *et al.*, 2014) and MinION (Mikheyev & Tin, 2014; Quick *et al.*, 2014).

DNA-DNA hybridization (DDH) versus Average Nucleotide Identity (ANI) and Average Amino acid Identity (AAI)

The principle of DNA-DNA hybridization (DDH) is to measure the extent to which single strands of different DNA molecules bind to form double helices (Lawrence, 2011). The phylogenetic definition of a species is defined as strains that have 70% or more DNA that is similar and 5% or less, difference in melting temperature (ΔT_m) (Bernardet *et al.*, 2002). In a comparative study, 97% of 16S rRNA gene sequencing similarity corresponded to 70% DDH (Stackebrandt & Goebel, 1994). It is, therefore, only necessary to perform DDH if the 16S rRNA value between two strains is higher than 97% (Tindall *et al.*, 2010). Higher 16S rRNA similarity values of 98.7–99.0% have also been used (Stackebrandt & Ebers, 2006). The recent values recommended by Meier-Kolthoff and co-workers (2013) are 98.2–99.0%.

The renaturation rate method through spectrophotometry has been the preferred method to determine the DNA relatedness of strains that belong to *Chryseobacterium* species (Bernardet *et al.*, 2002). The S1-nuclease method can also be used to determine DNA relatedness. This method involves the absorption of S1-resistant DNA to filters consisting of diethylaminoethyl-cellulose. The evaluation should include the type strain of the new species and that of related species. The hybridization study should also include all the strains of the new species (Bernardet *et al.*, 2002).

Although DDH has been known as the “gold” standard, it is also known to be labour-intensive and errors often occur during experiments. Therefore, genome sequence similarities will potentially replace DDH (Kim *et al.*, 2014). Overall genome-related indexes (OGRIs) are values identified as analogous values to DDH values (Chun & Rainey, 2014). Examples of OGRIs are Average Nucleotide Identity (ANI) and digital DNA-DNA hybridization (dDDH). Various software tools are available to calculate ANI and dDDH (Chun *et al.*, 2018). ANI can be calculated using Kostas lab ANI calculator (<http://enve-omics.ce.gatech.edu/ani/>) and dDDH can be calculated using the Genome-Genome Distance Calculator (<http://ggdc.dsmz.de/>).

The ANI value is the mean value of identity/similarity of two genomes that have homologous genomic regions (Kim *et al.*, 2014). 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). Advantages of using ANI are that it is not affected by lateral gene transfer or affected to recombination rates that are variable and the resolution is extended to the subspecies level (Konstantinidis & Tiedje, 2005).

Investigations of the correlation between 16S rRNA gene sequence and ANI was limited to a study performed on a small scale of 70 genomes (Konstantinidis & Tiedje, 2005) until Kim and co-workers (2014) investigated 6787 genomes. The results showed that the newly proposed threshold of 16S rRNA gene sequence similarity (98.65%) could accelerate the rate of which novel species are discovered especially combined with ANI. It is, however, essential to use high-quality sequence data (Kim *et al.*, 2014).

Another method used is Average Amino acid Identity (AAI). It is a robust method used to easily measure relatedness between prokaryotic taxa (Konstantinidis & Tiedje, 2005). Strains that have AAI values of 95% or higher are classified as part of the same species (Konstantinidis & Tiedje, 2005). Studies show that AAI and ANI may offer better resolution between species that are closely related than the 16S rRNA gene. Average Amino Acid Identity can be determined by the Newman lab ROSA calculator (<http://lycofs01.lycoming.edu/~newman/CurrentResearch.html>).

Mol% G+C

G+C content is one of the taxonomic markers most frequently used in microbiology (Mesbah *et al.*, 1989; Rosselló-Mora & Amann, 2001). Members of the family *Flavobacteriaceae* can have G+C values ranging from 27 to 44 mol% (Bernardet *et al.*, 2002) whereas members of the genus *Chryseobacterium* have G+C values ranging from 29-39 mol% (Bernardet *et al.*, 2011). Examples of methods used to determine base composition are high-performance liquid chromatography (HPLC)

and melting temperature profiles (Levy-Frebault & Portaels, 1992). The reference strain's G+C content is used to compare the new strain with known values and should, therefore, be included when describing a new species (Bernadet *et al.*, 2002).

Due to sequencing technology progressing rapidly, the G+C content can be calculated from a high-quality genome sequence, therefore, replacing traditional methods (Hahnke *et al.*, 2016).

2.3.2. Phenotypic characterization

Phenotypic characteristics are used to place a new taxon in the genus it belongs to and to differentiate between the new taxon and the other taxa within the genus (Bernadet *et al.*, 2002).

Conventional methods

Conventional phenotypic methods can be divided into three groups, namely, morphological, physiological and biochemical tests (Figure 2.1). These methods are the oldest tools used for the classification and characterization of prokaryotes (Tindall *et al.*, 2010).

Morphological characteristics include cell shape and size; the life cycle; formation of endospores or exospores; the presence or absence of flagella; the presence of motility caused by flagella and gliding or the lack thereof and lastly the characteristics of the colony (Tindall *et al.*, 2010). A Transmission Electron Microscope (TEM) can be used to investigate internal membrane structures, cytoplasmic inclusions and the cell envelope's infrastructure. A Scanning Electron Microscope (SEM) can be used to investigate the morphology of whole cells (Tindall *et al.*, 2010).

Biochemical tests include production of acid from different sugars (tested in ammonium salt medium) (Barrow & Feltham, 1993); production of L-phenylalanine deaminase (Richard & Kiredjian, 1995); nitrate and nitrite reduction (West & Colwell, 1984); production of indole and urease (Richard & Kiredjian, 1995; Hugo, 1997); β -galactosidase activity on O-nitrophenyl- β -D-galactopyranoside (ONPG) discs or API 20NE galleries; degradation of aesculin (Yabuuchi *et al.*, 1990); H₂S production on Kligler iron agar (Smibert & Krieg, 1994); hydrolysis of Tween 80 and starch (West & Colwell, 1984); precipitation on 10% egg yolk nutrient (Barrow & Feltham, 1993) or trypticase-soy agar and lastly hydrolysis of L-tyrosine on 0.5% L-tyrosine nutrient (Barrow & Feltham, 1993) or trypticase-soy agar.

Although chemotaxonomy is included under a separate heading, it forms a part of phenotypic characterization (Smibert & Krieg, 1994; Tindall *et al.*, 2008; Tindall *et al.*, 2010). Therefore, biochemical characterization also includes the outer cell layers (peptidoglycan and mycolic acids), the cytoplasm (polyamines) and the cell membrane(s) (polar lipids, fatty acids, respiratory lipoquinones and pigments).

Physiological characteristics include growth at different temperatures, i.e., 5, 37 and 42°C and on different media e.g., cetrimide and MacConkey agars; growth at different pH values; aerobic or anaerobic growth and growth at different salt concentrations (Bernardet *et al.*, 2002).

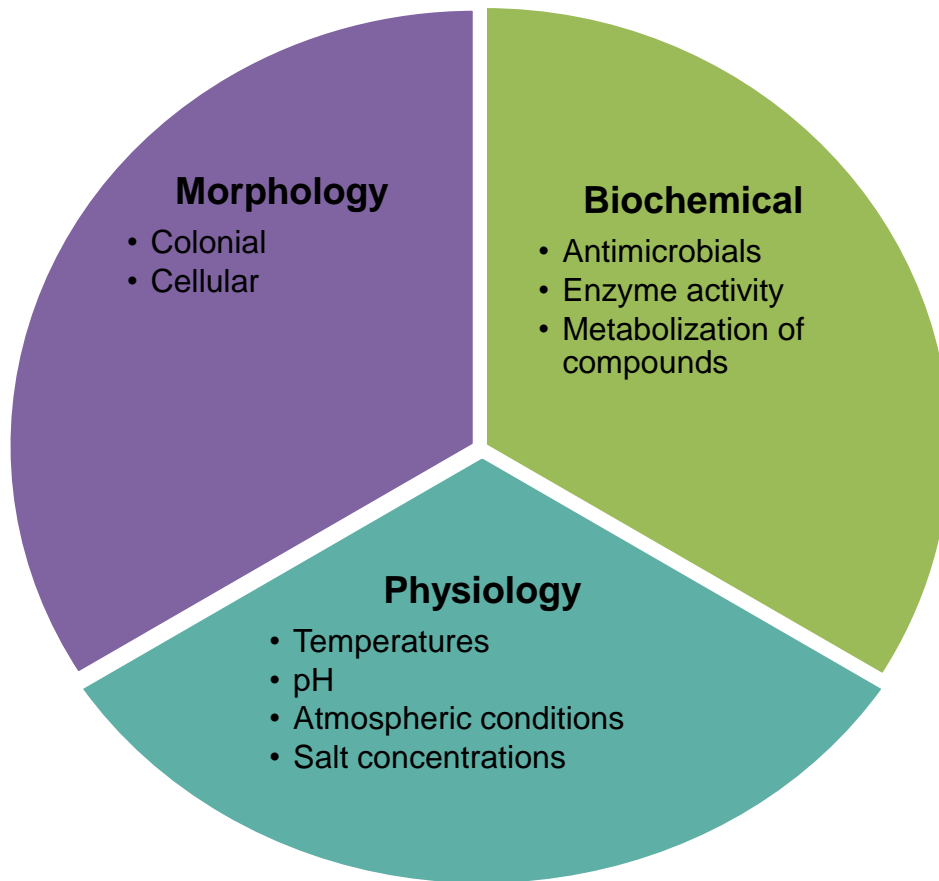


Fig. 2.1. Conventional phenotypic methods used in the characterization of a bacterial isolate.

Automated methods

For the description of novel bacteria, commercial systems are used in conjunction with the conventional methods mentioned before. The outcome of a test using a commercial system can be different from a conventional method used. It is, therefore, crucial that standardized conditions be used to obtain reproducible results (Vandamme *et al.*, 1996a). Examples of commercial systems are API ZYM, API 50CH, API 20E, API ID 32E, API 20NE, Biolog GEN III MicroPlate and BIOTYPE 100 (Bernardet *et al.*, 2002). The API test systems can contain up to 20 different biochemical tests that consist of microtube/cupules with substances that are dehydrated but changes colour when an enzymatic reaction takes place (The Global Health Network, 2013). The substrate can either be assimilated or fermented by the organism.

The BIOLOG GEN III MicroPlate system can contain up to 71 tests for the utilization of carbon sources and 23 tests for the determination of sensitivity to chemicals, therefore, 94 phenotypic tests in total (BIOLOG, 2013). The microplate wells contain tetrazolium redox dye and will turn purple due to a reaction when the organism oxidizes a specific carbon source or shows no sensitivity to a specific inhibitory substance (BIOLOG, 2013).

2.3.3. Chemotaxonomic methods

Analytical methods are used to provide valuable information in terms of chemotaxonomic markers or chemical constituents that are used to separate bacterial strains in taxonomic ranks (Vandamme *et al.*, 1996a; Mora & Amann, 2001).

Fatty acid methyl ester analysis

Lipids contain fatty acid constituents that consist of long-chain organic acids [$\text{CH}_3(\text{C}_n\text{H}_x)\text{COOH}$], where the chain is either unsaturated or saturated (Lawrence, 2011). Fatty acids can be used as fuel in respiration. Gas-liquid chromatography is the method that is used to determine the fatty acid composition of an organism (Bernardet *et al.*, 2002). Different species can only be compared with each other if the organisms were grown under the same nutritional conditions because different conditions will result in a different profile of fatty acids.

Fatty acid methyl esters should be prepared and separated according to a standard protocol (Paisley, 1996) of the Microbial Identification System (MIDI) (Sasser, 1990). Specific growth media and temperature have been chosen to minimize variables that may occur. Most bacteria that grow aerobically are grown either on Trypticase Soy Broth Agar (TSBA) or on the medium that is usually used to grow the organism in the laboratory. A temperature of 28°C is preferable when using TSBA to enable a wide range of organisms to grow. The harvesting of cells at a given turbidity when using

broth as a medium minimizes the effect of the physiological age of the cells. However, when using agar plates, the time of incubation ranges from 24 h for aerobic organisms to 48 h for anaerobic organisms. Organisms that grow slowly may be incubated for a longer time period until adequate growth is obtained. To ensure a standardized physiological age of cells, a sector of choice from a quadrant streak on the agar plate should be taken (Figure 2.2).

The family *Flavobacteriaceae* contains branched monounsaturated, branched saturated and branched hydroxyl C₁₅-C₁₇ fatty acids (Bernardet *et al.*, 2011). The major branched-chain fatty acids are iso-C_{15:0}, iso-C_{17:1}ω7c (which may have been incorrectly annotated in previous literature), 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).

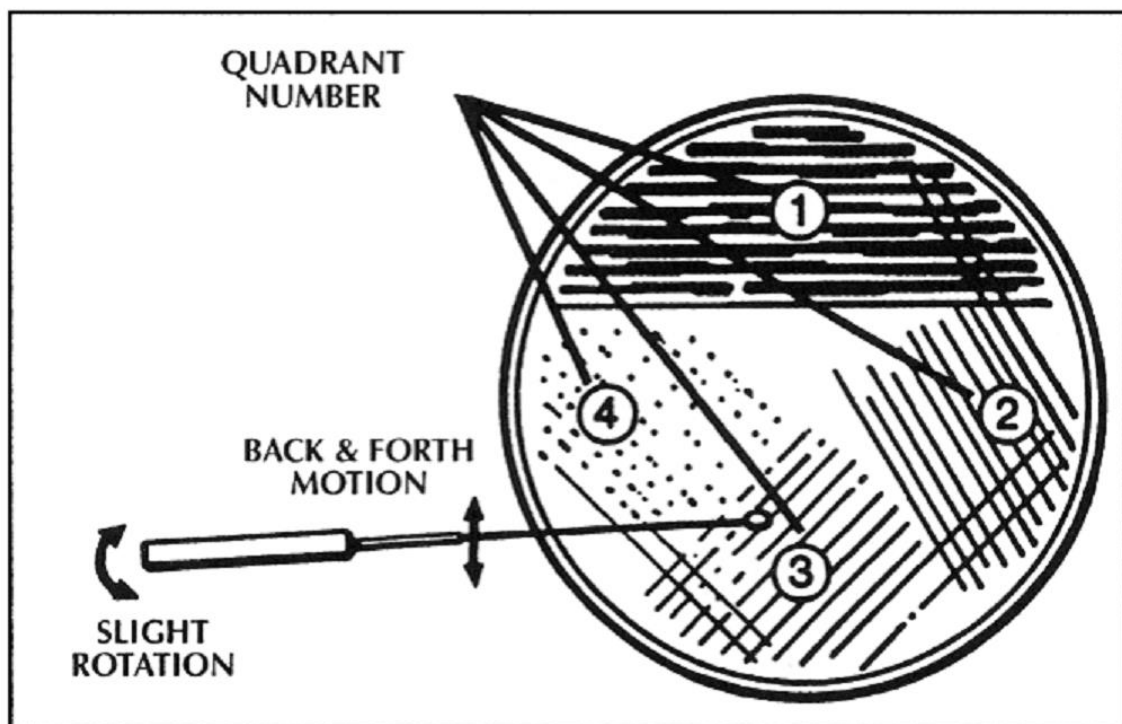


Fig. 2.2. Example of a quadrant streak on an agar plate to ensure a standardized physiological age for determination of fatty acid methyl esters (Sasser, 1990).

Polar lipid analysis

Lipids and proteins are components of biological membranes that aid in the in-and-out transport of molecules that are soluble in water (Barák & Muchová, 2013). Polar lipids can only dissolve in solvents that are organic and play an essential role in biological membranes as a fuel source (Lawrence, 2011). Gram-negative bacteria differ from Gram-positive bacteria in having two membranes where Gram-positive bacteria have a membrane and a thick layer of peptidoglycan. Thin layer chromatography (TLC) is usually used to determine the polar lipids of an organism. Sphingolipids (glyco- or phosphosphingolipids), aminophospholipids, glycolipids, phosphoglycolipids, amino acid derived lipids, phospholipids and hopanoids are examples of polar lipids present in bacteria (Tindall *et al.*, 2010). In *Chryseobacterium* species, phosphatidylethanolamine is the most abundant polar lipid (Wu *et al.*, 2013).

Menaquinone analysis

Respiratory quinones in a membrane-based electron-transport system act as mobile electron carriers that donate electrons on the acceptance of hydrogen (Lawrence, 2011). Quinones are soluble in lipids and can diffuse within the membrane because it comprises of aromatic hydrocarbons that are hydrophobic molecules. In the family *Flavobacteriaceae*, menaquinone-6 is reported to be the major or only respiratory quinone (Bernardet *et al.*, 2002) and can be determined through the method of high-performance liquid chromatography (Nakagawa & Yamasato, 1993).

2.4. Growth kinetics of *Chryseobacterium* species

2.4.1 Microbial growth phases

Microorganisms that grow in an enclosed vessel, e.g., a tube or flask, have a specific growth cycle consisting of mainly a lag, exponential, stationary and death phase (Figure 2.3) (Madigan *et al.*, 2015).

The lag phase begins when fresh media is inoculated with a microbial culture and growth only occurs after a period (Madigan *et al.*, 2015). The period of this phase depends on the inoculum used, e.g., if a culture that has been growing exponentially is transferred to media with the same conditions, there will be no lag phase but an immediate exponential phase. A lag phase can occur if the culture is old and depleted of nutrients or low in viable cells because of damage due to high/low temperature, toxic chemicals or radiation. A lag phase can also occur during a medium downshift, where the culture is transferred from a complex medium to a defined medium. Time is needed to produce new enzymes for the biosynthesis of essential metabolites (Madigan *et al.*, 2015).

The exponential phase is when the population multiplies at regular intervals for a short or extended period (Madigan *et al.*, 2015). This phase is dependent on environmental conditions e.g., temperature and nutrients as well as genetics. Enzyme or other cell component studies are usually done during this phase because the cells are in their healthiest state. Growth can be affected by the size of the cells because smaller cells have a higher capacity for waste and nutrient exchange than larger cells, which is an advantage. The slope of the exponential phase is used to determine the maximum specific growth rate (Zwietering *et al.*, 1990).

The stationary phase of a culture begins when waste products accumulate, or essential nutrients are depleted (Madigan *et al.*, 2015). The growth rate of the population is zero because the cell number does not increase or decrease. Cryptic growth occurs when the processes of cells dividing and cells dying, balance each other out.

The death phase occurs exponentially but at a slower rate than the exponential growth phase (Madigan *et al.*, 2015). Cells begin to die, and this causes a decrease in cell numbers (Buchanan, 1918).

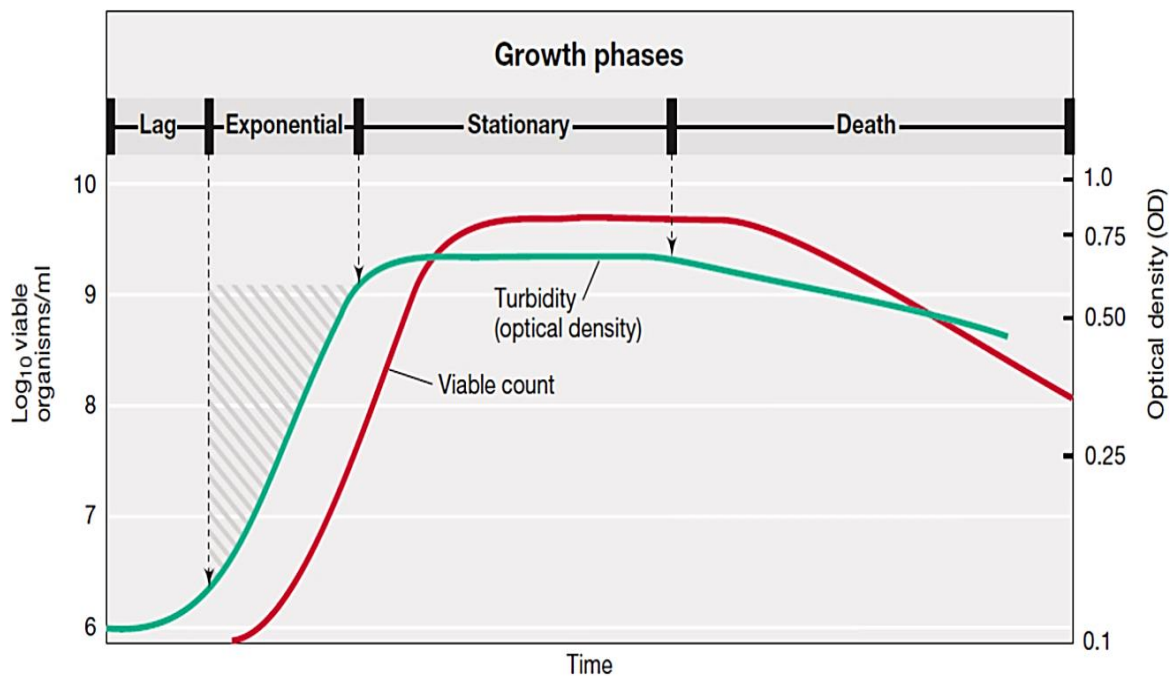


Fig. 2.3. Growth phases of a microbial culture in an enclosed vessel (Madigan *et al.*, 2015).

2.4.2. Methods for measuring microbial growth

Cell growth can be measured by two main methods namely cell count (viable and non-viable count) and turbidity that is also a function of cell mass (Madigan *et al.*, 2015). Viable counts for total numbers include standard plate counts (SPC) or aerobic plate counts (APC), most probable numbers (MPN) method, dye reduction techniques and direct microscopic counts (also used for non-viable counts) (Jay *et al.*, 2005). Viable cells are defined as cells that can divide and form an offspring (Madigan *et al.*, 2015). It is assumed that one viable cell will form one colony and, therefore, colony numbers are a mirror image of cell numbers. In the food industry, viable counting methods are essential in detecting microbial contamination (Madigan *et al.*, 2015).

Standard plate counts (SPC) or APC involve blending or homogenizing a food sample and then preparing serial dilutions of the sample; the sample is then plated in or onto agar plates (Jay *et al.*, 2005). Two methods, namely pour-plate method and spread-plate method can be used to determine the SPC (Madigan *et al.*, 2015). The pour-plate method is done by pipetting a known volume of sample into a sterile Petri dish and pouring molten agar that has cooled down to a temperature of 45°C onto the sample and mixing the agar and sample by a swirling movement of the plate. The spread-plate method is done by spreading a volume of diluted sample with a glass spreader over the surface of an agar plate. Plates that have colony numbers between 25 and 250 are regarded as statistically valid and this is obtained by diluting the sample. Cell count errors can occur due to inaccurate pipetting, insufficient mixing or heat intolerance only to name a few. Data are expressed as the number of colony forming units (CFU) especially when cell clumps are involved where more than one cell forms a colony (Madigan *et al.*, 2015).

For the MPN method, samples are diluted as in the SPC method (Jay *et al.*, 2005). Three dilutions are inoculated in nine to fifteen tubes that contain the appropriate medium (therefore replicas of three or five of the dilutions). The results are obtained by using a MPN table and are statistical in nature. Advantages of the MPN method are the simplicity of the method, it can be used for comparison between different laboratories, specific microorganisms can be identified through selective and differential media and it is mainly used to determine faecal coliform densities. It does, however, have drawbacks, e.g., high volumes of glassware are needed, the morphology of the colony cannot be observed and the method is not always precise (Jay *et al.*, 2005).

The dye reduction technique involves the reduction of dyes by microorganisms either using methylene blue that turns from blue to white when reduced or resazurin that turns from blue to pink/white when reduced (Jay *et al.*, 2005). This technique has mainly been used in determining the microbial quality of raw milk in the dairy industry. Dye reduction techniques are rapid and straightforward to use, inexpensive and reduction of the dyes can only be achieved by viable cells. Drawbacks include problems with food specimens that contain reductive enzymes and dyes that are not always reduced equally by all organisms (Jay *et al.*, 2005).

Microscopic cell count methods, also called direct microscopic counts (DMC) (Jay *et al.*, 2005), entail dry samples that are stained to create a contrast with their background or cells in liquid samples can be counted via counting chambers (Madigan *et al.*, 2015). Counting chambers consist of a grid with squares of which the area is known, etched on the surface of a glass slide. A flow cytometer can also be used to determine the number of cells in a liquid sample by employing a laser beam and sophisticated electronics. Limitations of microscopic cell count methods are that staining techniques are needed to distinguish dead cells from live cells; small cells are difficult to see; motile cells must first be killed and debris can be mistakenly be seen as microbial cells (Madigan *et al.*, 2015).

Turbidity is used to determine cell mass and cell mass is proportional to cell number (Madigan *et al.*, 2015). Light passing through a suspension is scattered by cells and, therefore, the suspension looks more turbid when more light is scattered due to more cells. A spectrophotometer is used to measure turbidity or in other words, the unscattered light that emerges from a cell suspension. Optical density (OD) is the unit of turbidity (Madigan *et al.*, 2015). A standard curve is used to relate cell number, protein content or dry weight to turbidity. Caution should be taken at high cell densities where the linearity of cell number/dry weight and OD can deviate due to light scattering away from the spectrophotometer's photocell by one cell and back again by another cell. The OD measurement then becomes less accurate. Optical density values will also be different between bacterial species with different shapes and sizes. Therefore, different standard curves are drawn for each organism. Optical density measurements are easy to use, do not destroy/disrupt the sample and are useful in determining parameters e.g., generation time and growth rate. It is, however, essential to maintain culture suspension either by shaking or stirring to ensure that OD measurements are accurate (Madigan *et al.*, 2015).

2.4.3. Factors influencing microbial growth and food spoilage

In a food processing environment, optimal conditions for the growth of microorganisms can occur (Stellato *et al.*, 2015). A microenvironment can develop

due to organic residues that aid in the growth, accumulation and cross-contamination of microorganisms in the food handling environment (McLandsborough *et al.*, 2006; Brooks & Flint, 2008; Simões *et al.*, 2010). Ecological factors (intrinsic and extrinsic) will determine which spoilage organism will mainly colonize the food product (Stellato *et al.*, 2015). Examples of intrinsic parameters include pH, osmolarity (water activity), oxidation-reduction potential (Eh) and nutrients. Examples of extrinsic parameters are temperature, gaseous atmosphere surrounding the food, relative humidity of the environment and time (Jay *et al.*, 2005). Linking the presence of microbiota in food and ecological factors together, play a significant role in food safety and quality (Stellato *et al.*, 2015). Fresh meat, e.g., chicken meat, can spoil in a relatively short time if it is not packaged, transported or stored at the appropriate temperatures (Koutsoumanis *et al.*, 2006).

Temperature

A microorganism's cardinal temperatures are the minimum temperature at which growth will occur; optimal temperature where growth is best and a maximum temperature where growth will not occur if the temperature is increased further (Madigan *et al.*, 2015). The temperature range of a microorganism can be related to the environment it inhabits. According to Madigan *et al.* (2015), microorganisms can be grouped into four classes, namely psychrophiles, mesophiles, thermophiles and hyperthermophiles.

Mesophiles have mid-range temperature optima and are commonly found in terrestrial and aquatic environments and warm-blooded animals (Madigan *et al.*, 2015). Thermophiles have high-temperature optima and are found in unusually hot environments. Hyperthermophiles have very high temperature optima and are found in e.g., hot springs and geysers. Psychrophiles have low-temperature optima (Madigan *et al.*, 2015). Psychrophiles grow optimally at 15°C or lower, maximally at 20°C and have a minimal temperature of 0°C or lower (Morita, 1975). Growth of microorganisms is possible even in solidly frozen materials. This is possible if small pockets of water with concentrated solutes that can be metabolized by the organism

are trapped inside the material. Adaptations of psychrophiles range from producing enzymes that function optimally in cold temperatures, cytoplasmic membranes that are more flexible due to more unsaturated and shorter-chain fatty acids and lastly containing cold-shock proteins. Cold-shock proteins maintain the activity of other proteins and bind specific mRNAs to facilitate their translation (Madigan *et al.*, 2015).

Psychrotolerant (also known as psychrotrophic) bacteria grow optimally at a temperature of 15°C or higher, have a maximum growth temperature of 20°C or higher but have the ability to also grow at low temperatures (Moyer & Morita, 2007). Psychrotolerant microorganisms can be found in soil, water, dairy products, meat, vegetables, cider and fruit stored at standard refrigeration temperatures of 4°C (Madigan *et al.*, 2015). A physicochemical environment is created especially in raw milk which favours the growth of a broad spectrum of psychrotolerant bacteria, predominantly *Pseudomonas* species (Sørhaug & Stepaniak, 1997; Pinto *et al.*, 2006; McPhee & Griffiths, 2011). *Chryseobacterium* species are widely distributed in food sources and most are classified as psychrotolerant and proteolytic spoilage micro-organisms (Hugo *et al.*, 2003; de Beer *et al.*, 2005, 2006; Charimba *et al.*, 2013).

pH

Neutrophiles grow at a pH of 5.5 to 7.9; acidophiles grow at a pH below 5.5 and alkaliphiles grow optimally at pH 8 or higher (Madigan *et al.*, 2015). Some alkaliphiles can be used to produce hydrolytic exo-enzymes, e.g., protease and lipase. Most *Chryseobacterium* species can grow at a pH range of 5 to 7 or others from pH 8 to 10 (Hugo & Jooste, 2012) and, therefore, it may be possible for some members to produce protease. Optimal pH refers to the extracellular environment of an organism, but in order to prevent the destruction of macromolecules the intracellular pH must remain neutral. Buffers are used in batch cultures to maintain the pH from shifting. Most microorganisms grow best at pH 6.6 to 7.5 (Jay *et al.*, 2005). Examples of approximate pH ranges in different food sources are 5.1 to 6.2 for beef; 5.4 to 6.7 for lamb; 5.3 to 6.9 for pork; 6.6 to 6.8 for fish and 6.2 to 6.4 for

poultry (Callow, 1949; Reay & Shewan, 1949). pH affects the functioning of a microorganism's enzymes and the transportation of nutrients into the cell (Jay *et al.*, 2005).

Osmolarity

The influence of water availability on the specific death rate of an organism was investigated by Scott (1936) and today it is defined as water activity (a_w) that states that zero is dry and one is wet. Water availability and solutes like sugar, salt and other substrates are important in an organism's environment (Madigan *et al.*, 2015). Halophiles require NaCl to grow whereas halotolerant organisms grow best in the absence of added solutes. Extreme halophiles can grow in environments with very high salt concentrations. Some halophiles can grow at a a_w as low as 0.75 (Jay *et al.*, 2005). Osmophiles grow in environments with high sugar concentrations and xerophiles live in dry environments (Madigan *et al.*, 2015).

Some osmophiles grow at a a_w of 0.61 while some xerophiles grow at a a_w as low as 0.65 (Jay *et al.*, 2005). The ability of a microorganism to adapt to low a_w by concentrating salts, amino acids and polyols to internal levels helps them extract water from the environment rather than losing water. Most fresh food has a_w above 0.99. Most spoilage bacteria cannot grow below a_w 0.91, therefore, drying food as preservation method prevents microorganisms that require moisture, from growing (Jay *et al.*, 2005).

Oxygen

Organisms that respire oxygen in their metabolism and grow at full oxygen tensions (air is 21% O₂) are called aerobes. In contrast, microaerophiles can only use oxygen when the O₂ is reduced from that present in air (Madigan *et al.*, 2015). Facultative aerobes can grow in the absence of oxygen when the appropriate nutrient and

culture conditions are present. In the laboratory environment, it is necessary to provide enough oxygen to aerobes by shaking a flask or tube on a shaker or by bubbling sterilized air into the medium. Aerotolerant anaerobes cannot use oxygen in their metabolism but can grow in the presence of it whereas obligate anaerobes are killed or inhibited by oxygen (Madigan *et al.*, 2015).

The ease with which a substrate gains or loses electrons is called the oxidation-reduction potential (O/R) of the substrate and is expressed by the symbol Eh (Jay *et al.*, 2005). Organisms show different sensitivities to the O/R potential of their growth medium (Hewitt, 1950). Positive Eh values (oxidized) are required for aerobic microorganisms to grow and negative Eh values (reduced) are required for anaerobic microorganisms to grow. Therefore, juices or plant products and minced meat tend to be spoiled by aerobic microorganisms due to their positive Eh values and solid meat and cheese tend to be spoiled by anaerobic microorganisms due to their negative Eh values (Jay *et al.*, 2005).

Nutrients

Microorganisms require the appropriate source of water, energy, nitrogen, vitamins, growth factors and minerals to grow in food (Jay *et al.*, 2005). Macronutrients, e.g., nitrogen, are required in high quantities and micronutrients such as vitamin B, are required in low quantities (Madigan *et al.*, 2015). Moulds have the lowest nutrient requirement followed by Gram-negative bacteria, yeasts and Gram-positive bacteria (Jay *et al.*, 2005). Sugars, amino acids, fats and alcohols may be utilized by microorganisms as energy sources. Cellulose and starches that are more complex carbohydrates can be degraded into simple sugars.

Microorganisms will firstly utilize simple nitrogen sources like amino acids and thereafter more complex sources, e.g., peptides and proteins (Jay *et al.*, 2005). Some microorganisms can produce their own vitamin B and others rely on the contents available in food. Gram-negative bacteria and moulds can synthesize most or all compounds that they require whereas Gram-positive bacteria rely on the

contents in food. Meat products tend to have more vitamin B and are, therefore, more vulnerable to spoilage by bacteria than moulds (Jay *et al.*, 2005).

2.5. Predictive microbiology

Predictive microbiology is used to develop mathematical equations to determine the behaviour of microorganisms when exposed to different environmental factors, e.g., physical, chemical and competitive (Fakruddin *et al.*, 2011). Microbial behaviour can be predicted under intrinsic environmental conditions, e.g., pH or extrinsic environmental conditions, e.g., salinity or temperature. Responses are evaluated under conditions that are controlled; then mathematical equations are used to explain the results and used to predict combinations of conditions that have not been tested (Hajmeer & Cliver, 2002).

Kinetic models describe the rate of response of either death or growth and examples include: the Gompertz and square root models that describe the rates of response (lag time, maximum population density and specific growth rate) (McMeekin *et al.*, 1993; Whiting & Buchanan, 1994) and inactivation or survival models that describe survival or destruction over time (Xiong *et al.*, 1999). Probability models describe the probability of growth or production of a toxin but do not specify the speed of occurrence (Roberts, 1989).

Mechanistic models are preferred rather than empirical models according to Draper (1988) because they contain fewer parameters, the extrapolation makes better sense and the data fits better. Examples of empirical models are first and second-degree polynomials used in curve fitting like the quadratic response surface model (Gibson *et al.*, 1988) and an example of a mechanistic model is the square root model (McMeekin *et al.*, 1993).

Primary models consider only a single set of conditions and the response can be measured as microbial population density or products produced by the microorganism's metabolism (Fakruddin *et al.*, 2011). Primary models include growth decline models (Whiting & Cyhnarowicz, 1992), exponential growth models (Gibson

et al., 1987; Buchanan *et al.*, 1989), D-values or thermal inactivation (Rodriguez *et al.*, 1988), growth rate values (McMeekin *et al.*, 1987), inactivation/survival models (Kamau *et al.*, 1990; Whiting, 1992) and estimation of lag time or time to formation of turbidity/toxin (Baker & Genigeorgis, 1990).

Secondary models describe how environmental or cultural factors e.g., pH or temperature can affect parameters of primary models (Fakruddin *et al.*, 2011). Secondary model examples are the response surface model (Buchanan & Philips, 1990) and the Arrhenius model (Broughall *et al.*, 1983). Temperature as the only or one of the parameters is most often used (Ross & McMeekin, 1994; Whiting, 1995). In 1889, Svante Arrhenius correlated temperature and the growth rate constant through one equation that is still named after him, the Arrhenius equation. The Arrhenius equation ($\mu_{\max} = Ae^{-(E/RT)}$) consists of μ which is the specific growth rate; A is an entropy constant; E is the activation energy (temperature coefficient); R is the universal gas constant ($8.314 \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$) and T which is the absolute temperature measured in Kelvin. The thermal inactivation theory assumes that the logarithm of the specific death rate decreases linearly as the temperature increases (Baranyi & Roberts, 2004).

Tertiary models use the combination of secondary models, e.g., the response of the microorganism under two salt levels or growth of different microorganisms at the same time (Buchanan, 1991).

A study performed by Bekker and co-workers (2015) determined the growth kinetics of *Chryseobacterium joostei* which was isolated from milk (Hugo *et al.*, 2003). A temperature gradient incubator (TGI) that consists of an aluminium bar that is heated at one end and cooled at the other to achieve a stable temperature gradient, was used. The organism was firstly grown in nutrient broth no. 2, transferred to sterile TGI tubes and then placed in the sample well of the TGI. The bar of the TGI rocks to provide aeration and mixing of the samples. Temperatures measured in the TGI can range from 14 to 50°C. Growth can be measured by means of OD measurements. Microsoft Excel (Microsoft Corporation, Washington, USA) can be used to determine the specific growth rate by linear regression analysis of the exponential phase of the growth curve.

The validation of models is done firstly by internal validation (curve fitting) where new data and new variables are tested to determine the accuracy and sufficiency of the model (Fakruddin *et al.*, 2011). The second validation, external validation, is used to predict the response of microorganisms in real food. Gill and co-workers (1997) report that models developed in laboratory broth systems are inappropriate for the description of growth in food. However, Ross and McMeekin (1994) and Whiting (1995) are of the opinion that as long as factors that are relevant, are equal, the effect of a factor is not dependent on whether the microorganism has been grown in broth or food.

Predictive models are limited by the fact that some cannot be extrapolated outside certain ranges in which they were derived; faster growth rates are predicted rather than observed; models are used in laboratory media, validated in food but cannot necessarily be used in the food industry (Fakruddin *et al.*, 2011). Models derived under conditions that are static are usually not applicable in fluctuating conditions and previous incubation conditions can affect the growth rate of the microorganism thereafter (Walker *et al.*, 1990; Fu *et al.*, 1991; Buchanan & Klawitter, 1991). It is a problem to model the lag phase duration in foods (Baranyi *et al.*, 1995), not because of the lack of a model that is suitable but because of limited knowledge of the physiological status of microorganisms (Fakruddin *et al.*, 2011).

Environmental factors can be measured in some situations but others, e.g., chilling of meat carcasses, are difficult (McMeekin *et al.*, 1997). Structure of food, microbial growth, and interactions between different microorganisms are the main factors that make predictions in food difficult (Koutsoumanis *et al.*, 2006). The microbial diversity and the composition of the growth substrate are the main differences between laboratory and real food conditions (Pin *et al.*, 1999). To eliminate the microbial diversity factor, e.g., meat may be irradiated and ultra-heat-treated (UHT) milk can be used in experiments focussed on real food (Pin *et al.*, 1999).

Predictive models can be applied in HACCP e.g., which is a system that combines the identification of pathogens, their routes of entry, a method of elimination and product handling to prevent potential food safety problems when manufacturing and distributing a product (Fakruddin *et al.*, 2011). Predictive modelling can be seen as an extension of HACCP.

The development of techniques to determine water activity, pH and redox potential might provide information that will be beneficial especially in food that is complex (Fakruddin *et al.*, 2011). The development of comprehensive models for spoilage organisms needs more attention (Whiting, 1997). Predictive models are research tools and not very effectively applied in the food industry (Koutsoumanis *et al.*, 2006) and, therefore, the accuracy of models should be improved so that realistic predictions can be made (Ross, 1996; Neumeyer *et al.*, 1997; Baranyi *et al.*, 1999).

2.6. Conclusions

The genus *Chryseobacterium* is still expanding today and represents a broad range of species. Since the inception of the family *Flavobacteriaceae*, it has been more organised and less challenging to classify genera in the family. The presence of the genus *Chryseobacterium* is indeed significant in different environments ranging from soil, diseased animals, humans and food to industrial premises. Food spoilage due to *Chryseobacterium* species has been observed in poultry, red meat, fish, milk and milk products.

In order for spoilage characteristics to be determined, the specific *Chryseobacterium* species should first be identified. Polyphasic techniques are important in describing new species and include genotypic methods, e.g., whole-genome sequencing; phenotypic methods, e.g., morphology and chemotaxonomic methods, e.g., fatty acid methyl ester analysis.

The rate at which an organism may spoil food can be determined by growth kinetic studies and can only be investigated if a thorough background of a microorganism's life cycle is understood. Life cycles of microorganisms generally consist of a lag phase, exponential phase, stationary phase and death phase. The microbial growth of viable cells can be determined either by different methods of cell counting or by turbidity measurements.

Colonization of a food product by microorganisms depends on intrinsic and extrinsic parameters. Intrinsic parameters include pH, osmolarity (water activity), oxidation-

reduction potential (Eh) and nutrients and an example of an extrinsic parameter is temperature. In predictive microbiology, equations are formulated to predict the behaviour of microorganism due to changes in ecological parameters. Although temperature is used as the only or one of the parameters in the Arrhenius equation, other models also exist. The Arrhenius equation will, however, be used to determine the effect of temperature on the growth of a novel *Chryseobacterium* strain and reference strains. Linking the presence of microbiota in food and ecological factors together, play a significant role in food safety and quality.

CHAPTER 3

POLYPHASIC STUDY AND SPECIES DESCRIPTION OF A NOVEL *Chryseobacterium* SPECIES ISOLATED FROM POULTRY FEATHER WASTE

Abstract

The strain 7_F195^T was previously isolated from chicken feather waste collected from an abattoir in Bloemfontein. A polyphasic approach was followed to determine if strain 7_F195^T belongs to the genus *Chryseobacterium* and if the organism can be classified as a new species. Whole genome and 16S rRNA gene sequencing were used to determine the closest neighbours of strain 7_F195^T using various phylogenetic methods and overall genome related indexes (OGRIs). Reference strains *C. flavum*, *C. gleum* (also the type species) and *C. arthrophaerae* were selected based on being the nearest phylogenetic neighbours and having the highest ANI, AAI and DDH values compared to strain 7_F195^T. The predominant fatty acids were iso-C_{15:0}, iso-C_{17:1} ω_{9c} and iso-C_{17:0} 3-OH and the most abundant polar lipid was phosphatidylethanolamine. Menaquinone-6 was the only respiratory quinone. These results supported the affiliation of strain 7_F195^T to the genus *Chryseobacterium*. The DNA G+C content of strain 7_F195^T was 38.6 mol%. The digital DNA-DNA hybridization values for strain 7_F195^T with *C. flavum*, *C. gleum* and *C. arthrophaerae* were 23.7, 24.9 and 24.7%, respectively. Based on the data generated from this polyphasic study, strain 7_F195^T represents a novel *Chryseobacterium* species for which the name *Chryseobacterium pennipullorum* sp. nov. is proposed.

3.1. Introduction

Chryseobacterium, a genus of the family *Flavobacteriaceae* was proposed in 1994 and six species previously included in the genus *Flavobacterium* were reclassified using a polyphasic study, as members of *Chryseobacterium* (Vandamme *et al.*,

1994a). The six species were *C. scophthalmum*, *C. indologenes*, *C. gleum*, *C. balustinum*, *C. indoltheticum* and *C. meningosepticum*. *Chryseobacterium* species isolated from different environments such as insects, milk, poultry, humans, fish, water, plants, beer-bottling factories, soil and lactic acid beverages are mostly saprophytic in habitats (Zhou *et al.*, 2007).

Classification is defined as placing organisms into different groups based on their different and similar characteristics (Lawrence 2011). Developing a process that makes identification and classification easy, stable and objective is the main goal in microbial taxonomy (Chun *et al.*, 2018). Classifications that were used in the past relied more on morphological and physiological characteristics, but the use of genetic information of an organism is becoming more common (Gevers *et al.*, 2006).

Molecular techniques are developing rapidly and among the criteria are 16S rRNA gene sequencing, DNA-DNA hybridization (DDH), DNA G+C content (Prakash *et al.*, 2007) and pairwise genome-sequence derived similarity (Chun *et al.*, 2018). DNA-DNA hybridization is still known as the 'gold standard' for delineating species of prokaryotes (Wayne *et al.*, 1987), but pairwise genome-sequence derived similarity has been proposed to replace this method. Overall genome related indexes (OGRIs) are values that are analogous to DDH values and can be used to determine if a strain belongs to a known species. Average nucleotide identity (ANI), amino acid identity (AAI) and digital DDH (dDDH) are examples of methods readily used (Chun *et al.*, 2018). Combining 16S rRNA similarity values and OGRIs can be used to identify a new species.

However, before the existence of molecular techniques, phenotypic methods were used in bacterial taxonomy (Prakash *et al.*, 2007) to place a new taxon in the appropriate genus for comparison with already described taxa (Bernadet *et al.*, 2002). Automated systems such as API 20 NE, API 20 E, API ZYM and BIOLOG GEN III MicroPlate system were developed to make the phenotypic testing more efficient and standardized. The API strips contain 20 biochemical tests and are an easy, reliable method (The Global Health Network, 2013). The BIOLOG GEN III MicroPlate system contains 94 phenotypic tests in total (BIOLOG, 2013).

Cellular morphology can be studied through scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Tindall *et al.*, 2010). Scanning electron

microscopy is used to examine the outer surface of the cell while TEM is more focused on the internal infrastructure of the cell, structure of membranes as well as the presence of inclusions in the cytoplasm.

Phenotypic methods involve judgement that is substantially subjective and it is, therefore, necessary to include other methods such as cellular fatty acid profiles (Von Graevenitz *et al.*, 1991; Stager & Davis, 1992). Analysis of cellular fatty acids, polar lipids and respiratory lipoquinones are added to provide a complete species description (Prakash *et al.*, 2007).

The aims of this study were as follows: perform whole-genome and 16S rRNA gene sequencing on strain 7_F195^T isolated from chicken feather waste; determine the closest neighbours of strain 7_F195^T using various phylogenetic methods and OGRIs; capture SEM images of the strain 7_F195^T and its reference strains including the type species of the genus and capture TEM images of strain 7_F195^T; use conventional methods and commercial system to determine the phenotypic characteristics of strain 7_F195^T and finally combine this with results from fatty acid profile, polar lipids and respiratory lipoquinone, of strain 7_F195^T in order to give a full description of this strain.

3.2. Materials and methods

3.2.1. Cultures used and maintenance

The strain 7_F195^T was previously isolated from chicken feather waste collected from an abattoir in Bloemfontein and the reference strains (Table 3.1) were obtained from international culture collections freeze-dried in ampoules. The cultures were revived in 10 ml nutrient broth no. 2 (Oxoid CM67) for 48 h at 25°C. The cultures were streaked out on nutrient agar (Oxoid CM003) and incubated for 24 h at 25°C, thereafter purity of the cultures was examined by Gram-staining. For short-term maintenance the cultures were freeze-dried on AA Whatman filter paper discs and stored at -20°C, the cultures were also stored on nutrient agar slants at 4°C and re-

streaked every four to six weeks. For long-term maintenance, cultures were freeze-dried in glass vials and stored at -20°C.

Table 3.1. Strain 7_F195^T and the nearest phylogenetic neighbours.

Genus and species	Culture collection^a	Source of isolation	Year of isolation	Reference
Strain 7_F195 ^T	UFSBC 710	Chicken feathers	2010	Charimba, 2012
<i>Chryseobacterium flavum</i>	KCTC 12877 ^T	Soil	2006	Zhou <i>et al.</i> , 2007
<i>Chryseobacterium gleum</i>	NCTC 11432 ^T	Vaginal swab	1983	Holmes <i>et al.</i> , 1984; Vandamme <i>et al.</i> , 1994a
<i>Chryseobacterium arthrophaerae</i>	DSM 25208 ^T	Pill millipede	2009	Kämpfer <i>et al.</i> , 2010a

^a, University of the Free State Bacterial Culture Collection (UFSBC); National Collection of Type Cultures (NCTC); Korean Collection of Type Cultures (KCTC); Deutsche Sammlung von Mikro-organismen (DSM).

3.2.2. 16S rRNA gene sequencing

The genomic DNA from strain 7_F195^T was extracted using the NucleoSpin® kit (Macherey-Nagel) and the protocol and buffers used were according to manufacturer's instructions. The steps were as follows: After the culture was revived and examined for purity by Gram-staining, the culture was transferred to 10 ml fresh nutrient broth no. 2 and incubated for 24 h at 25°C. The cell suspension was centrifuged at 3000 x g for 10 minutes at 4°C using an Eppendorf 5417R centrifuge

and the supernatant decanted. This was repeated until approximately 40 mg of wet microbial cell pellet was retrieved. The cells were resuspended using buffer BE. The cell suspension was transferred to a NucleoSpin® Bead Tube Type B. The cells were lysed by adding 40 µl MG buffer and 10 µl liquid proteinase K and vortexed for 20 minutes. The DNA binding conditions were adjusted by adding 600 µl MG buffer and mixed by vortex for three seconds.

The suspension was centrifuged at 11 000 x *g* for 30 seconds and the supernatant transferred onto the NucleoSpin® microbial DNA column, placed in a two millilitre collection tube. The supernatant was centrifuged at 11 000 x *g* for 30 seconds, the flowthrough was discarded and the column placed into a fresh collection tube. The column was firstly washed by adding 500 µl BW buffer and centrifuged at 11 000 x *g* for 30 seconds, the flowthrough was discarded. The wash was repeated by adding 500 µl B5 buffer to the column and centrifuged at 11 000 x *g* for 30 seconds, the flowthrough was discarded. Residual wash buffer was removed by centrifugation at 11 000 x *g* for 30 seconds. The DNA was eluted by placing the column in a 1.5 ml nuclease-free tube, adding 100 µl BE buffer, incubating the column at room temperature for 1 minute and finally centrifuged at 11 000 x *g* for 30 seconds.

The reaction mixture for the PCR-amplification of the 16S rRNA gene region was as follows: 2 µl (10 mM) dNTPs; 1 µl (10 mM) of the forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3'); 1 µl (10 mM) of the reverse primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991); 5 µl of 10x Taq Thermopol buffer (New England Biolabs); 1 unit (0.3 µl) Thermopol Taq polymerase (New England Biolabs); 1 µl Template DNA; 39.7 µl H₂O (total of 50 µl reaction volume). PCR-amplification was performed using an Applied Biosystems 2720 Thermal Cycler. The following reaction conditions were used: Initial denaturation at 94°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 115 seconds; final elongation stage at 72°C for 5 minutes. The PCR product was visualized on a 1% agarose gel. The amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions.

Sanger sequencing was done using the BigDye® Terminator v.3.1 Cycle Sequencing Kit on the Applied Biosystems 3130xl sequencer. Sequences obtained

were edited using Geneious 9. BLAST (Basic Local Alignment Search Tool) was used to compare the 16S rRNA sequence of strain 7_F195^T to sequences in Genbank database (<https://www.ncbi.nlm.nih.gov/>).

3.2.3. Whole-genome sequencing

After strain 7_F195^T was preliminarily identified as belonging to the genus *Chryseobacterium* using its 16S rRNA sequence and BLAST analysis, it was subjected to next-generation sequencing. The process was performed by the Next-Generation Sequencing Unit, Department of Medical Virology, Faculty of Health Sciences, University of the Free State, Bloemfontein, South Africa. The genomic material was prepared for sequencing according to the manufacturer's instructions using the Nextera® XT DNA Library Prep Reference Guide (Illumina). The genome was sequenced using an Illumina MiSeq sequencer and the assembly performed on PATRIC (<https://www.patricbrc.org/>) using the assembly method, Spades 3.10.0.

The genome of strain 7_F195^T was uploaded to the RAST (Rapid Annotation with Subsystems Technology) website (<http://rast.nmpdr.org>) for annotation. Genome related data including genome size, gene number, G+C content, coverage, number of contigs, N50 value, full 16S rRNA sequence etc., were obtained through RAST. The whole-genome shotgun project has been deposited at DDBJ/ENA/Genbank (<https://www.ncbi.nlm.nih.gov/>) under the following information: Accession, QNVV00000000; SUBID, SUB4224642; BioProject, PRJNA478529; BioSample, SAMN09519216. The version described in this paper is version QNVV01000000. Tools used to calculate digital DNA-DNA hybridization value (dDDH), average nucleotide identity (ANI), average amino acid identity (AAI) and creating the Venn diagram are shown in Table 3.2.

Table 3.2. Tools for calculating dDDH, ANI, AAI and creating the Venn diagram.

Method	Tool	URL/Reference
dDDH	Genome-Genome Distance Calculator (GGDC 2.0, formula 2)	German Culture collection DSMZ http://ggdc.dsmz.de/
ANI	Kostas lab ANI calculator	http://enve-omics.ce.gatech.edu/ani/
AAI	Newman lab ROSA calculator	http://lycofs01.lycoming.edu/~newman/ROSA.html
Venn diagram	Venn Diagram Data and Generator V 2.1	http://lycofs01.lycoming.edu/~newman/CurrentResearch.html

3.2.4. Phylogenetic tree construction methods

The 16S rRNA gene sequence of strain 7_F195^T obtained from whole-genome sequencing was compared to sequences of *Chryseobacterium* species obtained from EzTaxon (<http://www.ezbiocloud.net/>). Phylogenetic trees were constructed as described by Bernardet and co-workers (2002) by using the MEGA software version 7 (Tamura *et al.*, 2016). The Maximum Likelihood method (Tamura *et al.*, 2004) and the Neighbour-Joining method (Saitou & Nei, 1987) with the parameter distance measure Kimura two (KP2) was used. Bootstrap analysis was used to determine the confidence values at a 1000 replicates. 16S rRNA similarity values were not only obtained from EzBioCloud but were also calculated using CLUSTAL 2.1 (<https://www.ebi.ac.uk/Tools/msa/muscle/>).

3.2.5. Microscopy

Scanning and transmission microscopy were performed by the Centre of Microscopy at the University of the Free State.

Scanning electron microscopy (SEM)

The cell material was fixed for at least three hours in 0.1 M (3%) sodium phosphate-buffered glutardialdehyde (pH 7) and thereafter fixed for one hour in osmium tetroxide (1%). The cell material was collected on polycarbonate membrane filters of 0.2 μm . A graded ethanol series (50%, 70% and 95% for 20 minutes in each phase followed by two changes of 100% ethanol for one hour in each phase) was used to dehydrate the cell material. A critical point dryer (Tousimis, Maryland, U.S.A) was used to dry the cell material. The cell material was mounted on stubs (Cambridge pin type, 10 mm) by carbon tape after drying and coated with ± 60 nm gold using the Bio-Rad sputter coat (United Kingdom). A JSM-7800F Extreme-resolution Analytical Field Emission SEM (FE-SEM) was used to examine the cells.

Transmission electron microscopy (TEM)

The cell material was fixed for at least three hours in 0.1 M (3%) sodium phosphate-buffered glutardialdehyde (pH 7). The samples were gently pelleted, washed and re-suspended in the buffer after fixation. A drop of the cell suspension was transferred onto a formvar grid and air dried. The samples were negatively stained with 2% uranyl acetate dissolved in distilled water. A Philips CM100 Transmission Electron Microscope (FEI, The Netherlands) was used to examine the samples.

3.2.6. Phenotypic tests

Conventional phenotypic tests

The tests were performed according to Bernardet and co-workers (2002) described in “Proposed minimal standards for describing new taxa of the family *Flavobacteriaceae* and emended description of the family”. The cultures were streaked out on nutrient agar (Oxoid SR0054C) and incubated for 24 h at 25°C. Gram-staining, cell morphology, oxidase and catalase were determined according to MacFaddin (1980). The odour of the cultures was noted.

Gliding motility was determined by the hanging drop technique (Bernardet *et al.*, 2002) where a drop of cell suspension was placed on a coverslip and turned upside down. Observation of the bacteria took place through the coverslip and gliding was checked on the edge of the drop including the bottom surface of the coverslip. Determination of flexirubin-type pigment production was carried out by flooding a mass of cells smeared on a glass slide with 20% (w/v) potassium hydroxide (Bernardet *et al.*, 2002). If the flexirubin type pigment was present colonies turned from yellow to red, purple or brown. An acidic solution (1 N HCl) was used to remove the excess KOH and revert the cells to their initial colour (Reichenbach, 1989).

Biochemical tests were carried out by using cell suspensions that were grown under standard conditions of 25°C, in nutrient broth no. 2 for 24 h. The cell suspensions were centrifuged at 3000 x g for 10 minutes at 4°C using a Beckman Coulter, Avanti J-26 XPI centrifuge and the supernatant decanted. The cells were washed with 0.1 M phosphate buffer (pH 7) and suspended in 0.1 M phosphate buffer (pH 7) McFarland number 1 (Difco 0691326) density standard to standardize the cell suspension. A multi-inoculation device was used to perform the inoculations.

The following phenotypic tests were performed according to Cowan (1974) and MacFadden (1980) unless otherwise indicated: growth on MacConkey agar (Oxoid CM007), cetrimide agar (Oxoid CM0579) and β -hydroxybutyrate; growth in anaerobic conditions using a 2.5 L anaerobic jar and Anaerogen™ 2.5 L Thermo Scientific sachets; growth at different temperatures (5, 15, 25, 32, 37 and 42°C); growth at pH

4 – 7 (adjusted with citrate–phosphate buffer) and 7.5 – 8.5 (adjusted with TRIS-hydrochloride buffer), at intervals of 0.5; growth in sodium chloride (0 – 6% (w/v), in increments of 0.5%); H₂S production on Kligler iron agar (Oxoid CM003); hydrolysis of gelatin (liquefaction), esculin (Yabuuchi *et al.*, 1990), starch (West & Colwell, 1984), Tween 80 (West & Colwell, 1984), Tween 20, tyrosine (Barrow & Feltham, 1993); production of DNase, urease (Oxoid CM0053), β -galactosidase (ONPG), lecithinase, phenylalanine deaminase; nitrate and nitrite reduction; acid production from adonitol, L-arabinose, cellobiose, dulcitol, ethanol, D-fructose, D-glucose, glycerol, D-xylose, lactose, maltose, D-mannitol, raffinose, rhamnose, salicin, sorbitol, sucrose and trehalose.

Automated tests

API tests

The cultures were subjected to API 20 NE, API 20 E and API ZYM test systems (API[®], bioMérieux Inc., France). Cell suspensions for API test systems were prepared as follows: API 20 NE cell suspension in 2 ml 0.85% NaCl according to McFarland No. 0.5; API 20 E cell suspension in 5 ml 0.85% NaCl according to McFarland No. 1; API ZYM cell suspension in 2 ml 0.85% NaCl according to McFarland No. 5. The strips were inoculated according to the instructions on each kit and incubated at 25°C for the period specified on the kits (API 20 NE for 24 h, API 20 E for 24 h and API ZYM for 4 h).

BIOLOG Omnilog Gen III system

Strain 7_F195^T and reference strains were profiled in triplicate using the BIOLOG Omnilog Gen III identification system (BIOLOG Inc., Hayward, CA, USA) according to manufacturer's instructions. The steps were as follows: the cultures were

cultivated on nutrient agar for 24 h at 25°C (see 3.2.1); a cotton-tipped Inoculatorz swab was used to pick up a 3 mm diameter area of cell growth from the surface of the agar plate; the bacteria were released in the inoculating fluid (IF) by rubbing the tip of the swab against the bottom of the tube; the tube was stirred with the swab to obtain a uniform cell suspension; the cell density was read on the turbidity meter after it was calibrated and blanked with an uninoculated IF tube; the cell suspension was poured in a multichannel pipet reservoir and the microplate wells were each filled with 100 µl cell suspension using a 8-Channel Repeating pipettor. The microtitre plates were covered with a lid and incubated for 24 h at 25°C. The plates were read at a wavelength of 595 nm using a BIOLOG Omnilig Gen III station.

3.2.7. Chemotaxonomic methods

Fatty acid methyl ester analysis

Analysis of cellular fatty acids was carried out by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany. The cultures were grown on nutrient agar for 24 h at 25°C. Total culture growth of 40 mg of each isolate was scraped off from the agar plate, saponified, methylated and extracted using minor modifications of the method of Miller (1982) and Kuykendall *et al.* (1988). The Sherlock Microbial Identification System (MIS) (MIDI, Microbial ID, Newark, DE 19711 U.S.A.) was used to separate the fatty acid methyl ester mixtures. The system consists of an Agilent model 6890N gas chromatograph fitted with a 5% phenyl-methyl silicone 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: the carrier gas was ultra-high-purity hydrogen; column head pressure was at a value of 60 kPa; injection volume was 2 µl; column split ratio was 100:1; septum purge was 5 ml/min; column temperature ranged from 170 to 270°C at 5°C/min; injection port was at a temperature of 240°C; and temperature of the detector was at 300°C. The MIS

standard software (Microbial ID) version 6.1 was used to calculate the percentages and the names of the fatty acids. The peaks were automatically integrated.

Polar lipids

Analysis of polar lipids was carried out by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany. The two-stage method described by Tindall (1990a; 1990b) was used to first extract the respiratory lipoquinones followed by the polar lipids. Polar lipids were extracted from 100 mg of freeze-dried cell material of each isolate. Polar lipids were separated by two-dimensional silica gel thin layer chromatography (Macherey-Nagel Art. No. 818 135). The first direction was developed in chloroform:methanol:water (65:25:4, v/v/v) and the second in chloroform:methanol:acetic acid:water (80:12:15:4, v/v/v/v). Molybdotophosphoric acid was used to detect the total lipid material and spray reagents, specific for defined functional groups, were used to detect specific functional groups (Tindall *et al.*, 2007).

Respiratory lipoquinones

Analysis of respiratory lipoquinones was carried out by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany. As mentioned before, the two-stage method described by Tindall (1990a; 1990b) was used to extract the respiratory lipoquinones. Respiratory lipoquinones were extracted from 100 mg of freeze-dried cell material of strain 7_F195^T, using methanol:hexane, followed by phase separation into hexane. Analysis followed after the respiratory lipoquinones were extracted. Respiratory lipoquinones were separated into their different classes (e.g., menaquinones and ubiquinones) by thin layer chromatography on silica gel (Macherey-Nagel Art. No. 805 023), using the solvent hexane:tert-butylmethylether (9:1 v/v). HPLC was used to further analyse the UV absorbing bands corresponding to the different quinone classes. This was carried out on a LDC Analytical HPLC (Thermo Separation Products) fitted with a reverse phase column (Macherey-Nagel,

2 mm x 125 mm, 3 μ m, RP18) using methanol:heptane 9:1 (v/v) as the eluant. Detection of respiratory lipoquinones is at 269 nm.

3.3. Results and discussion

3.3.1. 16S rRNA sequencing

PCR-amplification and PCR product visualization (Figure 3.1) of the 16S rRNA gene region of strain 7_F195^T was successful. Results obtained from BLAST confirmed that strain 7_F195^T shows the highest similarity to the genus *Chryseobacterium*.

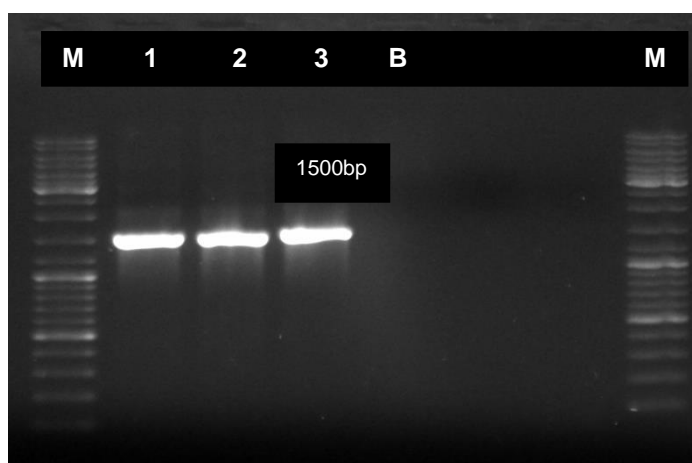


Fig. 3.1. Electropherogram of the 1500 bp PCR product of the 16S rRNA region of strain 7_F195^T. M, DNA molecular marker; 1, strain 1_F178^T (Not relevant to this study); 2, strain 5_R23647^T (Not relevant to this study); 3, strain 7_F195^T; B, No template control.

3.3.2. Phylogenetic trees

The phylogenetic trees were constructed using two different methods as can be seen in Figure 3.2 (Neighbour-joining method) and Figure 3.3 (Maximum Likelihood method). The reliability of the branching is validated by using the bootstrap analysis (Bernardet *et al.*, 2002). Although bootstrap values were very low, both phylogenetic

trees indicated that strain 7_F195^T formed a separate lineage from the other *Chryseobacterium* species in the trees. These results suggested that strain 7_F195^T belonged to the *Chryseobacterium* genus and the separate lineage indicated that this strain could be regarded as a new species in the genus.

The nearest neighbours based on 16S rRNA sequence similarity values (indicated in brackets) were *C. flavum* (98.42%), *C. indologenes* (98.24%), *C. tructae* (98.22%), *C. lactis* (98.19%), *C. vietnamense* (97.99%), *C. viscerum* (97.90%), *C. gleum* (97.71%), *C. cucumeris* (97.70%), *C. rhizoplanae* (97.69%) and *C. arthrosphaerae* (97.65%). Strains that have 16S rRNA similarity values of 98.7% or less are regarded as different species (Chun *et al.*, 2018).

Based on this data and the separate lineage in the phylogenetic trees, *C. flavum* (nearest neighbour), *C. gleum* (type species of *Chryseobacterium*) and *C. arthrosphaerae* (part of the closest lineage to strain 7_F195^T) were chosen as reference strains used in this study. The updated 16S rRNA gene sequence derived from the resequenced whole genome of *C. flavum* along with the previous sequence were included in both trees.

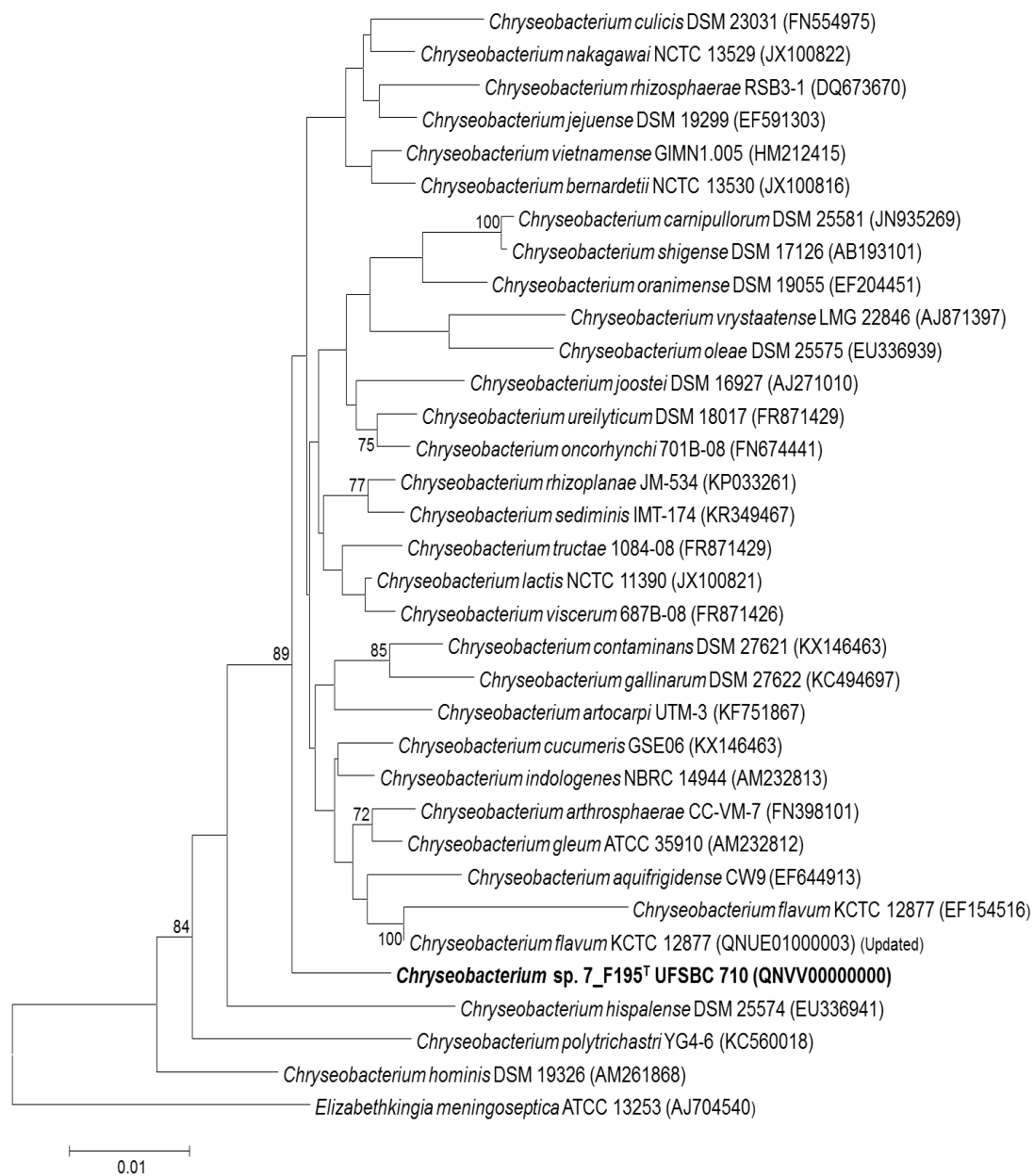


Fig. 3.2. The evolutionary history of strain 7_F195^T, nearest *Chryseobacterium* species and outgroup (*Elizabethkingia meningoseptica*) was inferred using the Neighbour-Joining method based on 16S rRNA gene sequences obtained from the EzTaxon database (accession numbers are given in parentheses). The bootstrap values >70%, based on 1000 replicates, are given as percentages at the branching points. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site, Bar 0.01. The analysis involved 34 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1319 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura *et al.*, 2016).

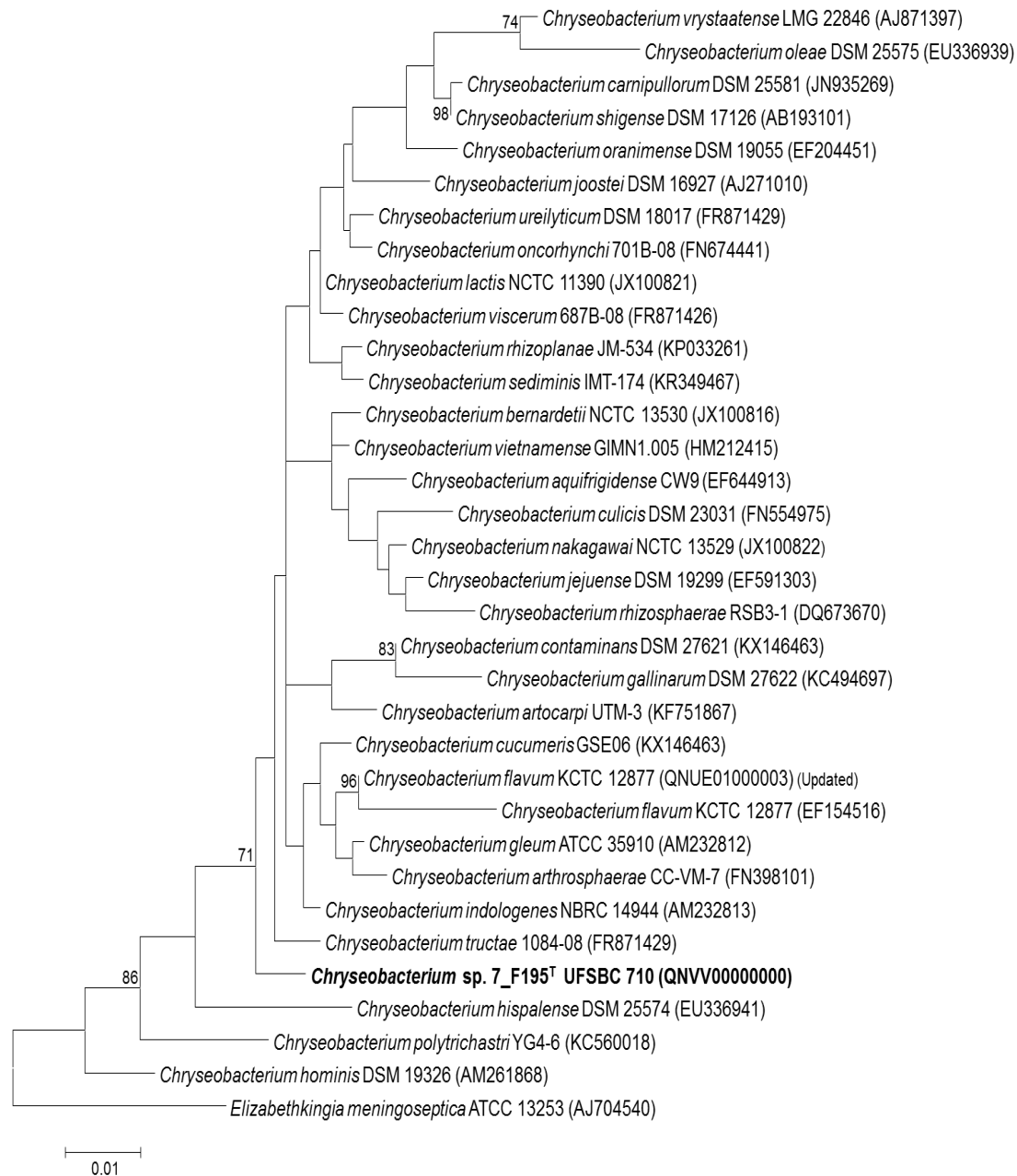


Fig. 3.3. The evolutionary history of strain 7_F195^T, nearest *Chryseobacterium* species and outgroup (*Elizabethkingia meningoseptica*) was inferred using the Maximum Likelihood method based on 16S rRNA gene sequences obtained from the EzTaxon database (accession numbers are given in parentheses). The bootstrap values >70%, based on 1000 replicates, are given as percentages at the branching points. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site, Bar 0.01. The analysis involved 34 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1319 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura *et al.*, 2016).

3.3.3. Whole-genome sequencing

General genome features of strain 7_F195^T were as follows: genome size, 4,796,535 bp; number of contigs, 61; coding sequences (CDS), 4348; N50 value, 208445 and coverage, 33.0x.

A summary of the G + C content, 16S rRNA similarity values, DDH, ANI and AAI values between strain 7_F195^T and the reference strains can be seen in Table 3.3. Strain 7_F195^T contains a G+C value of 38.6 mol% (Table 3.3) and can be classified as a member of the genus *Chryseobacterium* that has G+C values ranging from 29-39 mol% (Bernardet *et al.*, 2011).

Strains that have DNA-DNA similarity values of 70% or more are classified as part of the same species (Wayne *et al.*, 1987; Bernardet *et al.*, 2002). The digital DNA-DNA reassociation values from whole-genome sequencing for strain 7_F195^T with *C. flavum*, *C. gleum* and *C. arthrosphaerae* were 23.7, 24.9 and 24.7%, respectively. The DDH values obtained from DSMZ for strain 7_F195^T with *C. gleum* and *C. arthrosphaerae* were 22.9 and 31.2% respectively. *Chryseobacterium flavum* was not subjected to DDH at DSMZ with the other strains because at the time the similarity value between *C. flavum* and strain 7_F195^T was less than 97%.

Strains that have ANI (Average Nucleotide Identity) values of 95~96% or higher are classified as part of the same species (Richter & Rosselló-Móra, 2009). The ANI values of strain 7_F195^T compared to *C. flavum*, *C. gleum* and *C. arthrosphaerae* were 81.45, 82.38 and 82.38%, respectively.

Strains that have AAI (Average Amino acid Identity) values of 95% or higher are classified as part of the same species (Konstantinidis & Tiedje, 2005). The AAI values of strain 7_F195^T compared to *C. flavum*, *C. gleum* and *C. arthrosphaerae* were 84.94, 85.14 and 85.73%, respectively.

Table 3.3. 16S rRNA similarity values, OGRIs and G + C content of strain 7_F195^T and ten nearest *Chryseobacterium* strains. DDH, DNA-DNA hybridization; dDDH, digital DNA-DNA hybridization; ANI, Average Nucleotide Identity; AAI, Average Amino acid Identity; ND, not determined. Strains in bold were used as references in this study.

Strains	Compared to strain 7_F195 ^T (%)						
	16S rRNA similarity values		DDH	ANI	AAI	G + C	
	CLUSTAL 2.1	EzBio	dDDH	DDH (DSMZ)			
<i>C. flavum</i>	98.42	ND	23.7	ND	81.45	84.94	36.7
<i>C. indologenes</i>	98.24	98.26	23.7	ND	81.86	85.39	37.2
<i>C. tructae</i>	98.22	98.22	ND	ND	ND	ND	ND
<i>C. lactis</i>	98.19	98.19	24.2	ND	82.06	84.91	36.1
<i>C. vietnamense</i>	97.99	97.98	ND	ND	ND	ND	ND
<i>C. viscerum</i>	97.90	98.12	24.9	ND	82.40	85.16	36.2
<i>C. gleum</i>	97.71	97.78	24.9	22.9	82.38	85.14	36.8
<i>C. cucumeris</i>	97.70	97.85	24.8	ND	82.33	85.34	36.1
<i>C. rhizoplanae</i>	97.69	97.88	25.1	ND	82.47	85.45	36.4
<i>C. arthrosphaerae</i>	97.65	97.78	24.7	31.2	82.38	85.73	38.3

The Venn diagram (Figure 3.4) illustrates the number of genes shared between strain 7_F195^T and related *Chryseobacterium* species; the number of unique genes in each isolate and total genes amongst all isolates. Strain 7_F195^T has a total of 540 unique genes and there are 2786 genes shared by all the strains.

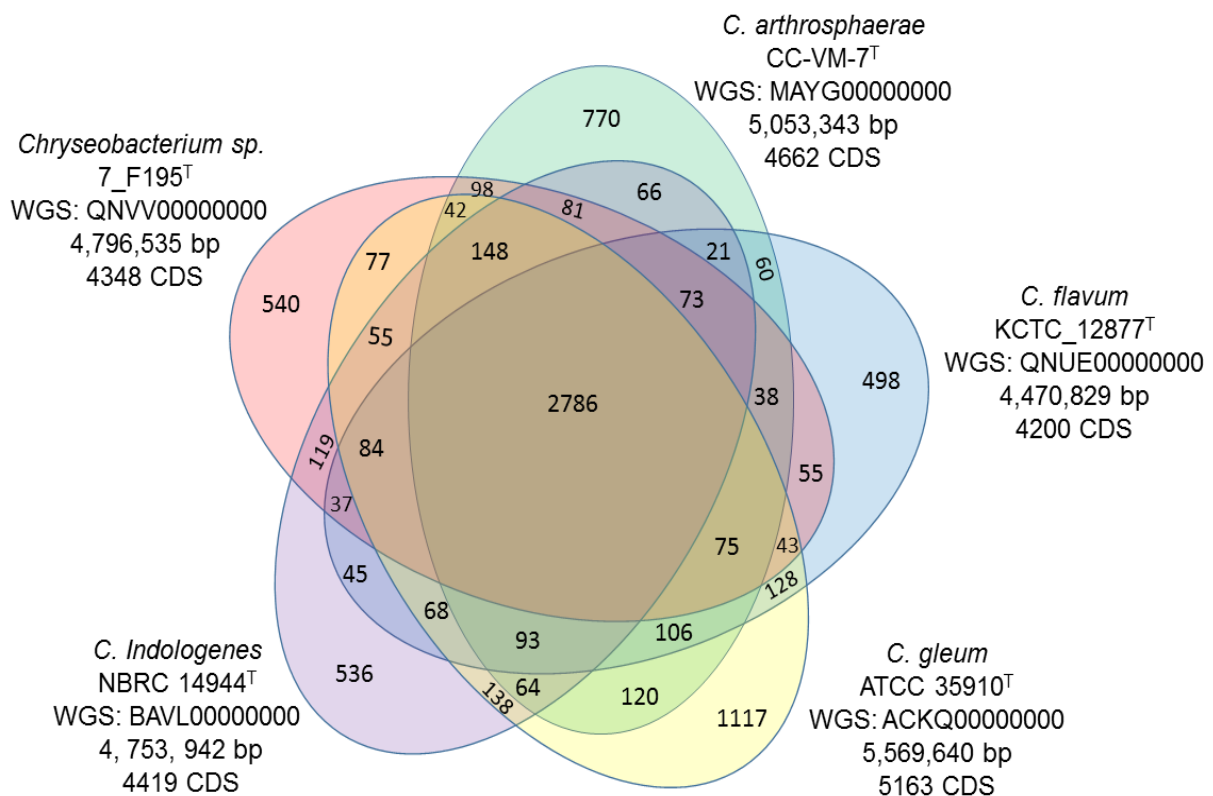


Fig. 3.4. Venn diagram illustrating the number of shared and unique coding sequences (CDS) among strain 7_F195^T, *Chryseobacterium flavum* KCTC 12877^T, *Chryseobacterium indologenes* NBRC 14944^T, *Chryseobacterium arthrosphaerae* CC-VM-7^T and *Chryseobacterium gleum* ATCC 35910^T.

Results obtained from 16S rRNA similarity values, DDH, ANI and AAI, conclude that strain 7_F195^T does not belong in the same species as *C. flavum*, *C. gleum* or *C. arthrosphaerae*. Based only on these results, strain 7_F195^T may possibly be identified as a new species, but further phenotypic and chemotaxonomic tests were nevertheless performed on strain 7_F195^T and its nearest neighbours.

3.3.4. Microscopy

Scanning electron microscopy (SEM)

The cell morphology of the cultures used can be seen in Figures 3.5 – 3.8. Strain 7_F195^T can be differentiated from *C. flavum* and *C. arthrospiraerae* by a less coarse surface and less string-like structures on the surface of strain 7_F195^T. Strain 7_F195^T can be differentiated from *C. gleum* by the presence of a coarser surface and string-like structures with *C. gleum* having a smoother surface.

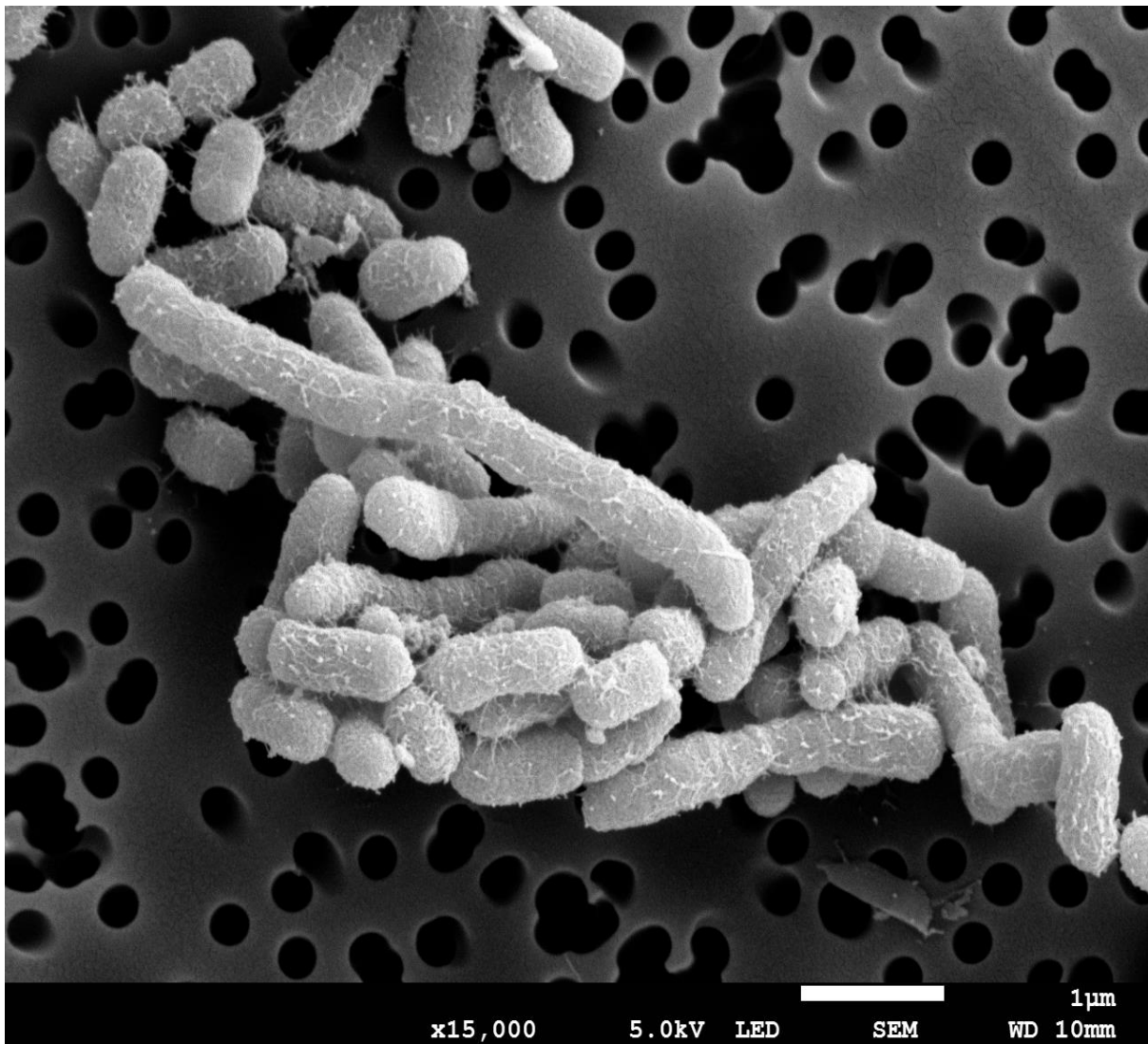


Fig. 3.5. Scanning electron microscopy image of strain 7_F195^T grown on nutrient agar at 25°C for 24 h; Bar, 1 μm.

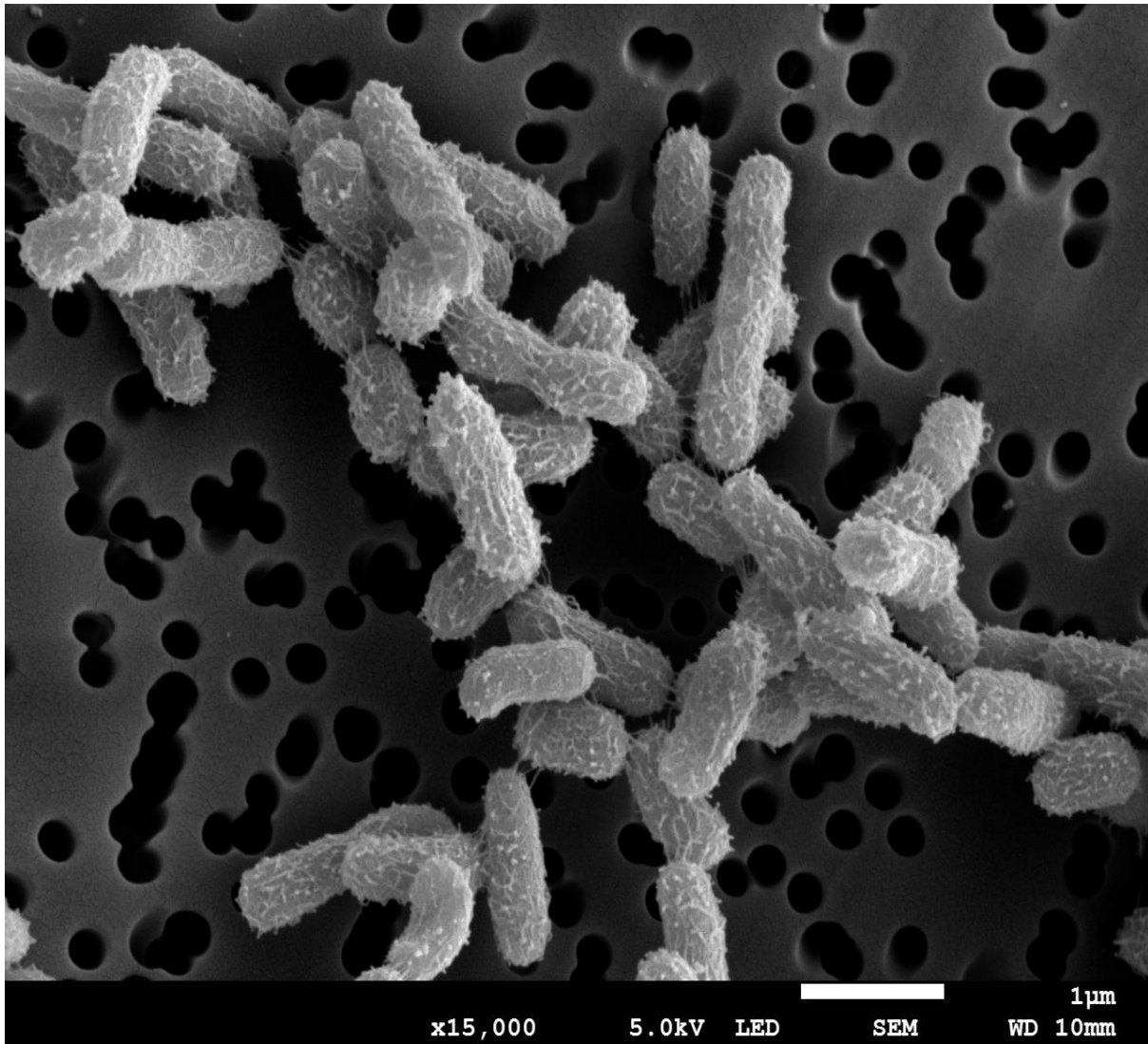


Fig. 3.6. Scanning electron microscopy image of *C. flavum* grown on nutrient agar at 25°C for 24 h; Bar, 1 μm.

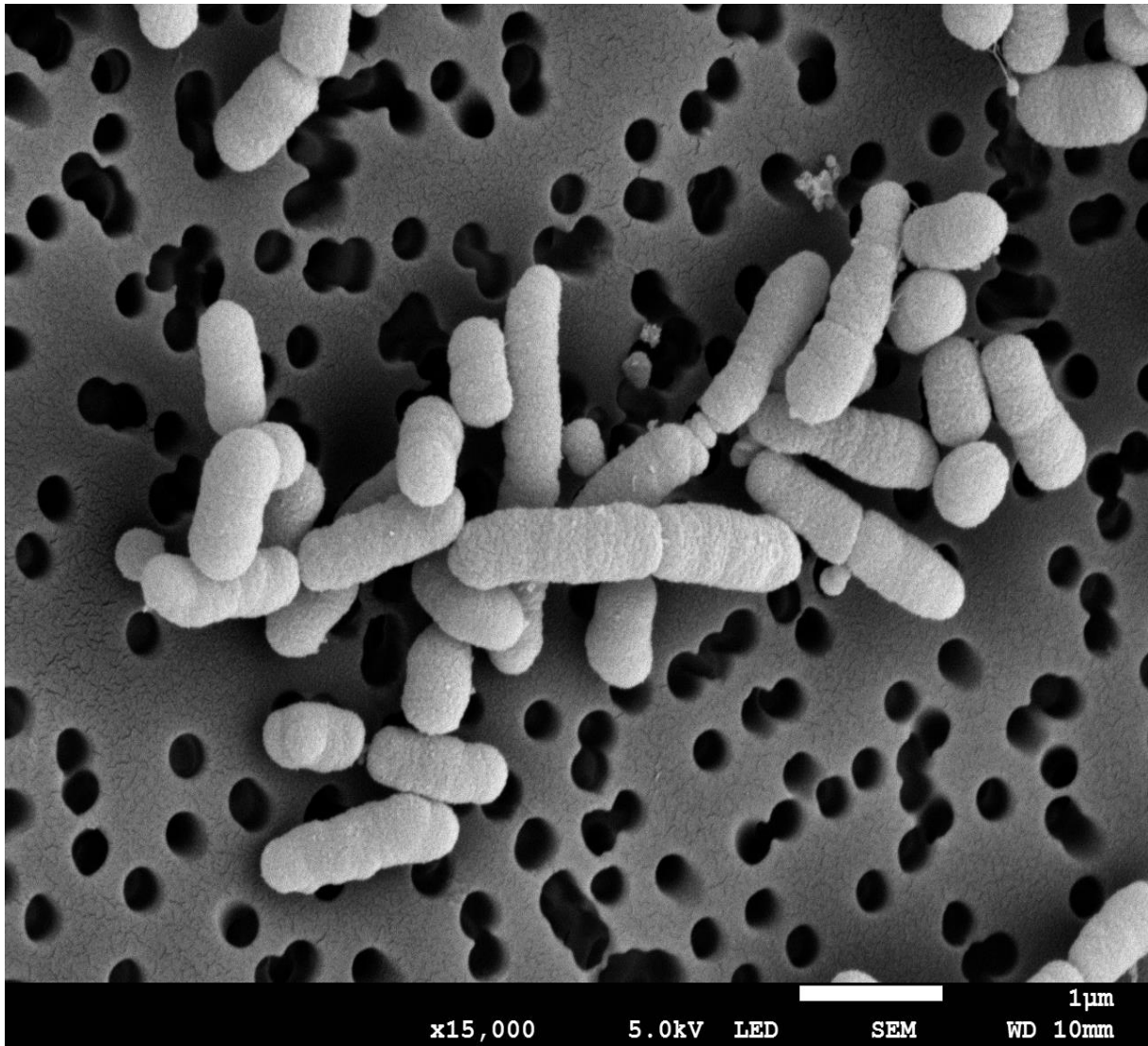


Fig. 3.7. Scanning electron microscopy image of *C. gleum* grown on nutrient agar at 25°C for 24 h; Bar, 1 μm.

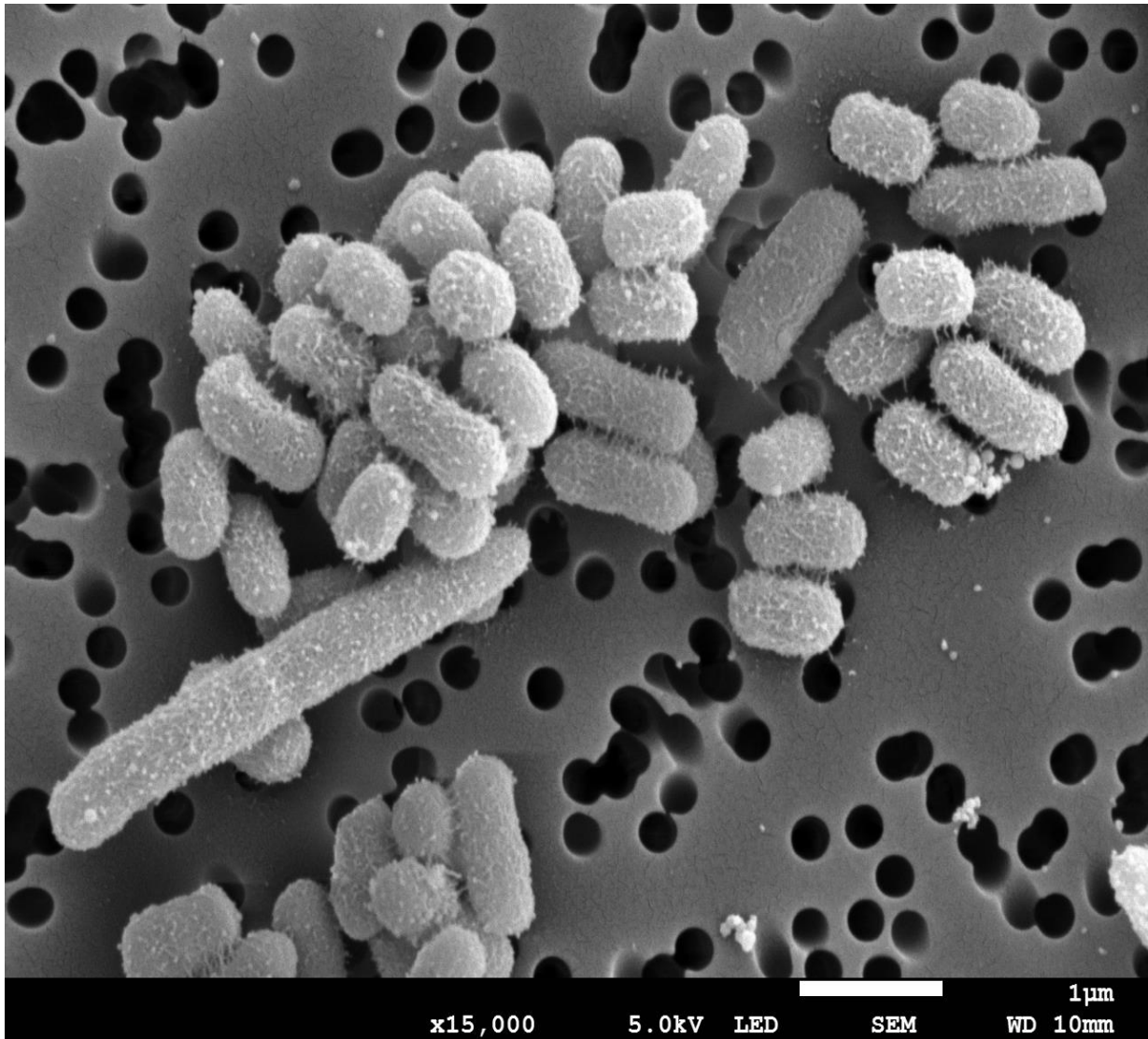


Fig. 3.8. Scanning electron microscopy image of *C. arthrophaerae* grown on nutrient agar at 25 °C for 24 h; Bar, 1 µm.

Transmission electron microscopy (TEM)

Figure 3.9 shows the TEM image of strain 7_F195^T. The cell size ranges from 2 – 2.3 µm in length and 0.4 µm in width. The cells feature a slimy appearance, but the exact composition and purpose of the extracellular slime is unknown.

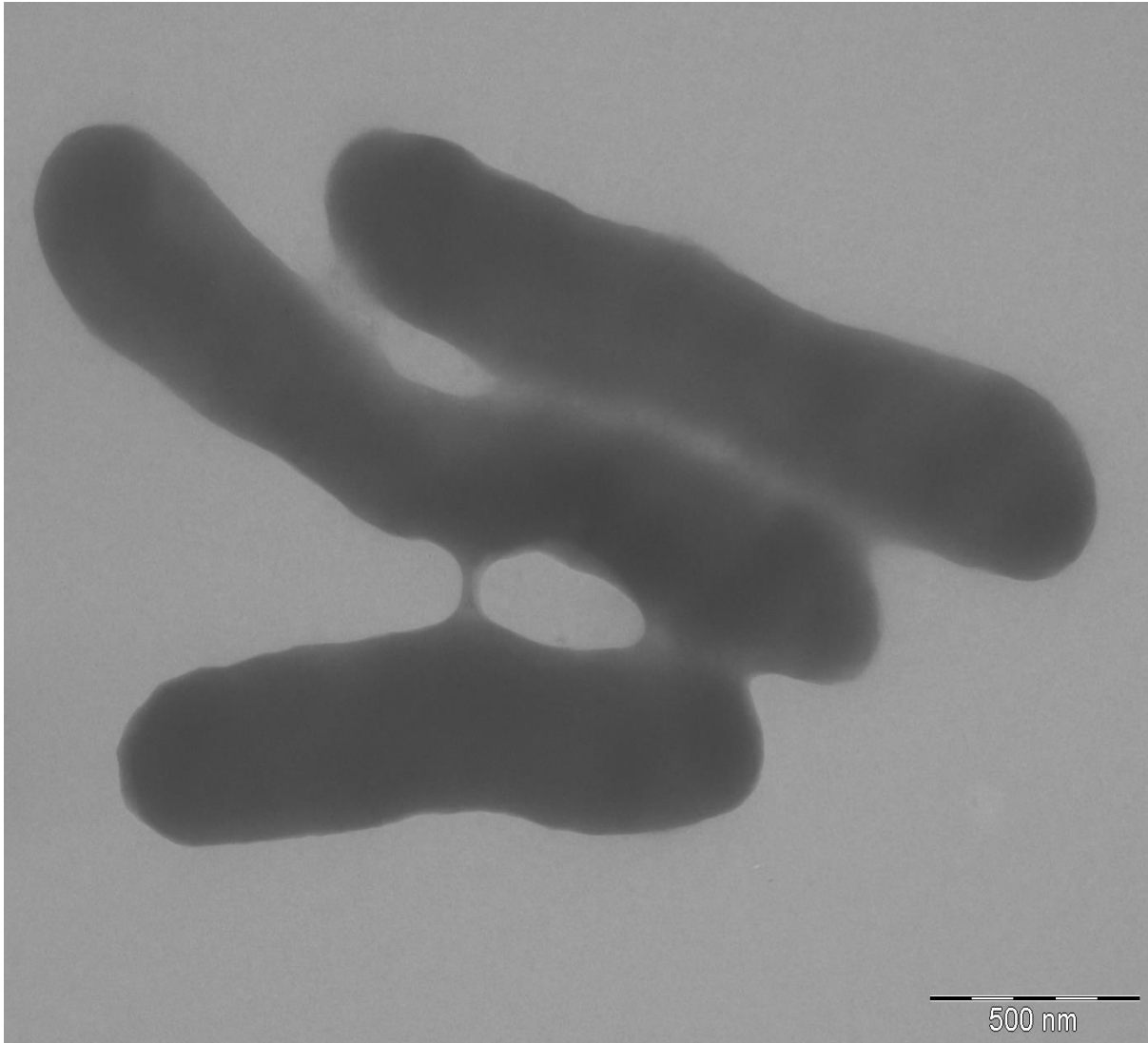


Fig. 3.9. Transmission electron microscopy image of cells of strain 7_F195^T grown on nutrient agar at 25°C for 24 h; Bar, 0.5 µm.

3.3.5. Phenotypic tests

The results of the phenotypic tests on strain 7_F195^T and the reference strains were as follows:

Conventional phenotypic tests

All the strains were positive for: a rod-shaped cell morphology, fruity odour and flexirubin-type pigment production; growth on MacConkey agar; growth at 25, 32°C and weak growth at 15°C; growth at pH 5.5 - 8; growth in 0 - 2.5% NaCl; hydrolysis of gelatin, esculin, starch and Tween 80; and DNase, oxidase, catalase and lecithinase production.

All the strains were negative for: Gram-reaction and gliding motility; growth on cetrimide and β -hydroxybutyrate agar; anaerobic conditions at 25°C; growth at 5 and 42°C; growth at pH 4 – 5 and 8.5; growth in 3 - 5% NaCl; H₂S production; hydrolysis of Tween 20; production phenylalanine deaminase; acid production from adonitol, L-arabinose, cellobiose, dulcitol, ethanol, glycerol, D-xylose, lactose, maltose, D-mannitol, raffinose, rhamnose, salicin, sorbitol and sucrose.

Table 3.4 summarizes the differential characteristics of the reference strains and strain 7_F195^T obtained from various conventional phenotypic tests. Strain 7_F195^T can be differentiated from *C. flavum* by the inability to grow at 37°C; to hydrolyse tyrosine; to produce β -galactosidase (ONPG) and to reduce nitrite. Strain 7_F195^T can be differentiated from *C. gleum* by the inability to grow at 37°C and to hydrolyse tyrosine; the lack of urease and β -galactosidase (ONPG) production; inability to reduce nitrate and by the lack of acid production from D-fructose and trehalose. Strain 7_F195^T can be differentiated from *C. arthrospiraerae* by the inability to grow at 37°C; to hydrolyse tyrosine; or to produce urease. In contrast with *C. arthrospiraerae*, strain 7_F195^T was able to produce acid from D-glucose.

Table 3.4. Differential characteristics of strain 7_F195^T and the reference strains.

Characteristic	Strain 7_F195 ^T	<i>C. flavum</i>	<i>C. gleum</i>	<i>C. arthrosporaerae</i>
Growth range of:				
Temperature (°C) (optimal)	15-32 (32)	15-37 (37)	15-37 (37)	15-37 (37)
pH	5.5-8	5.5-8	5.5-8	5.5-8
Optimum NaCl (w/v, %) (optimal)	0-2.5 (0)	0-2.5 (0)	0-2.5 (0)	0-2.5 (0)
Tyrosine hydrolysis	-	+	+	+
Production of:				
Urease	-	-	+	+
β-galactosidase (ONPG)	-	+	+	-
Reduction of:				
Nitrate	-	-	+	-
Nitrite	-	+	-	-
Production of acid from:				
D-Glucose	+	+	+	-
D-Fructose	-	-	+	-
Trehalose	-	-	+	-

+, positive; -, negative.

Automated tests

API test strips

Table 3.5 summarizes the differential characteristics of the reference strains and strain 7_F195^T obtained from the API 20 NE, API 20 E and API ZYM test strips.

With the API 20 NE, all the strains were positive for: indole production; hydrolysis of esculin and gelatin; assimilation of maltose and negative for: fermentation of glucose; arginine dihydrolase production; assimilation of N-acetyl-glucosamine, capric acid, adipic acid, malate and phenylacetic acid. Strain 7_F195^T can be differentiated from *C. flavum* by the lack of β -galactosidase production; ability to assimilate glucose, mannose, mannitol and potassium gluconate and the inability to assimilate arabinose. Strain 7_F195^T can be differentiated from *C. gleum* by the inability to reduce nitrate to nitrite and nitrate to nitrogen (N₂); lack of urease and β -galactosidase production; inability to assimilate arabinose and ability to assimilate mannitol. Strain 7_F195^T can be differentiated from *C. arthrospiraerae* by the lack of urease production and inability to assimilate arabinose.

With the API 20 E, all the strains were positive for: citrate utilization; production of tryptophan deaminase and indole and negative for: production of arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and H₂S. Strain 7_F195^T can be differentiated from *C. flavum* by the lack of β -galactosidase production. Strain 7_F195^T can be differentiated from *C. gleum* by the lack of β -galactosidase and urease activities; inability to ferment/oxidize glucose. Strain 7_F195^T can be differentiated from *C. arthrospiraerae* by the lack of urease production.

With the API ZYM, all the strains were positive for: the production of alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and negative for: the production of lipase, cystine arylamidase, α -galactosidase, β -glucuronidase, α -mannosidase and α -fucosidase. Strain 7_F195^T can be differentiated from *C. flavum* by the lack of trypsin, β -galactosidase and β -glucosidase production. Strain 7_F195^T can be differentiated

from *C. gleum* by the lack of esterase and β -glucosidase production. Strain 7_F195^T can be differentiated from *C. arthrospiraerae* by the lack of esterase production.

Table 3.5. Differential characteristics of strain 7_F195^T and the reference strains using API 20 NE, API 20 E and API ZYM test strips.

API 20 NE				
Characteristic	Strain 7_F195 ^T	<i>C. flavum</i>	<i>C. gleum</i>	<i>C. arthrospiraerae</i>
Reduction of:				
Nitrates to nitrites	-	-	+	-
Nitrates to nitrogen	-	-	+	-
Production of:				
Urease	-	-	+	+
β -galactosidase	-	+	+	-
Assimilation of:				
Glucose	+	W	+	+
Arabinose	-	+	+	+
Mannose	+	-	+	+
Mannitol	+	-	-	+
Potassium gluconate	W	-	-	-
Trisodium citrate	W	-	+	-

API E				
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Characteristic	<i>7_F195^T</i>	<i>C. flavum</i>	<i>C. gleum</i>	<i>C. arthrosporaerae</i>
Production of:				
β-galactosidase	-	+	+	-
Urease	-	-	+	+
Glucose fermentation/oxidization	-	-	+	-

API ZYM				
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Characteristic	<i>7_F195^T</i>	<i>C. flavum</i>	<i>C. gleum</i>	<i>C. arthrosporaerae</i>
Production of:				
Esterase	-	w	w	+
Esterase lipase	+	w	w	+
Trypsin	-	+	-	w
α-chymotrypsin	w	-	-	-
β-galactosidase	-	w	-	-
α-glucosidase	w	w	w	+
β-glucosidase	-	+	+	-
N-acetyl-β-glucosaminidase	w	+	+	-

+ , positive; - , negative; w , weakly positive.

BIOLOG Omnilog Gen III system – phenotypic profiling

All strains were positive for growth in: 1% NaCl, 1% sodium-lactate, acetic acid, aztreonam, dextrin, D-fructose, D-serine, gelatin, glycyl-L-proline, guanidine HCl, potassium-tellurite, lincomycin, pH 6, tetrazolium blue, tetrazolium violet, troleandomycin, Tween 40.

All strains were negative for growth in: 3-methyl glucose, 4% NaCl, 8% NaCl, α -D-lactose, α -hydroxy-butyric acid, α -keto-butyric acid, α -keto-glutaric acid, β -hydroxy-D,L-butyric acid, bromo-succinic acid, D-aspartic acid, D-cellobiose, D-fucose, D-galacturonic acid, D-gluconic acid, D-lactic acid methyl ester, D-malic acid, D-melibiose, D-raffinose, D-saccharic acid, D-serine, D-sorbitol, D-turanose, fusidic acid, γ -amino-butyric acid, inosine, L-fucose, L-galacturonic acid lactone, LiCl, L-lactic acid, L-malic acid, L-pyroglutamic acid, L-rhamnose, L-serine, methyl pyruvate, mucic acid, myo-inositol, Sodium bromate, N-acetyl neuraminic acid, N-acetyl-b-D-mannosamine, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, nalidixic acid, niaproof 4, pectin, pH 5, p-hydroxy-phenylacetic acid, propionic acid, quinic acid, stachyose, sucrose, vancomycin.

Table 3.6 summarizes the differential characteristics of strain 7_F195^T in comparison with its reference strains and will be further discussed. The Omnilog substrates were divided into the following groups: sugars (including miscellaneous substrates and hexose phosphates); amino acids; hexose acids; carboxylic acids, esters, fatty acids and inhibitory substances. Strain 7_F195^T can be differentiated from all the reference strains by showing negative results for susceptibility to minocycline and rifamycin SV.

Strain 7_F195^T can be differentiated from *C. flavum* in the sugar group by showing a positive result for D-mannitol and negative results for D-maltose, D-salicin and glycerol. In the carboxylic acids, esters and fatty acids group strain 7_F195^T can be differentiated from *C. flavum* by showing a positive result for formic acid.

Strain 7_F195^T can be differentiated from *C. gleum* in the sugar group by showing a positive result for D-mannitol and negative results for β -methyl-D-glucoside, D-fructose-6-PO₄, D-glucose-6-PO₄, D-maltose, D-salicin and glycerol. In the amino

acids group strain 7_F195^T can be differentiated from *C. gleum* by showing a negative result for L-aspartic acid. In the carboxylic acids, esters and fatty acids group strain 7_F195^T can be differentiated from *C. gleum* by showing a positive result for formic acid.

Strain 7_F195^T can be differentiated from *C. arthrospiraerae* in the sugar group by showing a positive result for D-mannose and a negative result for D-arabitol. In the amino acids group strain 7_F195^T showed a negative result for L-histidine.

Table 3.6. Phenotypic profile of strain 7_F195^T and reference strains using the BIOLOG Omnilog Gen III system.

Substrate	7_F195 ^T	<i>C. flavum</i>	<i>C. gleum</i>	<i>C. arthrospiraerae</i>
Sugars:				
α-D-glucose	w	+	+	w
β-methyl-D-glucoside	-	-	+	-
D-arabitol	-	-	-	+
D-fructose-6-PO ₄	-	-	+	-
D-galactose	w	-	w	-
D-glucose-6-PO ₄	-	-	+	-
D-maltose	-	+	+	w
D-mannitol	+	-	-	+

Substate	7_F195 ^T	<i>C. flavum</i>	<i>C. gleum</i>	<i>C. arthrosporaerae</i>
D-mannose	+	W	+	-
D-salicin	-	+	+	-
D-trehalose	+	+	+	W
Gentiobiose	W	+	+	W
Glycerol	-	+	+	W
Amino acids:				
L-alanine	-	-	W	-
L-arginine	W	-	+	-
L-aspartic acid	-	-	+	W
L-glutamic acid	W	-	+	+
L-histidine	-	-	-	+
Hexose acids:				
D-gluconic acid	W	-	-	W
Carboxylic acids, esters and fatty acids:				
Citric Acid	W	-	+	+
Formic Acid	+	-	-	+
Inhibitory substances:				
Sodium-butyrate	W	W	-	+

+, positive; -, negative; w, weakly positive.

3.3.6. Chemotaxonomic methods

Fatty acid methyl ester analysis

Cellular fatty acid profiles provide taxonomic information of high quality, mainly at the specific and generic level (Vandamme *et al.*, 1996a). The fatty acids predominantly found in members of the family *Flavobacteriaceae* can either characterise a genus and some can aid in the differentiation of species (Vandamme *et al.*, 1994b, 1996b; Bernardet *et al.*, 1996; Vancanneyt *et al.*, 1996; Bowman *et al.*, 1997, 1998; Gosink *et al.*, 1998; Barbeyron *et al.*, 2001). Table 3.7 contains the cellular fatty acid profiles of strain 7_F195^T, *C. flavum*, *C. gleum* and *C. arthrosphaerae*. The predominant fatty acids in strain 7_F195^T were as follows: iso-C_{15:0} (38.11%); iso-C_{17:1} ω_{9c} (25.32%); iso-C_{17:0} 3-OH (13.14%). The presence of these fatty acids supports the affiliation of strain 7_F195^T to the genus *Chryseobacterium*.

Table 3.7. Cellular fatty acid profiles of strain 7_F195^T and the reference strains. All data are from this study. Values are percentages of the total fatty acids. Fatty acids that are <0.5% in all strains are not shown. ECL, equivalent chain length; Tr, Trace (<0.5%). The major fatty acids (>10%) are indicated in bold.

Fatty acid%	7_F195 ^T	<i>C. flavum</i>	<i>C. gleum</i>	<i>C. arthrosphaerae</i>
C _{16:0}	1.00	0.82	1.13	1.43
iso-C _{13:0}	Tr	Tr	0.53	Tr
iso-C _{15:0}	38.11	33.21	35.00	34.95
iso-C _{17:0}	1.02	1.22	1.55	1.56

Fatty acid%	7_F195 ^T	<i>C. flavum</i>	<i>C. gleum</i>	<i>C. arthrospiraerae</i>
iso-C _{17:1} ω _{9c}	25.32	22.83	22.22	24.29
C _{18:1} ω _{5c}	0.65	0.60	Tr	0.50
iso-C _{15:0} 3-OH	2.80	2.66	2.87	2.59
iso-C _{16:0} 3-OH	0.68	0.62	0.61	0.59
C _{16:0} 3-OH	0.65	1.07	0.93	0.91
iso-C _{17:0} 3-OH	13.14	16.00	15.78	14.14
ECL 13.565	4.61	4.77	4.15	4.55
ECL 16.582	1.39	1.51	1.49	1.33
Summed Feature 3*	8.17	12.50	12.10	10.71
Summed Feature 4*	Tr	0.52	Tr	0.61

For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain while the *cis* isomer is indicated by the suffix *c*.

*Summed feature are groups of two or three fatty acids that are treated together for the 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 was listed as C_{16:1} ω_{7c} or iso-C_{15:0} 2-OH or both; Summed Feature 4 was listed as iso-C_{15:0} 2-OH or C_{16:1} ω_{7t} or both.

Polar lipids

In *Chryseobacterium* species, phosphatidylethanolamine is the most abundant polar lipid (Wu *et al.*, 2013). Figure 3.10 shows that strain 7_F195^T contains four unidentified lipids as well as a glycolipid and five unidentified aminolipids but the most abundant polar lipid is phosphatidylethanolamine. On the basis of the latter finding strain 7_F195^T can be classified as a member of the genus *Chryseobacterium*.

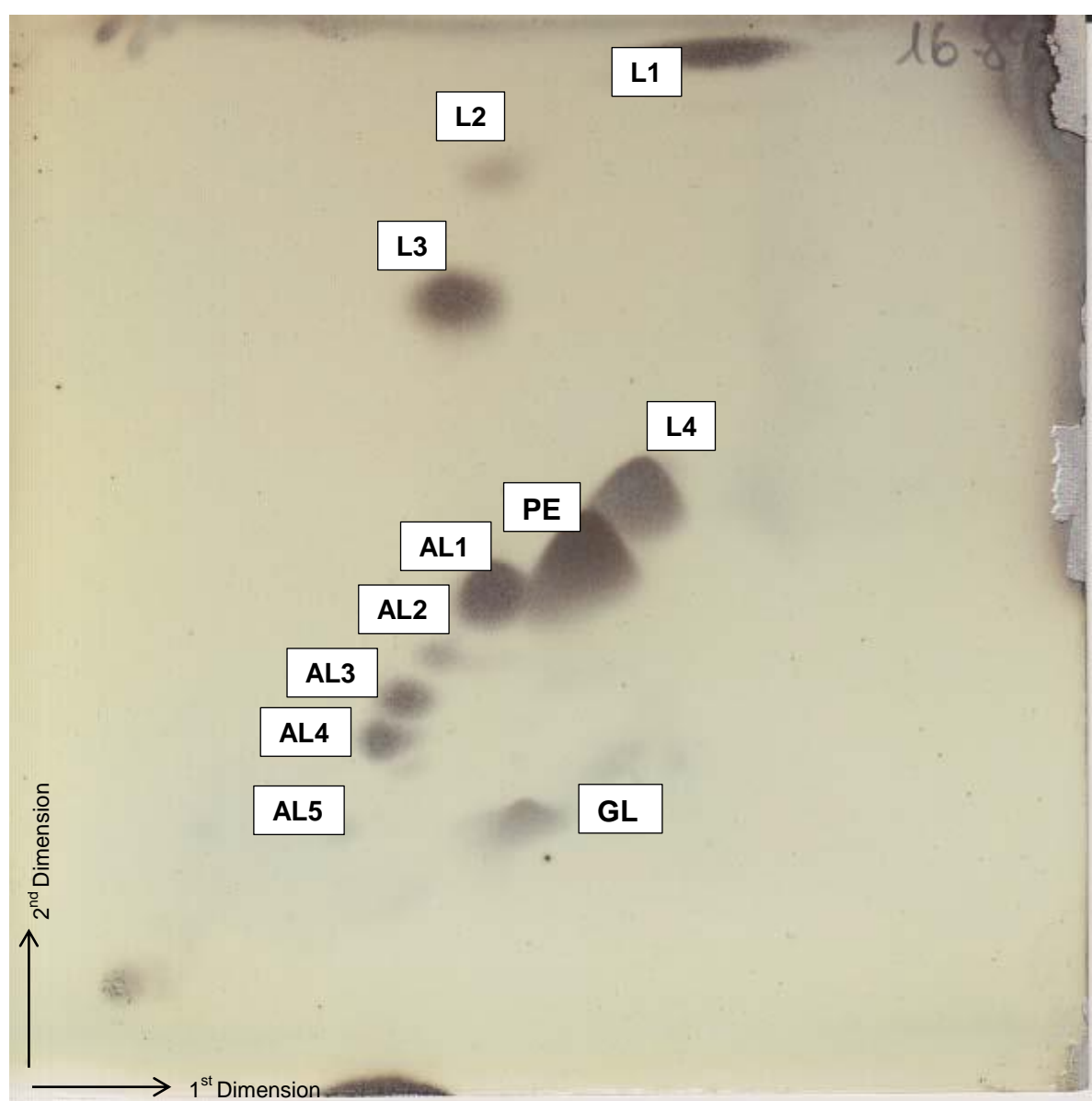


Fig. 3.10. Two-dimensional thin-layer chromatograms of polar lipids from strain 7_F195^T. L, unidentified lipid; GL, unidentified glycolipid; AL, unidentified aminolipid; PE, phosphatidylethanolamine.

Respiratory lipoquinones

In the family *Flavobacteriaceae* as well as in the genus *Chryseobacterium*, menaquinone-6 is reported to be the major or only respiratory quinone (Bernardet *et al.*, 2002, 2011). Results from DSMZ identification service confirms that strain 7_F195^T contains menaquinone-6 (100%) as the only respiratory quinone and can, therefore, be classified as a member of the family *Flavobacteriaceae* and of the genus *Chryseobacterium*.

3.4. Description of *Chryseobacterium pennipullorum* sp. nov.

Chryseobacterium pennipullorum (pen.ni.pul.lo'rum. L. fem. n. *penna* a feather; L. masc. n. *pullus* a chicken; N.L. gen. n. *pennipullorum* of chicken feathers).

Cells are Gram-negative; non-motile and non-gliding; obligate aerobic and rod-shaped (2 – 2.3 µm in length and 0.4 µm in width) without flagella, they also produce a slime capsule. The colony shape is circular with a diameter of 1 – 3 mm after cultivation at 25°C for 48 h on nutrient agar (Oxoid CM003). The colonies produce a flexirubin-type pigment and have a fruity odour; they are translucent, flat with a smooth and entire surface and become mucoid on prolonged incubation. Positive for growth on nutrient and MacConkey agar but do not grow on cetrimide and β-hydroxybutyrate agars; good growth at 25, 32°C and weak growth at 15°C; no growth at 5, 37 and 42°C; growth at pH 5.5 – 8 but not at pH less than 5 or more than 8; growth in 0 – 2.5% NaCl but not in 3 – 5% NaCl. Oxidase and catalase positive; positive for hydrolysis of gelatin, esculin, starch, and Tween 80; production of DNase and lecithinase; acid production from D-glucose. Negative for: H₂S production; hydrolysis of Tween 20 and tyrosine; production of urease, β-galactosidase and phenylalanine deaminase; nitrate and nitrite reduction; acid production from adonitol, L-arabinose, cellobiose, dulcitol, ethanol, D-fructose, glycerol, D-xylose, lactose, maltose, D-mannitol, raffinose, rhamnose, salicin, sorbitol, sucrose and trehalose.

With the API 20 NE test strip, reactions are positive for: indole production; hydrolysis of esculin and gelatin; assimilation of glucose, mannose, mannitol and maltose and negative for: the reduction of nitrate to nitrite and nitrate to nitrogen; fermentation of glucose; production of urease and β -galactosidase; arginine dihydrolase production; assimilation of arabinose, N-acetyl-glucosamine, capric acid, adipic acid, malate and phenylacetic acid. Weak positive reaction for assimilation of potassium gluconate and trisodium citrate.

With the API 20 E test strip, reactions are positive for: citrate utilization; production of tryptophan deaminase, indole and gelatinase and negative for: production of β -galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, H_2S and urease; fermentation/oxidation of glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, melibiose, amygdalin and L-arabinose.

With the API ZYM test strip, reactions are positive for: the production of esterase lipase, alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and negative for: the production of esterase, lipase, cystine arylamidase, trypsin, β -glucosidase α -galactosidase, β -galactosidase, β -glucuronidase, α -mannosidase and α -fucosidase. Weak positive reactions are reported for the production of α -chymotrypsin, α -glucosidase and N-acetyl- β -glucosaminidase.

Strain 7_F195^T was tested using the BIOLOG Omnilog Gen III system for its susceptibility to 23 inhibitory substances and its ability to oxidise 71 carbon sources. Strain 7_F195^T was not inhibited by following inhibitory substances: 1% NaCl, 1% sodium-lactate, aztreonam, D-serine, guanidine HCl, potassium-tellurite, lincomycin, pH 6, tetrazolium blue, tetrazolium violet and troleandomycin. Strain 7_F195^T showed weak growth in the presence of sodium-butyrate. Strain 7_F195^T showed susceptibility towards the following inhibitory substances: 4% NaCl, 8% NaCl, fusidic acid, LiCl, minocycline, sodium bromate, nalidixic acid, niaproof 4, pH 5, rifamycin SV and vancomycin.

Spoilage in food is caused by metabolites produced when the microorganism utilizes carbohydrates, amino acids and carboxylic acids (de Beer, 2005). Strain 7_F195^T was able to utilise the following carbon sources: gelatin, glycyl-L-proline, Tween-40, acetic acid, D-trehalose, dextrin, D-fructose, D-mannitol, D-mannose and formic acid.

It showed weak utilisation of the following carbon sources: acetoacetic acid, α -D-glucose, citric acid, D-galactose, D-glucuronic acid, gentiobiose, glucuronamide, L-arginine and L-glutamic acid. The strain could not utilise the following carbon sources: 3-methyl glucose, α -D-lactose, α -hydroxy-butyric acid, α -keto-butyric acid, α -keto-glutaric acid, β -hydroxy-D,L-butyric acid, β -methyl-D-glucoside, bromo-succinic acid, D-arabitol, D-aspartic acid, D-cellobiose, D-fructose-6-PO₄, D-fucose, D-galacturonic acid, D-gluconic acid, D-glucose-6-PO₄, D-lactic acid methyl ester, D-malic acid, D-maltose, D-melibiose, D-raffinose, D-saccharic acid, D-salicin, D-serine, D-sorbitol, D-turanose, γ -amino-butyric acid, glycerol, inosine, L-alanine, L-aspartic acid, L-fucose, L-galacturonic acid lactone, L-histidine, L-lactic acid, L-malic acid, L-pyroglutamic acid, L-rhamnose, L-serine, methyl pyruvate, mucic acid, myo-inositol, N-acetyl neuraminic acid, N-acetyl- β -D-mannosamine, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, pectin, p-hydroxy-phenylacetic acid, propionic acid, quinic acid, stachyose and sucrose.

General features of this genome assembly are as follows: genome size, 4,796,535 bp; number of contigs, 61; coding sequences (CDS), 4348; N50 value, 208445 and coverage, 33.0x; G+C value, 38.6 mol%. The predominant fatty acids are iso-C_{15:0}, iso-C_{17:1} ω _{9c} and iso-C_{17:0} 3-OH. The most abundant polar lipid is phosphatidylethanolamine. Contains menaquinone-6 as the only respiratory quinone.

The strain was isolated from chicken feather waste collected from an abattoir in Bloemfontein, Free State, South Africa.

3.5. Conclusions

New species can only be described when a phenotypic and a genetic coherence exists among the members of the new species as well as a diagnostic phenotype that will distinguish the new species from other relatives (Gevers *et al.*, 2006). Taxonomy provides a reference system and is, therefore, a discipline that is essential in biology (Gevers *et al.*, 2006).

Chryseobacterium strain 7_F195^T isolated from chicken feather waste collected from an abattoir in Bloemfontein was evaluated using genotypic, phenotypic and chemotaxonomic methods. The reference strains were determined by comparing 16S rRNA similarity values of all *Chryseobacterium* species known with that of strain 7_F195^T and further selection was done based on OGRI values including dDDH, ANI and AAI. The phylogenetic trees confirmed that strain 7_F195^T is part of the genus *Chryseobacterium*. Further phenotypic tests were performed to determine if strain 7_F195^T is a new species. The fatty acid methyl ester profile, polar lipids, respiratory lipoquinone of strain 7_F195^T was determined and compared to the reference strains.

Numerous common characteristics exist between strain 7_F195^T and the reference strains while other characteristics differ. Strain 7_F195^T can be differentiated from its nearest neighbours and could, therefore, be identified as a new species of the genus *Chryseobacterium* and the name *C. pennipullorum* is proposed and described in this study.

CHAPTER 4

TEMPERATURE KINETICS OF A NOVEL *Chryseobacterium* SPECIES IN COMPARISON WITH *Chryseobacterium carnipullorum* AND *Chryseobacterium vrystaatense*

Abstract

The temperature growth relationship of a novel *Chryseobacterium* species isolated from poultry feather waste in comparison to *C. carnipullorum* that was isolated from a raw chicken portion and *C. vrystaatense* that was isolated from chicken portions from a poultry abattoir in South Africa was investigated. The growth study was conducted in a temperature gradient incubator with a temperature range between 14 and 50°C and the optical density measured at different time intervals. Temperature profiles and Arrhenius plots were constructed. The results showed that *Chryseobacterium carnipullorum* had a higher optimum growth temperature than strain 7_F195^T and *C. vrystaatense*. Strain 7_F195^T showed a higher maximum specific growth rate of 0.67 h⁻¹ at its optimum temperature in comparison with the maximum specific growth rate of *C. carnipullorum* and *C. vrystaatense*. All the cultures showed least sensitivity to temperature change in the temperature range 25 - 32°C. Strain 7_F195^T showed the most sensitivity and *C. vrystaatense* showed the least sensitivity towards temperature change in the temperature range of about 15 – 26°C. The results of this study conclude that strain 7_F195^T may have the ability to cause spoilage of food at a faster rate than *Chryseobacterium carnipullorum* and *C. vrystaatense* at an optimal temperature.

4.1. Introduction

Members of the genus *Chryseobacterium* have been isolated from environments ranging from terrestrial, aquatic, diseased animals, humans and food (Bernardet *et al.*, 2002) and some species may play a significant role in food spoilage (Bekker *et*

al., 2016). Food sources from which *Chryseobacterium* species were isolated include poultry, red meat, milk and fish (Vandamme *et al.*, 1994b; Bernardet *et al.*, 2011).

The microbiota of a food product is dependent on the environment, nature of the raw materials, processing and storage conditions of the food product (Griffith *et al.*, 2000; Legnani *et al.*, 2003). Fresh meat, e.g., chicken meat, can spoil in a relatively short time if it is not packaged, transported or stored at the appropriate temperatures (Koutsoumanis *et al.*, 2006). Spoilage in poultry meat can be due to intrinsic factors e.g., changes in pH, but temperature as an extrinsic factor is the main cause of spoilage (Koutsoumanis *et al.*, 2006). Studies that focused on the temperature during transportation, retail storage and consumer handling of the raw product indicated that it is not uncommon for temperatures to rise above 10°C (Giannakourou *et al.*, 2001; Gill *et al.*, 2002). Temperature is an important factor that influences the rate of reactions in the cell, nutritional requirements, nature of the metabolism and biomass composition (Pirt, 1975).

The effect of environmental variables on pathogenic and spoilage bacteria can be better understood through predictive models (Fakruddin *et al.*, 2011). The potential growth of a microorganism under certain conditions can be predicted in the laboratory environment and then extrapolated to real food and environments (Fakruddin *et al.*, 2011). Kinetic models or predictive models are defined as mathematical models that contain characteristic kinetic parameters that capture changes e.g., rate constants and activation energies (Van Boekel, 2008). Activation energy is an important constant in the Arrhenius equation because it can be used to predict what effect temperature will have on an organism's growth rate over a normal temperature range (Pirt, 1975). Fluctuation of the activation energy indicates that differences in metabolism regulation and rate-controlling reactions can occur.

Predictive models are used to improve microbial food quality, safety and shelf-life. They can also contribute to better understanding the microbial ecology of foods. It is important, however, to remember that these models should only be used as a support tool (Fakruddin *et al.*, 2011). The models simplify the understanding of microbial behaviour in food systems that are much more complex. The purpose of this study was to compare the temperature growth relationship of a novel *Chryseobacterium* species isolated from poultry feather waste (Charimba, 2012) with

that of *C. carnipullorum* that was isolated from a raw chicken portion (Charimba *et al.*, 2013) and *C. vrystaatense* (de Beer *et al.*, 2005) that was isolated from chicken portions from a poultry abattoir in South Africa. All three strains were isolated during post-graduate studies at the University of the Free State, Food Science Section, Bloemfontein, South Africa.

4.2. Materials and Methods

4.2.1. Cultures used and maintenance

The cultures used in this study are indicated in Table 4.1. The cultures were revived in 10 ml nutrient broth no. 2 (Oxoid CM67) and the purity of the cultures was examined on nutrient agar (Oxoid CM003) by Gram-staining after incubation of all the cultures at 25°C for 24 h. The cultures were freeze-dried on AA Whatman filter paper discs and stored at -20°C for short-term maintenance and for long-term maintenance by freeze-drying in glass vials and stored at -20°C. The cultures were also stored on nutrient agar slants at 4°C and re-streaked every four to six weeks.

Table 4.1. Cultures used to determine the effect of temperature on growth.

Genus and species	Culture collection ^a	Source of isolation	Year of isolation	Reference
Strain 7_F195	UFSBC 710	Chicken feathers	2010	Charimba, 2012
<i>Chryseobacterium carnipullorum</i>	DSM 25581 ^T	Raw chicken	2005	Charimba <i>et al.</i> , 2013
<i>Chryseobacterium vrystaatense</i>	LMG 22846 ^T	Raw chicken	2005	de Beer <i>et al.</i> , 2005

^a University of the Free State Bacterial Collection, South Africa (UFSBC); Deutsche Sammlung von Mikro-organismen, Germany (DSM); Laboratory of Microbiology, University of Ghent, Belgium (LMG).

4.2.2. Preliminary growth study

The preliminary growth study was done to determine the late exponential phase of each strain and to eliminate/shorten the lag phase when transferred to fresh media. The lag phase depends on the inoculum used, e.g., if a culture that has been growing exponentially is transferred to media with the same composition and under the same conditions there will be no lag phase, but will immediately enter an exponential phase (Madigan *et al.*, 2015).

The cultures were cultivated for 48 h in 10 ml nutrient broth no. 2 at 25°C; 5 ml of culture was then transferred to 95 ml fresh nutrient broth no. 2 in a side arm flask. The flask was then shaken at 180 rpm for 24 h and the optical density (OD) was measured every three hours with a Photolab S6 spectrophotometer (WTW, Weilheim, Germany) at 620 nm, until the stationary phase was reached. At the end of the incubation period, the biomass was determined using 10 ml culture samples in triplicate. The tubes were centrifuged for 15 minutes at 4000 x g and the precipitated cells were washed with distilled water and dried at 105°C until a constant weight was obtained.

4.2.3. Determination of the effect of temperature on growth

4.2.3.1. Inoculation of broth and measurements

The cultures were each cultivated by inoculating 10 ml nutrient broth no. 2 with a freeze-dried disc of culture and incubated for 48 h at 25°C. A volume of 5 ml culture was transferred to fresh 95 ml nutrient broth no. 2 in a 500 ml Erlenmeyer flask and

incubated on a shaker at 180 rpm at 25°C until the late exponential phase was reached for each culture. In a 500 ml Erlenmeyer flask, 20 ml of culture from the shaker was added to 400 ml fresh nutrient broth no. 2. The suspension was mixed properly and divided in volumes of 10 ml into 30 sterile temperature gradient incubator (TGI) tubes which are L-shaped tubes (40 ml total volume and 17 mm in diameter) capped with loose-fitting metal caps.

The tubes were placed in 30 equidistant sampling wells on both sides of the TGI (Scientific Industries Inc., New York, USA) that consists of an aluminium bar that is heated at one end and cooled at the other to achieve a stable temperature gradient range of about 14 to 50°C with $2 \pm 0.2^\circ\text{C}$ increments between the 30 individual positions. The temperature between the central tubes varied about 0.2°C while the terminal tubes varied about 0.7°C . The one side of the bar contained tubes with bacterial culture and the other side contained corresponding tubes with water for the measurement of temperature. The bar of the TGI was rocked through a 30° arc at 60 oscillations min^{-1} to provide aeration and mixing of the samples.

Optical density was measured at 600 nm using a Biowave C0800 cell density meter (Walden Precision Apparatus Ltd., Cambridgeshire, UK). The tubes were given time to reach their separate incubation temperatures (approximately 30 minutes) and thereafter the first OD measurement was taken at 30 minutes intervals. The OD was measured until tubes reached an OD value of two at optimal temperature or until enough data points were gathered at lower/higher temperatures to determine the specific growth rate. The tubes were removed sequentially during measurement without stopping the TGI shaker to prevent disruption of growth. The experiment was performed in triplicate for each culture.

4.2.3.2. Data analysis

Microsoft Excel (Microsoft Corporation, Washington, USA) was used to determine the specific growth rate by linear regression analysis of the exponential phase of the growth curve. The following equation was used to calculate the slope: $\mu_{\text{max}} = (\ln x_t - \ln x_0)/t$; where x is the optical density of the cell concentration, and t is the time in hours (Baranyi *et al.*, 1993). The Huang rate model $[(\mu = \alpha (T - T_{\text{min}})^{1.5} (1 - e^{\beta(T - T_{\text{max}})})]$

was used to correlate growth rate with temperature and to mathematically predict the minimum growth temperature of the cultures (Huang *et al.*, 2011). The equation consists of μ which is the specific growth rate; α and β which are regression coefficients; T is the experimental temperature measured in °C; T_{\min} and T_{\max} are the minimum and maximum predicted temperatures, respectively, measured in °C; e is an exponential function.

The Arrhenius equation ($\mu_{\max} = Ae^{-(E/RT)}$) was used to describe the relationship between the growth rate and temperature and mainly to determine the activation energy of each microorganism in a specific temperature range (Broughall *et al.*, 1983). The equation consists of μ which is the specific growth rate; A is an entropy constant; E is the activation energy (temperature coefficient); R is the universal gas constant ($8.314 \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$) and T which is the absolute temperature expressed in ° Kelvin.

4.3. Results and Discussion

4.3.1. Preliminary growth study

The preliminary growth study was done to determine the late exponential phase of each strain and to eliminate/shorten the lag phase when transferred to fresh media. The late exponential phases for the cultures (Figure 4.1) were as follows: Strain 7_F195^T, 9 hours; *C. vrystaatense*, 9 hours and *C. carnipullorum*, 12 hours.

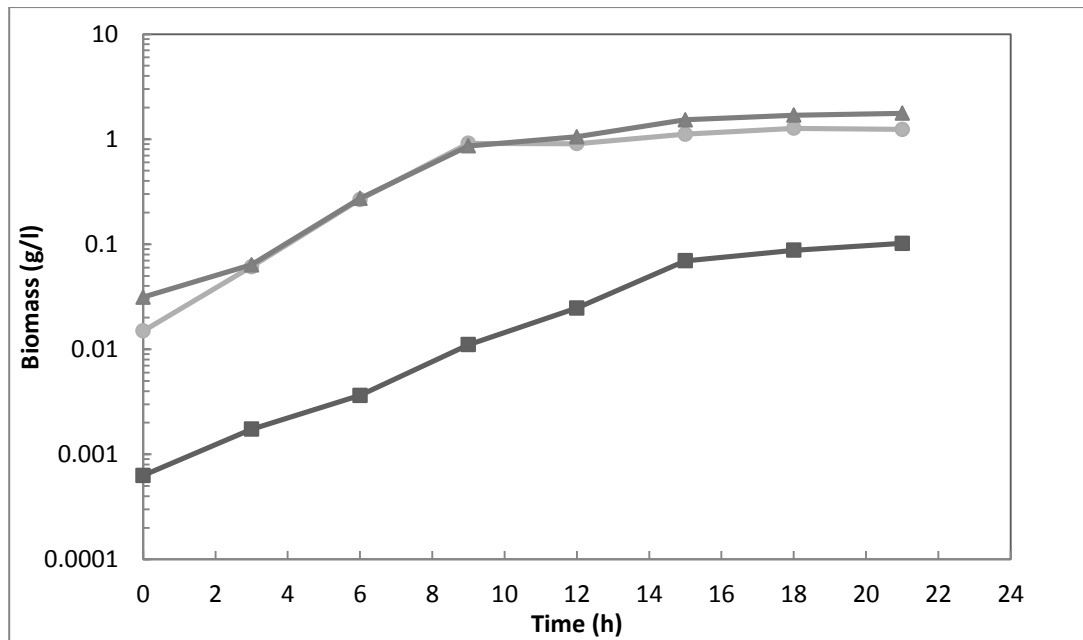


Fig. 4.1. Growth profiles of Strain 7_F195^T (●), *C. carnipullorum* (■) and *C. vrystaatense* (▲) cultivated in nutrient broth no. 2 at 25°C until the stationary phase was reached.

4.3.2. Growth kinetics - Temperature profiles

On the basis of growth temperatures, microorganisms can be grouped in four classes namely psychrophiles, mesophiles, thermophiles and hyperthermophiles (Madigan *et al.*, 2015). When the genus *Chryseobacterium* was first described, it was reported that all strains grow at 30°C and most grow at 37°C (Vandamme *et al.*, 1994a). *Chryseobacterium* species can, therefore, be classified as mesophiles, which have mid-range temperature optima (20 – 40°C) (Madigan *et al.*, 2015). Most *Chryseobacterium* species are also classified as psychrotolerant with an optimum temperature range of between 20 – 40°C but ability to also grow at 0°C (Hugo *et al.*, 2003; de Beer *et al.*, 2005, 2006; Charimba *et al.*, 2013). A previous study done by Bekker and co-workers (2015) reported that *C. joostei* and *C. bovis* had specific growth rates of 0.98 and 0.62 h⁻¹ respectively. No other growth kinetic information of the genus *Chryseobacterium* is available. A summary of the cardinal temperature ranges and the maximum specific growth rates for strain 7_F195^T, *C. carnipullorum* and *C. vrystaatense* are indicated in Table 4.2.

Table 4.2. The cardinal temperature ranges and the maximum specific growth rates for the *Chryseobacterium* cultures evaluated in this study.

Culture	Temperature, °C			Maximum specific growth rate (μ_{\max} , h ⁻¹) at optimum temperature
	Minimum	Maximum	Optimum	
Strain 7_F195 ^T	14.6	38.4	31.6	0.67
<i>C. carnipullorum</i>	14.4	37.7	31.8	0.59
<i>C. vrystaatense</i>	14.7	36.7	30.7	0.59

Strain 7_F195^T grew at a minimum and maximum temperature of 14.6 and 38.4°C respectively and had an optimum growth temperature of 31.6°C (Figure 4.2). *Chryseobacterium carnipullorum* grew at a minimum and maximum temperature of 14.4 and 37.7°C respectively and had an optimum growth temperature of 31.8°C (Figure 4.3). A study done by Charimba and co-workers (2013) reported that *C. carnipullorum* does not grow at 37°C and grows optimally at 25 – 30°C. *Chryseobacterium vrystaatense* grew at a minimum and maximum temperature of 14.7 and 36.7°C respectively and had an optimum growth temperature of 30.7°C (Figure 4.4). A study done by de Beer and co-workers (2005) on the description of *C. vrystaatense*, reported that the organism grows at 15, 25 and 32°C but no growth was observed at 37°C. The difference in temperature ranges in this study in comparison with previous studies can be due to the cultures being cultivated in nutrient broth rather than on nutrient agar plates and the additional shaking of the culture on a shaker during the incubation period and on the TGI, which provides extra aeration. The TGI also had the advantage that temperature ranges of growth could be determined more accurately.

Although not experimentally determined, extrapolation of the curves in Figures 4.2 – 4.4, through the Huang rate model [$\mu = \alpha(T - T_{\min})^{1.5}(1 - e^{\beta(T - T_{\max})})$] suggested that the minimum growth temperature of the cultures would be as follows: Strain 7_F195^T, 8.5°C; *C. carnipullorum*, 6.9°C; and *C. vrystaatense*, 6.7°C. The Huang

rate model, developed to correlate growth rate with temperature, is significant because it provides a closer estimation of the biological minimum temperature for microbial growth (Huang *et al.*, 2011). It is however, important that the true minimum temperature of a microorganism should also be determined experimentally.

Strain 7_F195^T grew at a higher maximum temperature of 38.4°C in comparison with *C. carnipullorum* and *C. vrystaatense* with maximum temperatures of 37.7 and 36.7°C respectively. *Chryseobacterium carnipullorum* had a higher optimum growth temperature of 31.8°C in comparison with strain 7_F195^T (31.6°C) and *C. vrystaatense* (30.7°C). *Chryseobacterium vrystaatense* will, therefore, be more likely to cause spoilage at a lower temperature in comparison with the other cultures. On the basis of its higher maximum growth temperature strain 7_F195^T could possibly be more likely to survive higher temperatures in comparison with the other cultures.

Strain 7_F195^T showed a higher maximum specific growth rate of 0.67 h⁻¹ at its optimum temperature in comparison with the maximum specific growth rate for both *C. carnipullorum* and *C. vrystaatense* being 0.59 h⁻¹ at their optimum temperatures. Strain 7_F195^T may, consequently, have the ability to cause spoilage at a faster rate than *C. carnipullorum* and *C. vrystaatense* if the temperature is optimal.

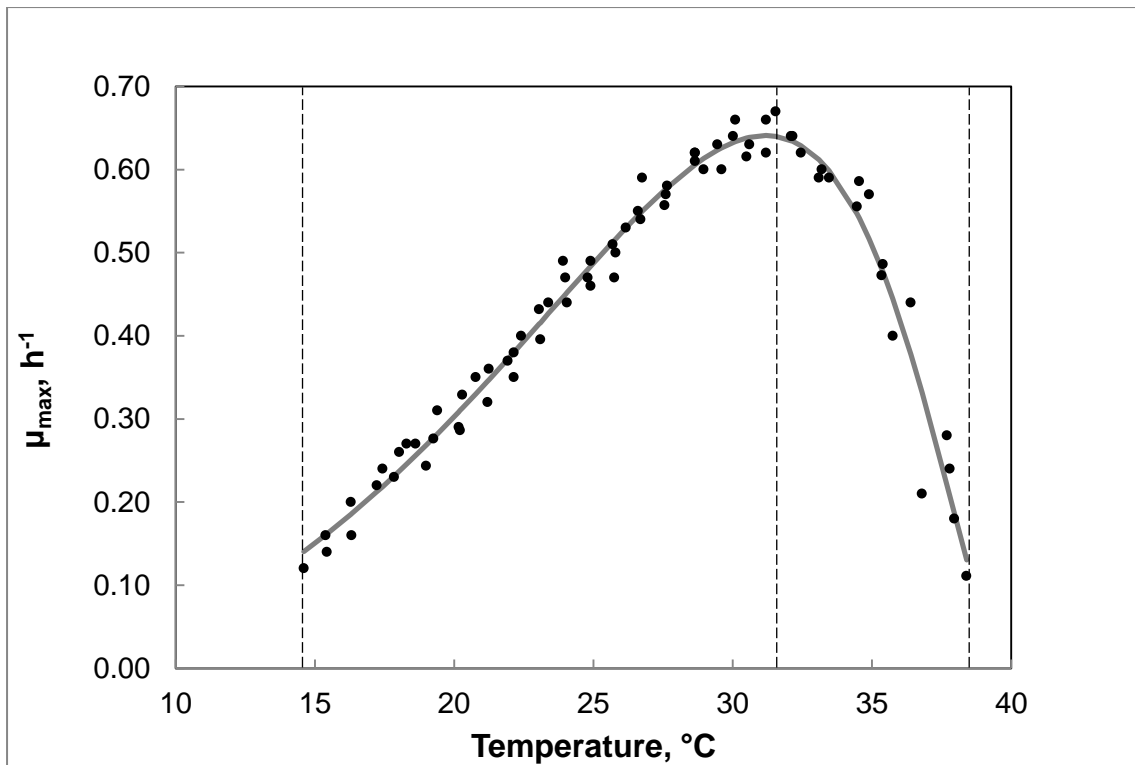


Fig. 4.2. Temperature profile of strain 7_F195^T. The broken vertical lines indicate the cardinal temperatures.

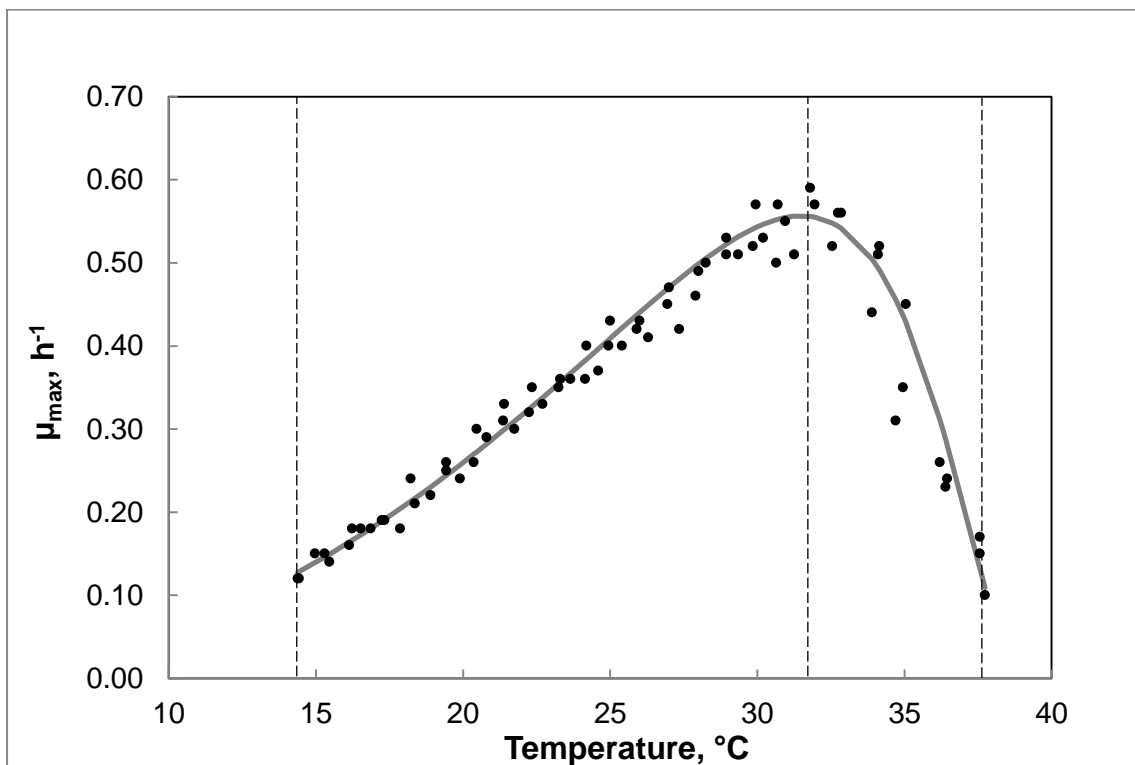


Fig. 4.3. Temperature profile of *Chryseobacterium carnipullorum*. The broken vertical lines indicate the cardinal temperatures.

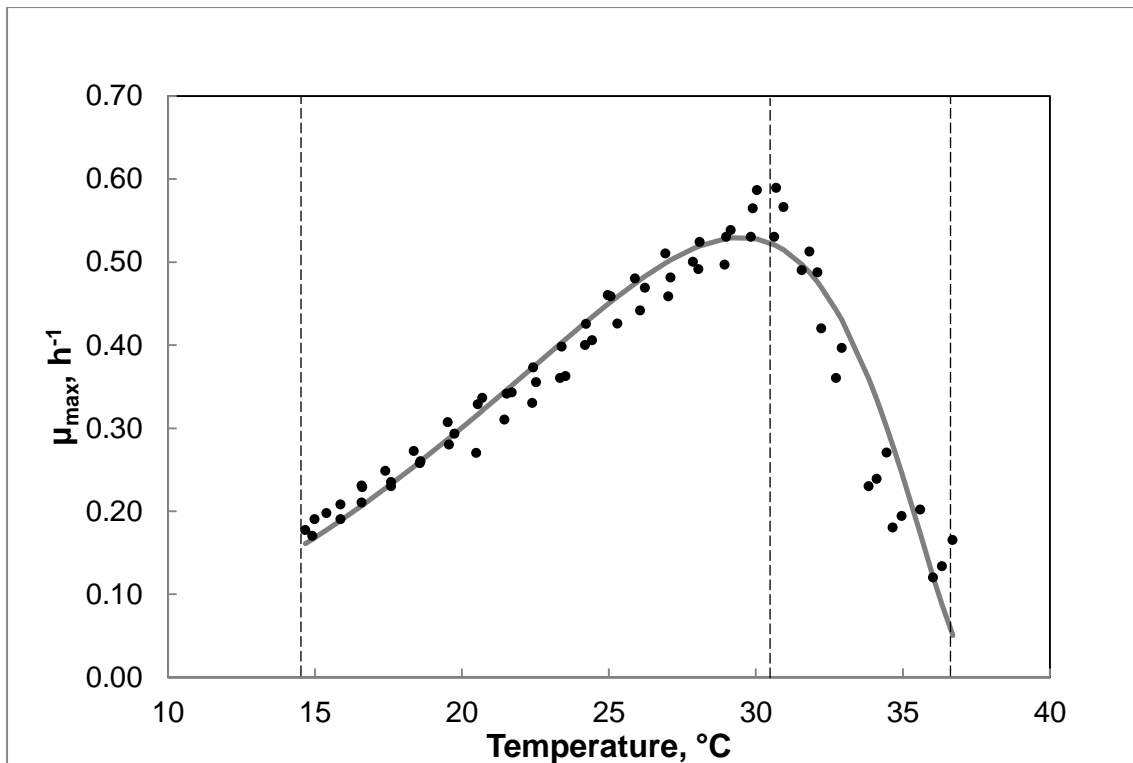


Fig. 4.4. Temperature profile of *Chryseobacterium vrystaatense*. The broken vertical lines indicate the cardinal temperatures.

4.3.3. Arrhenius plots

Activation energy is the energy barrier a microorganism needs to cross in order to react with food and without these barriers food spoilage will occur at a faster rate (Van Boekel, 2008). The higher the activation energy, the more difficult it becomes for the organism to grow optimally. The higher the activation energy at a certain temperature range the more sensitive the organism will be and will therefore, grow slower. Arrhenius plots are used to determine the activation energy of an organism (Pirt, 1975).

The activation energy is determined as follows: The slope ($\frac{\Delta \ln \mu_{max}}{\Delta \frac{1}{T}}$) of each zone e.g., A – B, B – C etc. on Figures 4.5 – 4.7 are determined and multiplied with $\frac{R}{1000}$ (R is the universal gas constant, $8.314 \text{ J}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$) to calculate activation energy in $\text{kJ}\cdot\text{mol}^{-1}$. According to the Arrhenius plots in Figures 4.5 – 4.7 (see summary in Table

4.3), strain 7_F195^T had an activation energy value of 33.90 kJ.mol⁻¹ in a temperature range of 25.7 – 31.6°C; *C. carnipullorum* had an activation energy value of 40.73 kJ.mol⁻¹ in a temperature range of 25.4 – 31.8°C and *C. vrystaatense* had an activation energy value of 31.39 kJ.mol⁻¹ in a temperature range of 25.9 – 30.6°C. These low activation energy values for each culture indicate that the organisms were less sensitive to temperature changes in these temperature ranges. Strain 7_F195^T showed the most sensitivity towards temperature change in the temperature range of 16.3 – 25.7°C with a higher activation energy value (75.99 kJ.mol⁻¹) than those of the other cultures. *Chryseobacterium vrystaatense* showed the least sensitivity towards temperature change in the temperature range of 16.6 – 25.9°C with an activation energy value of 57.07 kJ.mol⁻¹. *Chryseobacterium carnipullorum* had an activation energy value of 73.70 kJ.mol⁻¹ in the temperature range 15.3 – 25.4°C.

These results indicate that *C. vrystaatense* may have the ability to grow over a wider range of temperatures without being affected by the change of temperature. *Chryseobacterium vrystaatense* could, therefore, also possibly spoil food over a wider range of temperatures than strain 7_F195^T and *C. carnipullorum* can.

Table 4.3. Activation energies of strain 7_F195^T, *C. carnipullorum* and *C. vrystaatense* obtained from Arrhenius plots in Figures 4.5 – 4.7.

Culture	Zone	Temperature range (°C)	Slope	Activation energy (kJ.mol ⁻¹)
Strain 7_F195 ^T	A - B	16.3 – 25.7	-9139.51	75.99
	B - C	25.7 – 31.6	-4077.10	33.90
<i>C. carnipullorum</i>	D - E	15.3 – 25.4	-8865.05	73.70
	E - F	25.4 – 31.8	-4899.1	40.73
<i>C. vrystaatense</i>	G - H	16.6 – 25.9	-6864.10	57.07
	H - I	25.9 – 30.6	-3776.00	31.39

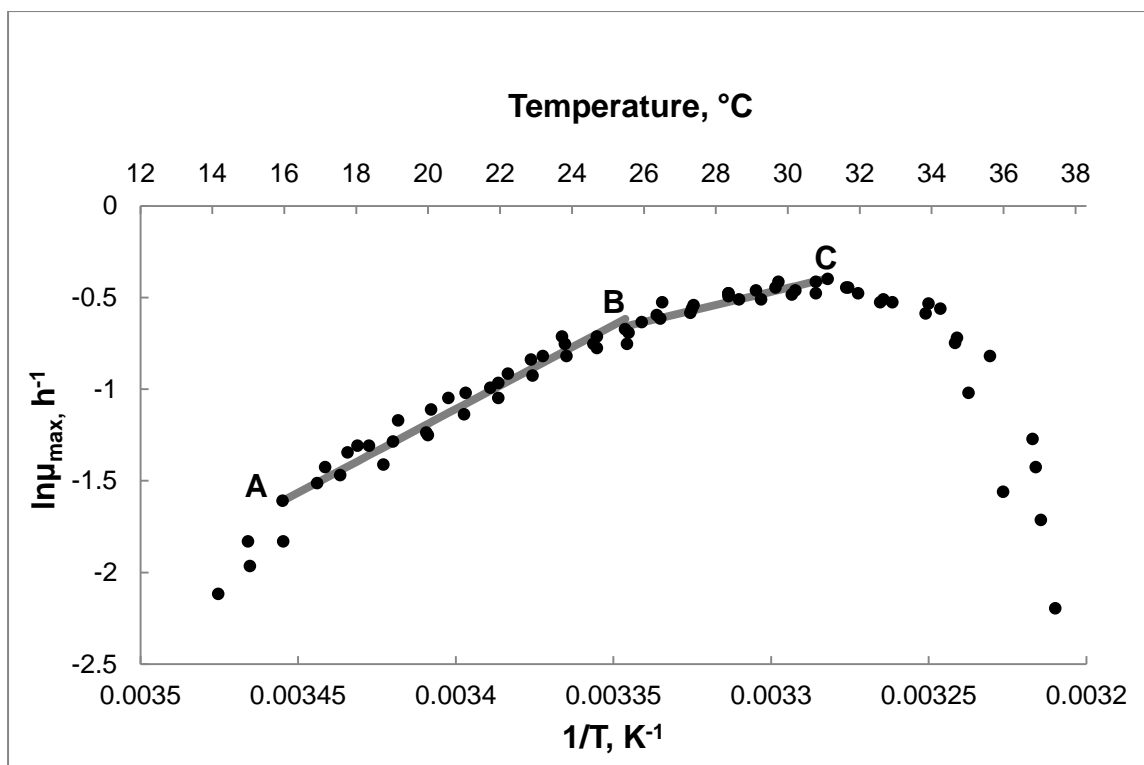


Fig. 4.5. Arrhenius plot of strain 7_F195^T. Activation energy values: A – B (16.3 – 25.7°C), 75.99 kJ.mol⁻¹; B - C (25.7 – 31.6°C), 33.90 kJ.mol⁻¹.

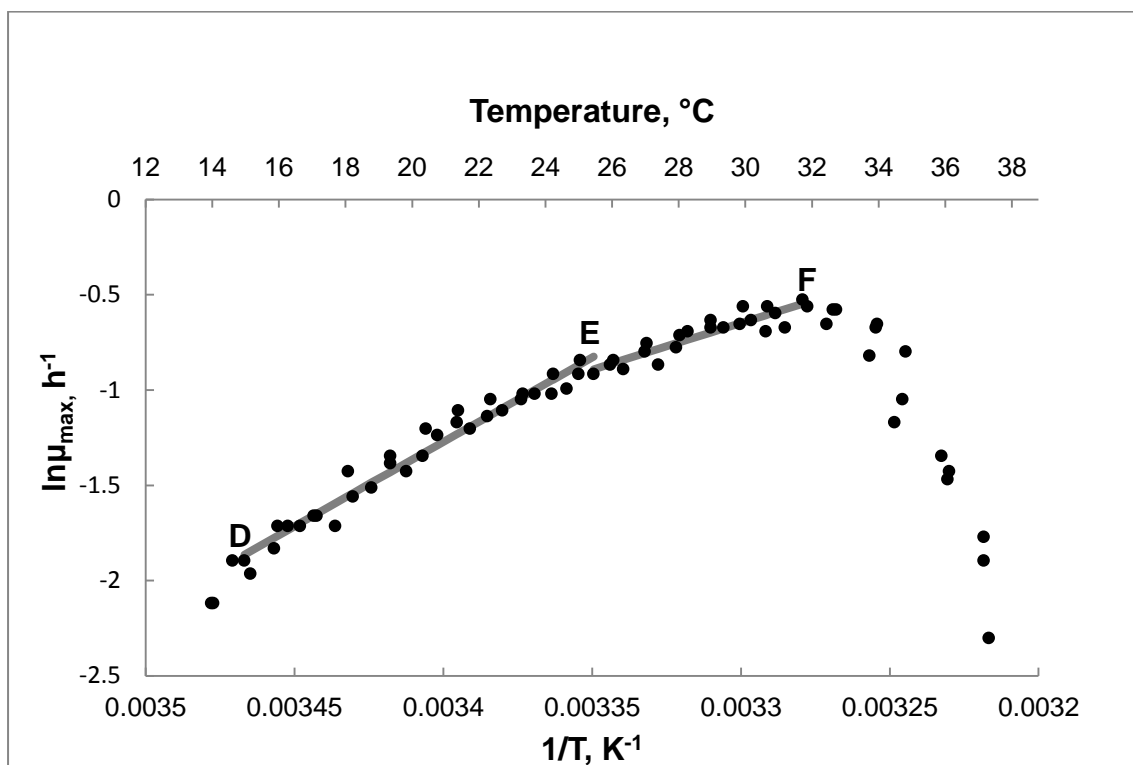


Fig. 4.6. Arrhenius plot of *Chryseobacterium carnipullorum*. Activation energy values: D – E (15.3 – 25.4°C), 73.70 kJ.mol⁻¹; E – F (25.4 – 31.8°C), 40.73 kJ.mol⁻¹.

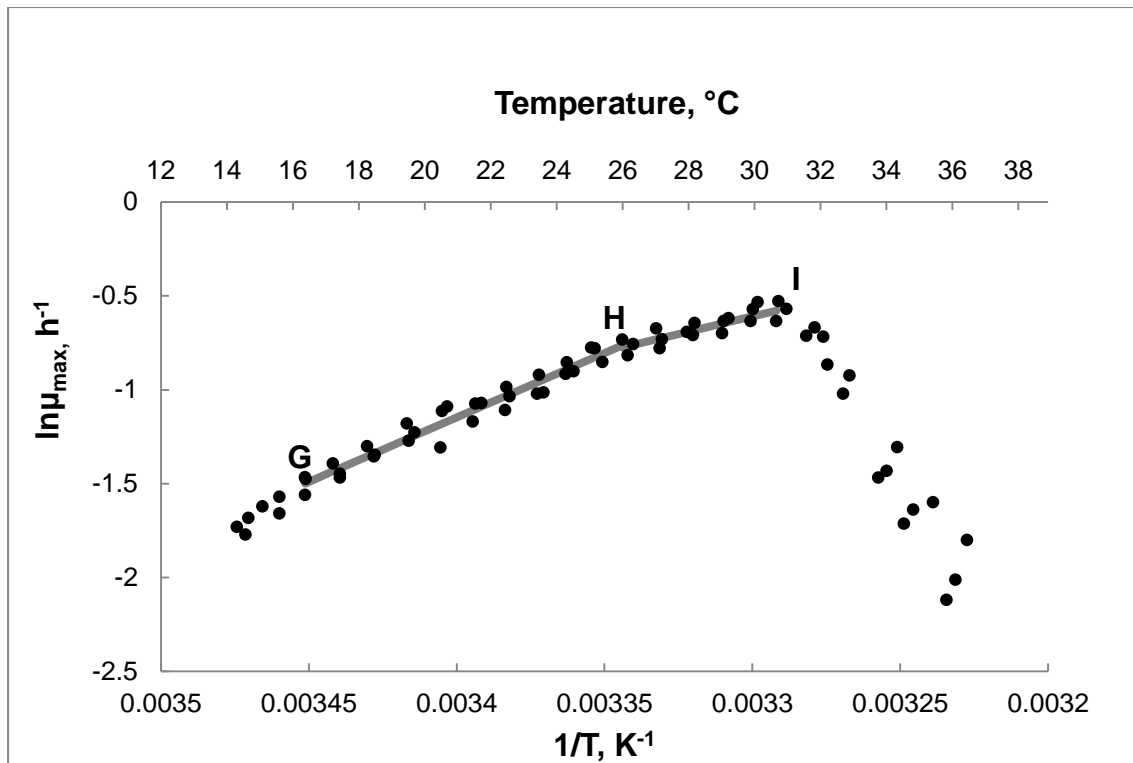


Fig. 4.7. Arrhenius plot of *Chryseobacterium vrystaatense*. Activation energy values: G – H (16.6 – 25.9°C), 57.07 $\text{kJ}\cdot\text{mol}^{-1}$; H – I (25.9 – 30.6°C), 31.39 $\text{kJ}\cdot\text{mol}^{-1}$.

4.4. Conclusions

The growth kinetics of a novel *Chryseobacterium* species, strain 7_F195^T, isolated from poultry feather waste (Charimba, 2012) in comparison with *C. carnipullorum* (Charimba *et al.*, 2013) and *C. vrystaatense* (de Beer *et al.*, 2005) isolated from raw chicken portions were determined. *Chryseobacterium carnipullorum* had a higher optimum temperature than strain 7_F195^T and *C. vrystaatense*. Strain 7_F195^T showed a higher maximum specific growth rate at its optimum temperature in comparison with the maximum specific growth rate of *C. carnipullorum* and *C. vrystaatense*.

After the maximum specific growth rates and cardinal temperatures were determined, Arrhenius plots were drawn of strain 7_F195^T in comparison with *C. carnipullorum* and *C. vrystaatense*. In the temperature range of about 25 - 32°C, all the cultures had low activation energies that indicated that these organisms are all

relatively insensitive to temperature change in these temperature ranges. In the temperature range of about, 15 – 26°C strain 7_F195^T showed the most sensitivity towards temperature change with a higher activation energy value while *C. vrystaatense* showed the least sensitivity towards temperature change with a lower activation energy value.

The results of this study conclude that strain 7_F195^T may have the ability to cause spoilage at a faster rate than *C. carnipullorum* and *C. vrystaatense* if the temperature is optimal. It is, however, more likely for *C. carnipullorum* and *C. vrystaatense* to cause spoilage at lower temperatures than for strain 7_F195^T according to the activation energies.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

The origin of the genus *Chryseobacterium* comes from an extensive history. In 1996 the family *Flavobacteriaceae* and the genus *Flavobacterium* were revised using polyphasic taxonomy (Bernardet *et al.*, 1996). The genus *Flavobacterium* was initially used to describe the family *Flavobacteriaceae* that included characteristics like having pigments ranging from yellow to orange or no pigments, no gliding movement and being strictly aerobic (Reichenbach, 1989). After the revision of the family *Flavobacteriaceae* and genus *Flavobacterium*, *Flavobacterium* (the type genus); *Bergeyella*; *Capnocytophaga*; *Chryseobacterium*; *Empedobacter*; *Ornithobacterium*; *Riemerella*; *Weeksella*; *Myroides* and *Tenacibaculum*, were included in the family.

The genus *Chryseobacterium* was first proposed in 1994 (Vandamme *et al.*, 1994a) and gained six species including *C. balustinum*, *C. gleum*, *C. indologenes*, *C. indoltheticum*, *C. meningosepticum* and *C. scophthalmum*, that were previously classified as members of the genus *Flavobacterium*. Today the genus *Chryseobacterium* has expanded to a total of 112 species (http://www.bacterio.net/c_hryseobacterium.html, accessed 2018/10/15).

Chryseobacterium species have been isolated from a wide variety of sources ranging from water, soil, plants, animals, humans and food (Bernardet *et al.*, 2002). *Chryseobacterium* species have especially become significant in the spoilage of food including poultry, red meat, milk, milk products and fish (Vandamme *et al.*, 1994b; Bernardet *et al.*, 2011) but have also been found in beer-bottling plants (Herzog *et al.*, 2008), lactic acid beverages (Shimomura *et al.*, 2005) and an edible sea urchin (Lin *et al.*, 2015). *Chryseobacterium* species are regarded as food spoilage organisms due to their proteolytic characteristics (Charimba *et al.*, 2013).

Polyphasic taxonomy integrates genotypic, phenotypic and chemotaxonomic methods to describe a microorganism (Prakash *et al.*, 2007) and then an organism can be evaluated for its role in food spoilage using predictive microbiology.

Prevention of food spoilage and loss of money, due to the growth of microorganisms are important in the food industry and to the consumers (Egan *et al.*, 2006; Cairo *et al.*, 2008).

The first aim of this study was to describe and name strain 7_F195^T previously isolated from chicken feather waste (Charimba, 2012) by using a polyphasic approach which included the following methods: whole-genome and 16S rRNA gene sequencing of strain 7_F195^T; determining the nearest neighbours of the strain 7_F195^T; using conventional and commercial system methods to determine the phenotypic characteristics of strain 7_F195^T and finally to combine this with results including the predominant fatty acid profile, polar lipids and respiratory lipoquinone, of strain 7_F195^T.

The second aim of this study was to determine the effect of temperature on strain 7_F195^T by determining the growth kinetics of the organism in comparison with *C. carnipullorum* that was isolated from a raw chicken portion (Charimba *et al.*, 2013) and *C. vrystaatense* (de Beer *et al.*, 2005) that was isolated from chicken portions from a poultry abattoir in South Africa. Knowing the spoilage potential and growth patterns of a microorganism may help in preventing food spoilage or, at the very least, assist in understanding its growth and spoilage characteristics.

The name *Chryseobacterium pennipullorum* was proposed for strain 7_F195^T after a polyphasic study was performed. The nearest neighbours of strain 7_F195^T were identified as *C. flavum*, *C. gleum* and *C. arthrosphaerae*. This was supported by constructing phylogenetic trees using two methods namely the Maximum Likelihood method and the Neighbour joining method. *Chryseobacterium pennipullorum* 7_F195^T was, however, distinct from its neighbours in having 16S rRNA similarity values of 98.42, 97.71 and 97.65% when compared to *C. flavum*, *C. gleum* and *C. arthrosphaerae*, respectively. Other methods including dDDH, ANI and AAI also confirmed that *C. pennipullorum* 7_F195^T does not belong in the same species as *C. flavum*, *C. gleum* or *C. arthrosphaerae*. The G+C value of 38.6 mol% for *C. pennipullorum* 7_F195^T also supports the affiliation of this organism to the genus *Chryseobacterium*.

The following chemotaxonomic markers also supported the affiliation of *C. pennipullorum* 7_F195^T to the genus *Chryseobacterium*: the predominant fatty

acids were iso-C_{15:0} (38.11%), iso-C_{17:1 ω9c} (25.32%) and iso-C_{17:0 3-OH} (13.14%); phosphatidylethanolamine were the most abundant polar lipid and menaquinone-6 (100%) was the only respiratory quinone. The identification and classification of *C. pennipullorum* 7_F195^T, as a new species of the genus *Chryseobacterium*, was supported by phenotypic tests performed using conventional and automated methods including API test strips and the BIOLOG Omnilog Gen III identification system.

The spoilage potential of *C. pennipullorum* 7_F195^T in comparison with *C. carnipullorum* and *C. vrystaatense* were determined by means of growth kinetics. The cultures were inoculated in nutrient broth no. 2 and incubated on a TGI with a temperature range of 14 to 50°C. The cardinal temperature ranges of *C. pennipullorum* 7_F195^T in comparison with *C. carnipullorum* and *C. vrystaatense* were determined. *Chryseobacterium carnipullorum* had a higher optimum temperature of 31.80°C than *C. pennipullorum* 7_F195^T (31.55°C) and *C. vrystaatense* (30.70°C). *Chryseobacterium pennipullorum* 7_F195^T showed a higher maximum specific growth rate of 0.67 h⁻¹ at its optimum temperature in comparison with the maximum specific growth rate of 0.59 h⁻¹ for both *C. carnipullorum* and *C. vrystaatense*, at their optimum temperatures. *Chryseobacterium pennipullorum* 7_F195^T may, therefore, have the ability to cause spoilage at a faster rate than *C. carnipullorum* and *C. vrystaatense* if the temperature is optimal.

Arrhenius plots were created for *C. pennipullorum* 7_F195^T in comparison with *C. carnipullorum* and *C. vrystaatense*. Results showed that *C. pennipullorum* 7_F195^T had the most sensitivity towards temperature change in the temperature range of 16.3 – 25.7°C with a higher activation energy value (75.99 kJ.mol⁻¹) than those of the other cultures. *Chryseobacterium vrystaatense* showed the least sensitivity towards temperature change in the temperature range of 16.6 – 25.9°C with an activation energy value of 57.07 kJ.mol⁻¹. *Chryseobacterium carnipullorum* had an activation energy value of 73.70 kJ.mol⁻¹ in the temperature range 15.3 – 25.4°C. These results indicated that *C. vrystaatense* may have the ability to grow over a wider range of temperatures without being affected by the change of temperature. *Chryseobacterium vrystaatense* could, as a result, also possibly spoil food over a wider range of temperatures than strain 7_F195^T and *C. carnipullorum*.

Chryseobacterium pennipullorum 7_F195^T was successfully classified, described and named as a new species of the genus *Chryseobacterium*. The growth kinetics of *C. pennipullorum* 7_F195^T was determined and will add value to future research on its food spoilage potential.

Future research:

The growth and food spoilage potential of *C. pennipullorum* 7_F195^T should be investigated under other parameters e.g., water activity, pH, oxygen concentration and nutrient composition of food.

Investigate enzyme production by *C. pennipullorum* 7_F195^T in terms of proteolytic enzymes that may potentially spoil food and keratinolytic enzymes for the degradation of feather waste.

Further investigation of the whole genome of *C. pennipullorum* 7_F195^T in terms of unique genes and functions may also prove useful.

CHAPTER 6

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SUMMARY

Animals, humans and food environments are major sources for the isolation of *Chryseobacterium* species. Spoilage of food by these organisms have become significant in poultry, red meat, milk and fish. Other sources include Beer-bottling plants and lactic acid beverages. Spoilage of food by *Chryseobacterium* species is mainly due to its proteolytic characteristics. The genus *Chryseobacterium* was proposed in 1994 and has since expanded to a total of 112 species today.

The first aim of this study was to use a polyphasic approach to describe and name strain 7_F195^T previously isolated from chicken feather waste collected from an abattoir in Bloemfontein. Methods used for describing strain 7_F195^T included: whole genome and 16S rRNA gene sequencing; phylogenetic treeing methods, conventional and commercial system methods for phenotypic characterization; fatty acid profile, polar lipid and respiratory lipoquinone analysis.

Chryseobacterium flavum, *C. gleum* and *C. arthrosperae* were identified as being the nearest neighbours. The DNA G+C content of strain 7_F195^T supported the affiliation of the strain to the genus *Chryseobacterium*. The digital DNA-DNA hybridization, ANI and AAI values confirmed that strain 7_F195^T does not belong in the following species: *C. flavum*, *C. gleum* or *C. arthrosperae*. Various similar phenotypic characteristics were reported between strain 7_F195^T and the reference strains, but many differences were also noted. Results obtained from the fatty acid profile, polar lipid and respiratory quinone analysis supported the affiliation of strain 7_F195^T to the genus *Chryseobacterium*. Based on the data generated from this polyphasic study, strain 7_F195^T represents a novel *Chryseobacterium* species for which the name *Chryseobacterium pennipullorum* sp. nov. is proposed.

The second aim of this study was to investigate the temperature growth relationship of a novel *Chryseobacterium* species, strain 7_F195^T, isolated from poultry feather waste in comparison to *C. carnipullorum* and *C. vrystaatense* that were isolated from raw chicken portions. The growth study was conducted using a Temperature Gradient Incubator and growth rates and cardinal temperatures were determined through optical density measurements. Temperature profiles and Arrhenius plots were constructed.

Strain 7_F195^T showed a higher maximum specific growth rate at its optimum temperature in comparison with *C. carnipullorum* and *C. vrystaatense*. All the cultures showed relatively low sensitivity to temperature change in the temperature range 25 - 32°C. Strain 7_F195^T was the most sensitive while *C. vrystaatense* was the least sensitive towards temperature change in the temperature range of about 15 – 26°C. The results of this study concluded that strain 7_F195^T may have the ability to cause spoilage at a faster rate than *C. carnipullorum* and *C. vrystaatense* at its optimal temperature.

Chryseobacterium pennipullorum 7_F195^T was successfully classified, described and named as a new species of the genus *Chryseobacterium*. The growth kinetics of *C. pennipullorum* 7_F195^T was determined and will add value to future research on its food spoilage potential.

Annexure 1

Chryseobacterium species and their isolation sources

Species	Sources	Reference(s)
<i>C. aahli</i>	Lake trout (<i>Salvelinus namaycush</i>) and brown trout (<i>Salmo trutta</i>)	Loch & Faisal, 2014
<i>C. angstadtii</i>	Environmental (freshwater)	Kirk <i>et al.</i> , 2013
<i>C. antarcticum</i>	Antarctic soil	Kämpfer <i>et al.</i> , 2009a
<i>C. anthropi</i>	Human	Kämpfer <i>et al.</i> , 2009b
<i>C. aquaticum</i>	Water reservoir	Kim <i>et al.</i> , 2008
<i>C. aquifrigidense</i>	Water-cooling system	Park <i>et al.</i> , 2008
<i>C. arachidiradicis</i>	Soil	Kämpfer <i>et al.</i> , 2015a
<i>C. arachidis</i>	Soil	Kämpfer <i>et al.</i> , 2014a
<i>C. arothri</i>	Pufferfish (<i>Arothron hispidus</i>)	Campbell <i>et al.</i> , 2008
<i>C. arthrosphaerae</i>	Pill millipede	Kämpfer <i>et al.</i> , 2010a
<i>C. artocarp</i>	Soil	Venil <i>et al.</i> , 2014
<i>C. balustinum</i>	Gold sand/dust	Vandamme <i>et al.</i> , 1994a
<i>C. bernardetii</i>	Human	Holmes <i>et al.</i> , 2013
<i>C. bovis</i>	Raw cow's milk	Hantsis-Zacharov <i>et al.</i> , 2008a

Species	Sources	Reference(s)
<i>C. caeni</i>	Bioreactor sludge	Quan <i>et al.</i> , 2007
<i>C. camelliae</i>	Plant (<i>Camellia sinensis</i>)	Kook <i>et al.</i> , 2014
<i>C. carnipullorum</i>	Raw chicken	Charimba <i>et al.</i> , 2013
<i>C. carnis</i>	Human	Holmes <i>et al.</i> , 2013
<i>C. chaponense</i>	Atlantic salmon	Kämpfer <i>et al.</i> , 2011
<i>C. contaminans</i>	Soil	Kämpfer <i>et al.</i> , 2014b
<i>C. cucumeris</i>	Plant root (<i>Cucumis sativus</i> L.)	Jeong <i>et al.</i> , 2017
<i>C. culicis</i>	Mosquito	Kämpfer <i>et al.</i> , 2010b
<i>C. daecheongense</i>	Freshwater lake sediment	Kim <i>et al.</i> , 2005a
<i>C. daeguense</i>	Wastewater	Yoon <i>et al.</i> , 2007
<i>C. defluvii</i>	Wastewater	Kämpfer <i>et al.</i> , 2003
<i>C. echinoideorum</i>	Edible sea urchin (<i>Tripneustes gratilla</i>)	Lin <i>et al.</i> , 2015
<i>C. elymi</i>	Wild rye (<i>Elymus</i>)	Cho <i>et al.</i> , 2010
<i>C. endophyticum</i>	Maize leaf	Lin <i>et al.</i> , 2017
<i>C. flavum</i>	Soil	Zhou <i>et al.</i> , 2007
<i>C. formosense</i>	Plant (<i>Lactuca sativa</i>)	Young <i>et al.</i> , 2005
<i>C. frigidisoli</i>	Soil	Bajerski <i>et al.</i> , 2013

Species	Sources	Reference(s)
<i>C. frigidum</i>	Soil	Kim <i>et al.</i> , 2016
<i>C. gallinarum</i>	Animal (Chicken)	Kämpfer <i>et al.</i> , 2014b
<i>C. gambrini</i>	Beer-bottling plants	Herzog <i>et al.</i> , 2008
<i>C. geocarposphaerae</i>	Soil	Kämpfer <i>et al.</i> , 2014a
<i>C. ginsengisoli</i>	Soil	Nguyen <i>et al.</i> , 2013
<i>C. ginsengiterrae</i>	Soil	Hahnke <i>et al.</i> , 2016
<i>C. ginsenosidimutans</i>	Soil	Im <i>et al.</i> , 2011
<i>C. glaciei</i>	Soil (ice)	Pal <i>et al.</i> , 2018
<i>C. gleum</i>	Vaginal swab	Holmes <i>et al.</i> , 1984; Vandamme <i>et al.</i> , 1994a
<i>C. greenlandense</i>	Greenland ice core	Loveland-Curtze <i>et al.</i> , 2010
<i>C. gregarium</i>	Decaying plant material	Behrendt <i>et al.</i> , 2008
<i>C. gwangjuense</i>	Soil	Park Y.J. <i>et al.</i> , 2013
<i>C. hagamense</i>	Rhizosphere of coastal sand dune plants	Cho <i>et al.</i> , 2010
<i>C. haifense</i>	Raw milk	Hantsis-Zacharov & Halpern, 2007b
<i>C. halperniae</i>	Food	Hahnke <i>et al.</i> , 2016

Species	Sources	Reference(s)
<i>C. hispalense</i>	Rainwater pond in an olive plant nursery	Montero-Calasanz <i>et al.</i> , 2013
<i>C. hispanicum</i>	Drinking water distribution system	Gallego <i>et al.</i> , 2006
<i>C. hominis</i>	Human	Vaneechoutte <i>et al.</i> , 2007
<i>C. humi</i>	Industrially contaminated sediments	Pires <i>et al.</i> , 2010
<i>C. hungaricum</i>	Hydrocarbon-contaminated soil	Szoboszlay <i>et al.</i> , 2008
<i>C. indologenes</i>	Human trachea at autopsy	Vandamme <i>et al.</i> , 1994a
<i>C. indoltheticum</i>	Marine mud	Vandamme <i>et al.</i> , 1994a
<i>C. jejuense</i>	Soil	Weon <i>et al.</i> , 2008
<i>C. jeonii</i>	Antarctic moss	Kämpfer <i>et al.</i> , 2009a
<i>C. joostei</i>	Raw milk	Hugo <i>et al.</i> , 2003
<i>C. koreense</i>	Human	Kämpfer <i>et al.</i> , 2009b
<i>C. kwangjuense</i>	Pepper (<i>Capsicum annuum</i> L.) root	Sang <i>et al.</i> , 2013
<i>C. lactis</i>	Milk	Holmes <i>et al.</i> , 2013
<i>C. lathyri</i>	Coastal sand dune plants	Cho <i>et al.</i> , 2010
<i>C. limigenitum</i>	Dehydrated sludge	Kämpfer <i>et al.</i> , 2015d
<i>C. lineare</i>	Freshwater	Zhao, Z., <i>et al.</i> , 2017

Species	Sources	Reference(s)
<i>C. luteum</i>	Phyllosphere of grasses	Behrendt <i>et al.</i> , 2007
<i>C. marinum</i>	Antarctic seawater	Kämpfer <i>et al.</i> , 2009a
<i>C. meningosepticum</i>	Human	Vandamme <i>et al.</i> , 1994a
<i>C. miricola</i>	Water (Space station Mir)	Li <i>et al.</i> , 2003
<i>C. molle</i>	Beer-bottling plants	Herzog <i>et al.</i> , 2008
<i>C. montanum</i>	Mountain soil	Guo <i>et al.</i> , 2016
<i>C. nakagawai</i>	Human	Holmes <i>et al.</i> , 2013
<i>C. nepalense</i>	Soil	Chaudhary & Kim, 2017
<i>C. oleae</i>	Olive tree (<i>Olea europaea</i> L.)	Montero-Calasanz <i>et al.</i> , 2014
<i>C. oncorhynchi</i>	Rainbow trout, <i>Oncorhynchus mykiss</i>	Zamora <i>et al.</i> , 2012a
<i>C. oranimense</i>	Raw cow's milk	Hantsis-Zacharov <i>et al.</i> , 2008b
<i>C. pallidum</i>	Beer-bottling plants	Herzog <i>et al.</i> , 2008
<i>C. palustre</i>	Industrially contaminated sediments	Pires <i>et al.</i> , 2010
<i>C. piperi</i>	Freshwater creek	Strahan <i>et al.</i> , 2011
<i>C. piscicola</i>	Diseased salmonid fish	Ilardi <i>et al.</i> , 2009

Species	Sources	Reference(s)
<i>C. piscium</i>	Fish	de Beer <i>et al.</i> , 2006
<i>C. polytrichastri</i>	Moss (<i>Polytrichastrum formosum</i>)	Chen <i>et al.</i> , 2015
<i>C. profundimaris</i>	Deep sea sediment	Xu <i>et al.</i> , 2015
<i>C. psychrotolerans</i>	Environmental	Hahnke <i>et al.</i> , 2016
<i>C. reticulitermitis</i>	Animal	Zhao, Y., <i>et al.</i> , 2017
<i>C. rhizoplanae</i>	Rhizoplane environment	Kämpfer <i>et al.</i> , 2015c
<i>C. rhizosphaerae</i>	Rhizosphere of coastal sand dune plants	Cho <i>et al.</i> , 2010
<i>C. rigui</i>	Estuarine wetland	Park <i>et al.</i> , 2013
<i>C. salipaludis</i>	Environmental	Divyasree <i>et al.</i> , 2018
<i>C. scophthalmum</i>	Gills of diseased turbot (<i>Scophthalmus maximus</i>)	Vandamme <i>et al.</i> , 1994a
<i>C. sediminis</i>	Freshwater	Kämpfer <i>et al.</i> , 2015b
<i>C. shandongense</i>	Soil	Yang <i>et al.</i> , 2015
<i>C. shigense</i>	Lactic acid beverage	Shimomura <i>et al.</i> , 2005
<i>C. soldanellicola</i>	Roots of sand-dune plants	Park <i>et al.</i> , 2006
<i>C. solani</i>	Field-grown eggplant rhizosphere soil	Du <i>et al.</i> , 2015
<i>C. soli</i>	Soil	Weon <i>et al.</i> , 2008

Species	Sources	Reference(s)
<i>C. solincola</i>	Soil	Benmalek <i>et al.</i> , 2010
<i>C. taeanense</i>	Roots of sand-dune plants	Park <i>et al.</i> , 2006
<i>C. taichungense</i>	Contaminated soil	Shen <i>et al.</i> , 2005
<i>C. taihuense</i>	Eutrophic lake	Wu <i>et al.</i> , 2013
<i>C. taiwanense</i>	Soil	Tai <i>et al.</i> , 2006
<i>C. takakiae</i>	Plant (<i>Takakia lepidozoides</i>)	Zhao <i>et al.</i> , 2015
<i>C. taklimakanense</i>	Soil	Holmes <i>et al.</i> , 2013
<i>C. tenax</i>	Plant	Hahnke <i>et al.</i> , 2016
<i>C. treverense</i>	Human	Yassin <i>et al.</i> , 2010
<i>C. tructae</i>	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Zamora <i>et al.</i> , 2012b
<i>C. ureilyticum</i>	Beer-bottling plants	Herzog <i>et al.</i> , 2008
<i>C. vietnamense</i>	Soil	Li & Zhu, 2012
<i>C. viscerum</i>	Fish	Zamora <i>et al.</i> , 2012c
<i>C. vrystaatense</i>	Raw chicken	de Beer <i>et al.</i> , 2005
<i>C. wanjuense</i>	Soil	Weon <i>et al.</i> , 2006
<i>C. xinjiangense</i>	Alpine permafrost	Zhao <i>et al.</i> , 2011
<i>C. xixisoli</i>	Soil	Hahnke <i>et al.</i> , 2016

Species	Sources	Reference(s)
<i>C. yonginense</i>	Mesotrophic artificial lake	Joung & Joh, 2011
<i>C. zeeae</i>	Plant	Kämpfer <i>et al.</i> , 2014a
